Investigating the potential of bacteriophage induction and phage-derived enzymes as alternative antibacterial approaches

by

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Declaration

I, Ji-Yun Lee, declare that the research thesis that I herewith submit for the Doctoral Degree qualification in Microbiology at the University of the Free State is my independent work, and that I have not previously submitted it for a qualification at another institution of higher education.

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List of Abbreviations

APEC	Avian Pathogenic *Escherichia coli*
CAI	Codon Adaptation Index
CAS	CRISPR-associated genes / proteins
CBD	Cell wall binding domain
CRISPR	Clustered regularly interspaced short palindromic repeats
CT	Cholera toxin
CWP	Cell wall protein
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EAD	Enzymatically active domain
EDTA	Ethylenediaminetetraacetic acid
ESCMID	European Society of Clinical Microbiology and Infectious Diseases
EtBr	Ethidium bromide
EUCAST	European Committee for Antimicrobial Susceptibility Testing
FPLC	Fast protein liquid chromatography
GFP	Green fluorescent protein
GPI	Glycosylphosphatidylinositol
GRAS	Generally regarded as safe
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HHP	High hydrostatic pressure
HRP	Horse radish peroxidase
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Lysogeny broth
LC-MS/MS	Liquid-chromatography Tandem Mass Spectrometry
LPS	Lipopolysaccharide layer
LTR	Long terminal repeats
MCS	Multiple cloning site
MRSA	Meticillin-resistant *Staphylococcus aureus*
MWCO	Molecular weight cut off
ORF	Open reading frame
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline-Tween
PCA	Plate count agar
PCR	Polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate Polyacrylamide Gel Electrophoresis
SEM	Scanning electron microscopy
TCA	Trichloroacetic acid
TSB	Tryptic Soy Broth
VRE	Vancomycin-resistant *Enterococcus*
YNB	Yeast nitrogen base
YPD	Yeast potato dextrose

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Abstract

Since their discovery in the 1920s, antibiotics have saved generations of people from succumbing to bacterial infections. Antibiotic usage has resulted in increased antibiotic resistance which has become a problem in various fields and industries. Without effective antibiotics, even simple bacterial infections can be fatal and this has the potential to create a post-antibiotic era where fatalities from currently controlled bacterial infections will become normal. This also extends to the poultry industry where Avian Pathogenic *Escherichia coli* (APEC) are known to affect the market due to colibacillosis and resulting in poor quality meat and egg products. As bans against antibiotics used in the growth of food animals are established there is a necessity for alternative treatment options. One of the potential solutions is harnessing the power of bacteriophages which are viruses that infect bacteria and proliferate within them. Bacteriophage therapy has been developing and the diverse use of bacteriophages in treatment of bacterial infections ranges from using the whole bacteriophage within its packaged virion, to only parts of the bacteriophage such as proteins/enzymes. Some of the challenges of using bacteriophage cocktails for therapy, extended also to the treatment of APECs, are the myriad environmental conditions that bacteriophages require to proliferate in a host.

In this thesis, the use of lysogenic bacteriophages and the heterologous expression of bacteriophage endolysins were investigated as alternative treatments against bacteria.

Screening PCRs in combination with induction of prophages within lysogenic bacteria were used to determine whether temperate bacteriophages could be used and genetically manipulated to remain in lysis and therefore be used in therapy or treatment. Potential lysogens were induced using UV-, heat- and mitomycin C inducers. Heterologous expression of bacteriophage proteins was performed through both bacterial expression and yeast expression systems. Bacterial expression was achieved using pETDuet-1 and pET28b expression vectors transformed into BL21 (DE3) competent *E. coli* cells. The proteins expressed in the pET28b vector contained a 6 x Histidine-tag and were subsequently purified on gravity-flow protein purification and fast protein liquid chromatography (FPLC). Yeast expression was performed using yeast expression vectors pINA1317 and pINA1317-CWP and transformed into *Yarrowia lipolytica* Po1h competent cells. Proteins visualization was done using 12 % SDS-PAGE and identified with LC-MS/MS. The proteins were tested against bacterial cells, both in the presence and absence of permeabilising agents to determine their efficacy. This included commercial products (Biotronic® Top Line and Virukill®) to determine whether the expressed proteins could improve the products.

PCR screening for the presence of prophages resulted in a single strain containing *cro* and *c*I genes while about one third of screened strains tested positive for the *int* gene. This makes it likely that this strain harbours an intact lambda phage that is potentially inducible from lysogeny into the lytic pathway. Bacteriophage proteins were successfully produced using the bacterial expression

systems and confirmed through LC-MS/MS results. Although the proteins were purified, they did not elicit an antibacterial effect when applied to the permeabilised bacteria. The yeast expression system was not as successful, although integration into the yeast was confirmed using PCR and sequencing. The treatment assays against bacterial cells were not as significantly effective as expected, whether used in combination with permeabilising agents or not even though initial results showed log differences when compared to the control.

The heterologously expressed and identified bacteriophage lambda endolysin from this study can be further tested for efficacy against bacteria. Ideally, this study should be expanded to include endolysins and virolysins from bacteriophages that target other bacteria.

Keywords

Antibiotic resistance, bacteriophage therapy, lysogenic phage, bacteriophage endolysin, bacteriophage holin, lipopolysaccharide permeabilising agents

Chapter 1 Avian Pathogenic *Escherichia coli* (APEC): Review on the control and prevention of colibacillosis

Preface

This chapter has been submitted as a journal review manuscript to Avian Pathology. This manuscript is the basis of the literature review of this thesis and is written according to the style of the journal. The specific contributions of the manuscript contributed by the thesis author are listed. Appendix A is supplementary to the literature review and provides additional information on the genetic bi-switch of bacteriophage lambda's lysogenic-lytic lifecycle as well as bacteriophage endolysin and holin proteins that facilitate breaking of the cell from within.

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- 1.4.7 Advantages of bacteriophage therapy:
- 1.7 Conclusions

Avian Pathogenic *Escherichia coli* (APEC): Review on the control and prevention of colibacillosis

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Abstract

Avian pathogenic *Escherichia coli* (APEC) is the causative agent of avian colibacillosis which has economic implications for the poultry industry. Control of APEC has mostly been done using antibiotics. However, many strains have now become multi-drug resistant. Alternative control and preventative measures are thus required, and these are investigated in this review. These include development of novel antibiotics, improved vaccine development, bacteriophage therapy, bacteriophage-encoded enzymes and finally improved biosecurity measures. Understanding the mechanisms of disease will thus become invaluable in the future as novel therapeutic agents are to be developed. The current knowledge of common virulence genes associated with APEC is therefore also discussed, outlining their functions in pathogenesis. Each of the discussed alternative measures have their benefits and pitfalls, therefore a combination of these will most likely be possible options to use in the poultry industry, especially since a post-antibiotic era is looming with antibiotics being banned from use in animal production altogether.

Key words

Bacteriophage therapy, vaccine, colibacillosis, APEC, antibiotic resistance, endolysin therapy

1.1 Avian Pathogenic *Escherichia coli* (APEC)

1.1.1 Background:

Avian Pathogenic *Escherichia coli* (APEC) cause the disease avian colibacillosis in many avian species, resulting in economic losses for the poultry industry (Barnes *et al.*, 2013). Clinical symptoms of avian colibacillosis include swollen head syndrome, septicaemia, air sacculitis, pericarditis and cellulitis; which lead to decreased egg yields and an increase in mortalities. Antibiotics are widely used throughout the poultry industry for the control of colibacillosis. However, this has led to multiple antibiotic-resistant strains becoming problematic in the poultry industry (Asai *et al.*, 2011). This could result in products contaminated with multi drug-resistant bacteria entering the food chain and could lead to foodborne antibiotic-resistant bacteria spreading among people in a community (Apata, 2009). This is one of the major reasons for abolishing sub-therapeutic antibiotic usage in poultry and livestock.

There are also concerns that bacteria such as APEC, which are generally only found in avian species, could have the zoonotic potential to infect humans, since they share common virulence genes to those found in human extra-intestinal pathogenic *E. coli* (Ewers *et al.*, 2007).

The virulence of *E. coli* is related to genes found in the genome, prophage remnants as well as on episomal structures, such as plasmids (Dozois *et al.*, 2003; Johnson *et al.*, 2008a). Many varieties of virulence-encoding genes exist and are associated with colibacillosis. The genes either act

individually or polygenically with varying frequencies in clinical isolates (Delicato *et al.*, 2003; Vandekerchove *et al.*, 2005). Currently the association of a gene with virulence is not completely understood, because different sets of virulence genes in different strains can lead to colibacillosis symptoms (Delicato *et al.*, 2003). Therefore, no single gene-product has been used as a feasible drug-target for the treatment of avian colibacillosis. In recent years it has been found that some genes occur in higher frequencies in pathogenic strains of APEC compared to commensal *E. coli* isolates from “healthy” chickens, and the relevant gene products are currently being researched for the development of possible vaccines against APEC (Lynne *et al.*, 2006).

1.1.2 Virulence genes:

A clear understanding of the virulence of the APEC is required for the development of specific therapeutics to combat colibacillosis. Substantial research has been done on the correlation of virulence genes present in APEC to the pathogenicity of the bacterium (Delicato *et al.,* 2003; Vandekerchove *et al.,* 2005). Non-pathogenic isolates are often obtained from chicken faecal matter of clinically healthy birds, while droppings, organs or gastrointestinal isolates from chickens showing clinical signs of colibacillosis are used to represent APEC isolates (Delicato *et al.,* 2003; Vandekerchove *et al.,* 2005).

Comparisons between suspected pathogenic and non-pathogenic isolates are made regarding the frequencies of virulence genes, the diversity and combination of virulence genes, and the effects of the genes on pathogenicity of the strains; to determine whether an isolate is a potential APEC strain (Mellata *et al.*, 2003).

To cause disease, a pathogen must be able to survive inside the host through resistance to or evasion from compounds found within the host or overcoming the host defence mechanisms. Survival of the bacterium could be attributed to serum resistance genes and capsule formations (Merino & Tomás, 2015). The pathogen can also adhere to specific tissues within the host, which is accomplished by means of adhesins, such as fimbriae, pilli and the hemagglutinins of the red blood cells in the circulatory system (Klemm & Schembri, 2000; Esko & Sharon, 2009). Furthermore, the pathogens invade the host cells, allowing the pathogens to spread through the host tissues (Ewers *et al.*, 2007). The pathogens grow within the host cells, chelating iron in the process to promote growth within the host and thus promoting disease (Andrews *et al.*, 2003). In addition, the pathogen can also produce disease-causing toxins, normally in the form of exotoxins, in the host, which either increase the availability of nutrients to the pathogen or facilitate the spread of the pathogen in the host.

Characteristics and proteins associated with the described virulence of *E. coli* include: colicin production, capsule formation, invasin production, serum resistance, iron chelators, adhesion factors

such as haemagglutinin and toxin formation (Delicato *et al.*, 2003; Vandekerchove *et al.*, 2005). These factors are reviewed in the following sections.

Invasivity in human intestinal epithelial cells (carcinoma T84 cell line) simulates the ability to spread into “healthy” tissue by invading host cells, in this case the host chicken cells (Ewers *et al.*, 2007). The invasivity of a bacterium is influenced by adhesion capabilities and general ability to survive outside of the host cells (Pizarro-Cerdá & Cossart, 2006). Pathogens should be highly invasive to survive long enough while in circulation to reach suitable tissues for infection. Genes coding for invasins include *gimB*, *ibeA* and *tia* (Ewers *et al.*, 2007).

Adhesion is required for pathogens to bind to specific host tissues and to prevent their physical removal from the infected areas, allowing for infection to occur (Klemm & Schembri, 2000). P-fimbriae, type-1 fimbriae and curli play important roles in adhesion to host cell membranes (Mellata *et al.,* 2003). P-fimbriae are encoded by *papC* and *papG* genes, type-1 fimbriae by the *fim gene* and curli by the *csgA* gene (Mellata *et al.,* 2003). Haemagglutinin is also important for the adhesion to host red blood cells, allowing the pathogen to easily circulate throughout the host (Esko & Sharon, 2009).

In order to evade the host defence mechanisms, a protective capsule consisting of a polysaccharide layer can be formed around the cell through the actions of proteins encoded by the *kps* and *neu*C genes (Delicato *et al.*, 2003). These capsules have been associated with complement resistance and decreased association with phagocytes (Dho-Moulin & Fairbrother, 1999; Mellata *et al.*, 2003).

Serum resistance allows bacterial pathogens to evade the host complement and antibody-mediated defence mechanisms (Williams *et al.*, 2001). Genes such as *iss* and *ompA* code for serum resistance in APEC (Prasadarao *et al.*, 2002; Miajlovic & Smith, 2014), while the above-mentioned capsule formation around the cell also offers resistance against serum (Hansen & Hirsh, 1989).

Iron acquisition is important for survival of the bacterium since various bacterial proteins require iron as a cofactor, therefore making it an essential and limiting nutrient (Andrews *et al.*, 2003). The proteins involved in iron uptake, such as siderophores, chelate free iron from the environment for use in the cell for metabolic activities, and this has adverse effects on the host as red blood cells also require iron for their function as a vital component of haeme (Barber & Elde, 2015).

Toxinogenicity involves the production of toxins by pathogenic bacteria to increase virulence. The gene encoding vacuolating autotransporter toxin, *vat*, has been observed in APEC isolates but the frequency is often low (Vandekerchove *et al.*, 2005). Bacteriocins, such as colicin V are coded by genes such the *cvaC* gene present on the ColV plasmid (Vandekerchove *et al.*, 2005). Colicin-sensitive bacterial cells are destroyed by colicins, allowing the bacteria to outcompete commensal bacteria and indirectly giving the bacteria access to host tissues.

1.1.3 Detection of virulence genes in APEC:

Various multiplex PCRs have been developed to screen *E. coli* isolates for virulence genes to identify potential APEC strains (Ewers *et al.*, 2005; Johnson *et al.*, 2008b; Van der Westhuizen & Bragg 2012). Johnson *et al.* (2008b) investigated potential minimum predictors of APEC by looking at the most prevalent genes present in the significant majority of APEC isolates. They concluded that five virulence genes, *ompT* (outer membrane protease VII), *iroN* (TonB-dependent siderophore receptor protein), *iss* (increased serum survival), *hlyF* (originally putative avian haemolysin F but thereafter described as a short-chain dehydrogenase/reductase enzyme according to Murase *et al.*, 2016) and *iutA* (ferric siderophore receptor), are the most significantly associated genes within the large sample of APEC isolates in their study.

1.1.4 Colibacillosis impact on the poultry industry:

Chickens of all ages are susceptible to colibacillosis, but it has been found that the severity of the disease is greater in younger chickens (Barnes *et al.*, 2013). It affects both the breeder and layer industries, leads to decreased productivity in layers and can lead to bird mortalities. Antibiotic resistance and bans on antibiotic use in the poultry industry, due to public safety concerns, can lead to outbreaks of colibacillosis, with great economic impact, leaving few alternative treatments available for use (Barnes *et al.*, 2013).

1.2 Antibiotics: from celebrated discovery to imposed restrictions

1.2.1 History:

The production of the antibiotic penicillin from the fungus *Penicillium notatum* was discovered by Alexander Fleming in 1928 (Assadian, 2007). While lethal to bacteria, penicillin was found to be non-toxic to animal and human tissues, resulting in the development and wide use of the penicillin-based “miracle cure” treatment during the Second World War. This naturally led to increased research in antibiotics and their consequent widespread use over the years toward the benefit of human and later animal health against infections.

A study by Moore and colleagues (1946) revealed accelerated growth of chicks that were fed streptomycin at sub-bactericidal levels along with their feed. They proposed that this was due to the inhibition of toxin-producing bacteria or bacteria that compete with the bird for nutrients, such as vitamins (Bird, 1969). This was followed by further demonstrations of the growth-promoting effects of antibiotic supplementation, which fuelled the extensive use of antibiotics as growth promoters in the agricultural industry.

The large-scale use of antibiotics as growth promoters, as well as their incorrect and irresponsible use to prevent and treat disease outbreaks in the poultry industry, however, have led to the selective

generation of antibiotic-resistant bacteria (Luangtongkum *et al.*, 2009). Consequently, the large-scale mismanagement of various antibiotics has led to multiple antibiotic-resistant strains, representing a significant problem for the poultry industry, as well as for human health.

As a result, bans against the use of certain antibiotics in the poultry industry are being imposed. One such example is the ban of fluoroquinolones in poultry by the Food and Drug Administration in the United States of America in 2005 (U.S. Food and Drug Administration, 2012). The ban was imposed due to increased resistance to fluoroquinolone by *Campylobacter* sp., which is a commensal bacterium in poultry but a human pathogen (Price *et al.,* 2007). The acquired resistance impaired the treatment of people suffering from *Campylobacter* sp*.* infection. Similarly, the European Union imposed a ban on the non-therapeutic use of antimicrobials in animal feeds, which came into effect in 2006 (European Union, 2005). It is thus clear that the discovery and development of alternatives to antibiotics are becoming more urgent.

1.2.2 Antibiotic resistance: mechanisms and origins:

Bacterial resistance mechanisms toward antibiotics can either involve physical, protection or substitution of molecular targets of the antibiotic, antibiotic exclusion and / or expulsion from the cells; or enzymatic detoxification of the antibiotic (Bennett, 2008).

Bacteria gain resistance mechanisms to antibiotics genetically, either through adaptation or acquisition (Brüssow *et al.*, 2004). Bacteria can rapidly adapt genetically to gain resistance to toxic compounds including antibiotics, as they have been doing during their existence in nature. They can achieve this by means of convergent evolution within a population and through selectively regulated sequence amplification (Laehnemann *et al.*, 2014).

In addition, bacteria regularly exchange resistance-related genes through horizontal transfer of genetic material such as genes or chromosomal fragments via plasmids, transposable elements, bacteriophages or integrons from other bacteria. This transfer is achieved by means of transduction, transformation, and particularly conjugative events (Bennet, 2008). Furthermore, this transfer is not limited to intra-species transfer, as it is well established that bacteria are also capable of interspecies horizontal gene transfer (Courvalin, 1994).

Bacteriophages (Balcazar, 2014) and phage-related mobile elements (Brown-Jaque *et al.*, 2015) are additional vectors for transfer of antibiotic resistance genes (Penadés *et al.*, 2015). Despite being insusceptible to antibiotics, phages have been shown to frequently carry various genes capable of conferring antibiotic resistance to the bacterium which they infect. Such genetic content is carried on mobile genetic elements, and are prone to donation to and uptake by bacterial hosts. Indeed, it has been further demonstrated that these genes are transferred to bacteria and lead to acquired resistance (Balcazar, 2014). Strikingly, bacteriophages extracted from environments with little to no

contamination by human-distributed antibiotics still possessed antibiotic resistance genes, potentially indicating bacterial evolution of these genes independent of exposure to human-spread antibiotics; prior to uptake of these genes by the phages (Muniesa *et al.*, 2013). Nevertheless, the spread of antibiotic resistance to bacterial hosts by phages is amplified by the presence of antibiotics (Ross & Topp, 2015).

Aside from transference of resistance genes directly to bacterial hosts, lytic phages can also contribute to horizontal gene transfer by lysing plasmid-containing bacterial cells, thereby releasing resistance gene-containing plasmids into the environment for potential uptake by other bacterial cells (Keen *et al.*, 2017).

Therefore, through means of chromosomal mutations, uptake of mobile genetic elements carrying resistance genes from other bacteria, transfer of resistance genes from phages, chromosomal recombination events, and combinations of these processes, bacteria can rapidly adapt to unfavourable conditions such as the presence of toxic compounds. The development of antibiotics therefore needs to evolve at a similar rate, or effective alternative treatments need to become readily available.

1.2.3 Development of new antibiotics:

Recent developments in the field of antibiotic discovery have been aided by the work of Ling and colleagues (2015), who have developed a method of growing previously unculturable bacteria. This development is the iChip, which enables soil microorganisms to grow separately from other microorganisms in their natural habitat. Results have shown up to 50 % of the iChip displaying growth compared to the 99 % unculturable bacteria that is known to be present in soil. This approach may contribute to the discovery and production of potential new antibiotics.

Despite such innovation, the actual development of new antibiotic compounds is not the only limiting factor contributing to the lack of new antibiotics. The FDA approved 56 % less novel antibacterial agents during the period 1998–2002 than it did during the years 1983–1987; with no new agents approved in 2002 (Spellburg *et al.*, 2004). This is compounded by the fact that it takes at least 8 years to develop novel antibiotics and get them onto the market (Institute of Medicine, 2003). Furthermore, the proportion of new antibacterial agents being developed by pharmaceutical companies relative to their total products was found to be at an alarming 1 %, as disclosed by 7 of the world's largest biotechnology companies (Spellburg *et al.*, 2004). While there are smaller companies that have products in the pipeline for FDA approval, there is a clear deficit of novel antibacterial agents that are available on the market, emphasizing the need for alternative treatment options considering the impending post-antibiotic era.

One of the main reasons for the lack of antibiotics being developed is the time (realistically 10 years or more) and financial (approximately US$ 0.8 million) costs which go into development of a new

drug from research and development to release onto the market (Conly & Johnston, 2005). This extensive process was comprehensively outlined by Hughes and Karlén (2014). Taken together with the low probability of the product finally reaching the market, and beyond that the product actually being successful on the market; results in a fear of failure to achieve return on investment, which likely deters companies from actively searching for new antibiotics. Thus, there either needs to be an incentive for companies to continue research into novel antibiotics and their mechanisms or a funding programme to allow companies to develop new antibiotics without concerns over economic loss (Höjgård, 2012).

1.2.4 Surveillance, monitoring and stewardship of antibiotics:

After bans were implemented by Denmark in 1995 against antibiotic use in agriculture (DANMAP, 1997), there has been constant monitoring by Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (DANMAP). DANMAP is responsible for the national surveillance of antimicrobial consumption and resistance in bacteria from animals, food and humans and has been active for the past two decades. Their first report, in 1996 (English version published in 1997), covered the antimicrobial resistance of human and animal pathogenic, zoonotic and indicator bacteria from the previous year. There are other antimicrobial resistance monitoring programmes such as NORM-VET (Norway; http://www.vetinst.no/), SVARM (Sweden, http://www.sva.se/), CIPARS (Canada, http://www.phac-aspc.gc.ca/cipars-picra/index-eng.php), JVARM (Japan, http://www.maff.go.jp/nval/tyosa_kenkyu/taiseiki/monitor/e_index.html), NARMS (United States, https://www.cdc.gov/narms/), GERM-VET (Germany), NETHMAP/MARAN (Netherlands) and ITAVARM (Italy). A difficult decision needs to be made with regards to antibiotic therapy; either the large-scale administration of antibiotics in poultry and other livestock should be completely banned, which could possibly lead to lower yields and quality of food for the consumer; or continuation of the use of antibiotics, potentially increasing the cases of nearly untreatable bacterial diseases in humans and animals. This also has implications on food prices, as antibiotics are currently in use in many countries to improve production in various livestock industries (Butaye *et al.*, 2003). While it is improbable to completely eradicate antibiotic resistance, it is possible to retain the use of antibiotics by not promoting the acquisition of further resistance by bacteria. This requires stewardship over antibiotic use, as a coordinated, multidisciplinary approach including the efforts of scientific researchers, veterinarians, agricultural industry, food animal producers, medical doctors and importantly, the general public (Goff *et al.*, 2017).

1.2.5 Potential alternative treatment and prevention options:

The development of alternative treatments to antibiotics is a valuable approach to alleviate the reliance on antibiotics. In the following sections, the potential use of vaccination strategies, bacteriophage therapies and endolysin treatments, as well as antibody therapy to combat APEC will be discussed.

1.3 Vaccines

1.3.1 Background:

Vaccines consist of agents that can elicit immune responses in a host, intended for proactive protection against future infections, during the process of immunization (Madigan & Martinko, 2006). Vaccines can be prepared from inactivated causative agents of diseases such as bacteria and viruses, or alternatively a synthetic substitute can be used. First-generation vaccines make use of attenuated or deactivated / dead pathogens to elicit protective immune responses (Alarcon *et al.*, 1999). With the continual development of genetic engineering techniques, much safer and more specific second-generation vaccines have been developed. These vaccines include sub-unit vaccines and genetically modified organisms (Alarcon *et al.*, 1999). Various vaccines exist for the prevention of disease in farm animals, although so far second-generation vaccines are mainly used for viral rather than for bacterial infections (Meeusen *et al.*, 2007). Theoretically, vaccines against viruses are more effective, as antibodies produced against viral antigens have the capability of neutralising the viral particle, rendering it non-infectious. However, with bacteria the antibodies merely mark the cells for phagocytosis and to attach complement, which is not nearly as rapid as with viral neutralisation (Robbins *et al.*, 1996). More specific bacterial vaccines, such as sub-unit vaccines, can contain several antigens to illicit a specific immune response which can neutralise toxins and mark the bacteria and therefore lead to a safe and efficacious vaccine against a bacterial pathogen (Strugnell *et al.*, 2011).

Major advantages of using vaccines in production animals include an improvement in animal health combined with a decreased reliance on antibiotics as growth promotors, preventing carry-over of antibiotics into humans (Nisha, 2008). Various vaccines are currently available for use in animal production consisting of different antigens and formulations for the respective animal species.

1.3.2 Vaccine use in the poultry industry:

Important diseases controlled by vaccination include Marek's disease, infectious bronchitis, Newcastle disease, salmonellosis and colibacillosis, among other diseases (Marangon & Busani, 2006; Gregersen *et al.*, 2010). Vaccines may also prevent the spread of emerging pathogens with zoonotic potential such as avian influenza (Marano *et al.*, 2007). Avian pathogenic *Escherichia coli*, as discussed, also have zoonotic potential (Ewers *et al.*, 2007; Ewers *et al.*, 2009). Vaccines in the poultry industry therefore play important roles in both flock health and potentially to prevent human diseases.

Aside from different types of *E. coli* vaccines available (live, inactivated and subunit; comprehensively reviewed by Ghunaim *et al.*, 2014), there are also different routes of vaccination. Practically feasible routes for simultaneous immunization of thousands of broilers are

required, with particular focus being paid to vaccine addition to feed or drinking water, or via aerosol spray. In this line, interesting research was conducted on the development of genetically altered corn that produces recombinant *E. coli* antigens, thereby achieving oral vaccination together with feeding (Lamphear *et al*., 2002; Streatfield *et al*., 2002).

Even when high efficacy vaccines are used, good hygienic practices and biosecurity are still required to maintain the health of the flock by preventing the introduction of other pathogenic organisms or genetically different strains for which the vaccine is ineffective (Velkers *et al.*, 2017). Bragg (2004) conducted a study during which vaccinated and unvaccinated chickens were challenged with *Avibacterium paragallinarum* in an experimental setup which included a control layout without continuous disinfection and a layout where a continuous disinfection programme was in place (Bragg, 2004). Less severe symptoms were observed in all experimental challenges that received the continuous disinfection programme and in some cases the duration of infection was reduced, showing the importance of improved hygienic practices even when vaccination programmes are in place.

1.3.3 Commercial *E. coli* vaccines for animal production:

The *E. coli* O157 bacterial extract vaccine (Epitopix) is marketed for use in bovine species to reduce infection and prevent the spread of *E. coli* strain O157, which is pathogenic to both bovines and humans (Armstrong *et al.*, 1996; Elder, 2000). The antigens present in the vaccine are siderophores and porins derived from *E. coli* O157 (http://epitopix.com/prod-cattle-ecoli). Nobilis® *E. coli* inac (MSD Animal Health) is an inactivated vaccine for the passive immunisation of broiler chickens against colibacillosis through the vaccination of broiler breeders. The antigens present are *E. coli* flagellar antigen (FT) and fimbrial antigen (F11) (MSD Animal Health, 2011).

The first commercially available modified live vaccine against *E. coli* is Poulvac® *E. coli* (Zoetis, 2017), which is intended for use in broilers, breeders and layers in both chickens and turkeys. The gene *aroA* was deleted from an APEC strain, rendering it avirulent while retaining the ability to stimulate protective immunity against various APEC serotypes through the presence of pathogen-associated molecular patterns (PAMPs).

1.4 Bacteriophages

1.4.1 History:

In 1917, Fèlix Hubert d'Hèrelle observed *Shigella* sp. cells being lysed in a broth culture. The bacterial lysate containing the virus-like causative agent, later termed bacteriophages, was used to treat dysentery (Ackermann, 2003). This was the first reported therapeutic use of bacteriophages. The discovery seemed ideal since the treatment killed bacteria without any negative effect on the

host. However, with the advent and development of antibiotics in the antibiotic era, research on bacteriophage therapy nearly came to a standstill, although in 1970, the Society of Friends of d'Hèrelle, was founded to continue bacteriophage research (Alisky *et al.*, 1998).

Through the years 1981-1986, Dr Stefan Slopek in Wroclaw, Poland, started investigating the use of bacteriophage therapy in clinical trials to treat human patients infected with antibiotic resistant bacteria (Ślopek *et al.*, 1987). With Western researchers preoccupied with antibiotic-research, bacteriophage research was confined to other countries like the former Soviet Union and Eastern Europe (Sulakvelidze *et al.*, 2001). The recent surge in antibiotic resistance has however seen a return of interest in bacteriophage therapy in the rest of the world, with renewed attempts toward phage therapy by the Western world starting around 1980 (Clokie & Kropinski, 2009). In a pioneering study, Smith and Huggins (1982) successfully eradicated an experimentally-induced *Escherichia coli* infection in a mouse infection model.

1.4.2 The importance of bacteriophages in disease-causing bacteria:

As mentioned earlier, phages are among transgenic elements similar to plasmids, transposons and genetic islands. This is due to features of their genomes that allow for easy exchange of genetic information; especially when integrated into the host genome (Wagner & Waldor, 2002). One of the chief factors of genetic exchange between bacteriophage and their host is the acquisition of toxicity and / or immune evasion mechanisms by the host. Studies have shown that bacteriophages not only encode for toxicity genes such as *tst* (toxic shock syndrome toxin) and *bor* (serum resistance lipoprotein), but also serve as a vehicle for these genes between host cells (Wagner & Waldor, 2002). A very early demonstration of the transfer of virulence genes by bacteriophages was the observed conversion of avirulent strains of *Corynebacterium diphtheriae* to virulent strains by infection with bacteriophages (Freeman, 1951). It has been hypothesised that the APEC-associated virulence gene *iss* is derived from the bacteriophage-encoded *bor* gene due to their highly homologous sequences (Johnson *et al.*, 2008a). It is thus likely that bacteriophages play a role in virulence gene evolution and transmission as transgenic elements in APEC.

1.4.3 Bacteriophage Therapy: Alternative to Antibiotics:

The multiple antibiotic resistant bacteria of the 1990s were a possible sign of an impending post-antibiotic era and in 2014, the World Health Organization published a report acknowledging the threat of antibiotic resistance around the world (WHO, 2014). Potential alternatives to antibiotics for the treatment of bacterial diseases are therefore required. One such alternative could be the therapeutic usage of bacteriophages. An ideal replacement therapy to cure bacterial diseases must be highly effective while having no toxic effects to the host. Bacteriophages, unlike antibiotics, have shown few toxic effects in hosts except for some rare reversible allergic reactions (Alisky *et al.*, 1998). There are however, some concerns about the non-linear pharmacokinetics of bacteriophages

when used as therapeutic agents (Tsonos *et al.*, 2014b).

1.4.4 Lysogenic phages:

The main different lifecycles of bacteriophages are the lytic and lysogenic lifecycles (see also A.1. in Appendix A). In the former, the host cells are infected by the bacteriophage which in turn reroutes its replication mechanism, leading to assembly of virions that upon maturation are released from host cells, usually by lysing the host cells (Haq *et al.*, 2012). This lifecycle is ideal for bacteriophage therapy due to the inherent abilities of bacteriophage to infect and lyse host. Naturally, the challenge is to identify and isolate phages which can be used against pathogenic bacteria. This can be time-consuming, although recent technological developments have made screening for bacteriophages easier (Gillis & Mahillon, 2014).

The lysogenic lifecycle is a mean by which bacteriophages primarily aim to preserve their genomic data (Weinbauer & Suttle, 1996). In this pathway, bacteriophages infect the host cell and the viral genome is integrated into the genome of the host (Mittler, 1996). The phage genome is then replicated along with the host genome until an environmental trigger, such as DNA damage, induces genome excision and viral replication (Osterhout *et al.*, 2007). Thus, the lysogenic lifecycle can be induced to become lytic, but some temperate phages that have a lysogenic lifecycle will be able to revert to lysogeny upon environmental stability through reversible active lysogeny which allows for the phage genome to reintegrate into the host genome (Feiner *et al.*, 2015). Therefore, temperate phages are not useful for therapy, unless genetic manipulation can result in maintained lytic lifecycles. Understandably this is an immense amount of work, which is less favourable than identifying alternative sources of lytic phages.

1.4.5 Possible shortcomings and adverse effects of bacteriophage therapy:

Allergies to antibiotics in humans are common and can lead to effects such as tissue damage (Khalili *et al.*, 2013; Weisser & Ben-Shoshan, 2016). Naturally, due to historic priorities, there is a much larger database of clinical trials for antibiotics in comparison to bacteriophage therapy. Therefore, the potential and severity of side-effects during bacteriophage therapy is not well established. It has been speculated that the crude lysates used in some bacteriophage therapies could be the cause of allergic reactions in humans due to the immunogenicity of released cellular components (Henein, 2013). Indeed, bacteriophage-released bacterial toxins and cellular components such as Gram-negative lipopolysaccharide (LPS) layer can be potent immunostimulators and inflammation response stimulators leading to side effects for the host, although such side effects are not uncommon with antibiotic treatments either (Drulis-Kawa *et al.*, 2012).

Additionally, although it has been demonstrated that certain phages preferentially attack plasmid-containing bacterial cells, which may in turn mean cells more prone to antibiotic resistance

(Davidson & Harrison, 2002); there is recent evidence that indicates that plasmids survive lytic events and become available for uptake by other bacterial cells (Keen *et al.*, 2017). Such "superspreader" phages would therefore potentially contribute to overall antibiotic resistance (Keen *et al.*, 2017).

Another potential disadvantage is the narrow host range of bacteriophages, meaning that some pathogens might not be susceptible to bacteriophage infection (Weinbauer, 2004). This specificity also necessitates improved diagnosis of disease in poultry, as phages specific for treating certain infections (for example colibacillosis) will be ineffective against other infections (for example *Salmonella* infections). This contrasts with the common broad-spectrum nature of antibiotics. If the specificity of a bacteriophage mixture is broadened by using phage cocktails containing a wider variety of bacteriophages, there may potentially be undesirable consequences for normal host microbiota. This is however also encountered with antibiotic treatment. In a recent study, Kauffmann *et al*. (2018) have shown that dsDNA non-tailed bacteriophage–under the proposed name of *Autolykiviridae*–have a broad host range compared to tailed viruses. The *Autolykiviridae* on average kill 34 hosts in four *Vibrio* species of the Vibrionaceae bacteria while tailed viruses kill only two hosts in one species. The *Autolykiviridae* offer a broad-spectrum target which is ideal for therapeutic use. This validates the broadened search for bacteriophages outside of the dsDNA tailed bacteriophages which can be used successfully as bacteriophage therapy options.

1.4.6 Bacteriophage resistance:

As with antibiotic resistance, bacteriophage therapy is also plagued with resistance development by the bacteria. Quorum sensing in bacteria has been shown to induce antiphage mechanisms which enable cells to be phage receptor-free for certain periods which ensure the survival of resistant cells (Høyland-Krogsbo *et al.*, 2013). One such mechanism is the prevention of phage adsorption/attachment by blocking the phage receptors on the cell surface as well as phase variation which alters the cell surface as with *Bordetella* spp. which are able to switch between Bvg + and Bvg - phases to be phage susceptible and resistant respectively (Labrie *et al.*, 2010). *Staphylococcus aureus* produces protein A which has been found to prevent adsorption of bacteriophage to the receptor (Nordström & Forsgren, 1974); where mutants which produced less protein A had increased bacteriophage adsorption when compared to their counterpart mutants which produced more protein A.

Phage DNA entry into the bacterial cell post-adsorption may also be blocked, such as in the case of superinfection exclusion (Sie) protein systems, which stop the phage DNA from entering the host. Numerous Sie systems have been found associated with prophages and it may be geared for phage-phage interactions (Labrie *et al.*, 2010). Furthermore, restriction-modification systems (R-M) utilize restriction endonucleases to degrade unmethylated foreign / phage DNA before methylation by bacterial methylase can occur (Weigele & Raleigh, 2016). There are also abortive infection systems by which infected cells sacrifice themselves for the sake of the rest of the bacterial

population (Örmälä & Jalasvuori, 2013). These mechanisms are comprehensively reviewed by Allocati and colleagues (2015).

A highly interesting mechanism occurs in *Staphylococcus aureus* for example, involving pathogenicity islands that can interfere with phage reproduction and parasitize the phage (Ram *et al.*, 2012). Basically, the bacteria hijack the already hijacking phage, through packaging of the genetic material of the pathogenicity island in phage particles, replacing phage DNA (Ram *et al.*, 2012). The cells still lyse, but genes for *S. aureus* virulence, antibiotic resistance etc. are spread by the compromised phages, instead of phage-encoding genes (Ram *et al.*, 2012; Shousha *et al.*, 2015).

An adaptive immunity-based resistance mechanism that has received considerable recent attention is the CRISPR-CAS system, consisting of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated (CAS) genes / proteins, previously thought to be limited to vertebrates (Marraffini, 2015). In a simplified overview, unique recognition sequences of viruses (and in some cases other mobile genetic elements) are incorporated as spacers into CRISPR sequences, thereby storing this information for use in recognition of similar viruses later (Karginov & Hannon, 2010). The resultant CRISPR regions are transcribed and expressed, and in response to foreign DNA matching the stored spacers, interference occurs by destruction of the recognized foreign DNA by crRNA and Cas proteins. These processes were comprehensively reviewed by Rath and co-workers (2015).

Of course, in response, viruses have in turn developed anti-CRISPR strategies (Wiedenheft, 2013; Bondy-Denomy *et al.*, 2015). The ability of phages to produce proteins capable of interfering with CRISPR-Cas complex formations or components has been demonstrated (Bondy-Denomy*et al.*, 2015). An astonishing discovery was made when viruses encoding their own CRISPR-Cas system that targets a phage-inhibitory genetic island within the host (Seed *et al.*, 2013). A cruder mechanism simply relies on mutations to the spacer recognition sequence, thereby reducing binding affinity (Semenova *et al.*, 2011).

As pathogenic bacteria and their hosts are in constant attempts to out-strategize each other on a molecular level, so do bacteriophages and their host bacteria. In fact, it is even more so between the latter pair, due to their rapid evolutionary rates.

1.4.7 Advantages of bacteriophage therapy:

Unlike antibiotics, bacteriophages evolve naturally alongside their bacterial hosts (Hendrix, 1999). Therefore, phage resistance seems to be a more temporary, potentially self-solving problem than antibiotic resistance. In addition, due to the host-phage relationship specificity, resistance to one bacteriophage would likely still mean susceptibility to different bacteriophages, whereas resistance to antibiotic classes can develop (Loc-Carrillo & Abedon, 2011; Nóbrega & Brocchi, 2014; Shaikh *et*

al., 2015). This high specificity for the host also results in safety of the treatment toward the host microbiota. Another advantage of bacteriophage therapy is that it has been demonstrated that when administered intravenously, bacteriophages can be found in nearly all organs (Dabrowska *et al.*, 2005), which is ideal when treating localized infections in different parts of the body.

Viruses, of which phages constitute a significant portion, represent the most abundant biological entities in the biosphere, and phages are routinely isolated from a diversity of environmental sources (Breitbart & Rohwer, 2005). Bacteriophage numbers are self-sustaining, as they reproduce rapidly during their lytic life cycle, allowing for exponential growth in numbers (Carlton, 1999). Some bacteriophages can release approximately 100 new bacteriophages on average per lytic infection cycle, which takes about 25 min in the case of the bacteriophage T4's lifecycle (Madigan & Martinko, 2006). This means that there will be approximately 0.1 billion bacteriophages after the fourth replication cycle. Bacteriophages themselves have been found to be non-toxic to the host during therapeutic use and cause low occurrences of fully-reversible allergic reactions, making bacteriophage therapy potentially very safe (Alisky *et al.*, 1998; Jończyk-Matysiak *et al.*, 2015).

1.4.8 Current bacteriophage-based products:

The majority of the developed bacteriophage-based products are used in food, animal or surface applications, although there have been clinical trials in which bacteriophage products have been used in human treatment (Drulis-kawa *et al.*, 2012). One such trial was performed using a bacteriophage preparation (Biophage-PA) which was used on antibiotic-resistant *Pseudomonas aeruginosa* in chronic otitis or ear inflammation in humans (Wright *et al.*, 2009). The bacteriophage was applied directly to the biofilm and successfully degraded biofilms, whereas antibiotics cannot destroy biofilms.

There have been several companies globally that have created or are in the development process of bacteriophage-based products. These include: AmpliPhi Biosciences (US), Enbiotix (US), Fixed Phage (UK), Intralytix (US), Novolytics (UK), Pherecydes Pharma (FR), Sarum Biosciences (UK) and Technophage (PT) amongst others. Some products, such as the LMP-102 phage cocktail (approved for use in 2006 by the FDA), have been approved to be used on ready-to-eat meat and poultry products to guard against *Listeria monocytogenes* (Lang, 2006). Another example is the biopesticide "AgriPhage", which was registered with the EPA in 2005 and contains bacteriophages of *Xanthomonas campestris* pv. vesicatoria and *Pseudomonas syringae* pv. tomato (Parracho *et al.*, 2012). This product is to be used on tomato and pepper crops against the bacteria that cause spot disease in these plants.

1.4.9 Initial development of bacteriophage therapy of APEC:

The ideal objective of APEC bacteriophage therapy would be to use bacteriophages that specifically

target avian pathogenic strains of *E. coli* and not strains from the normal microbiota, including non-virulent strains of *E. coli*. To do this, the phage would need to target factors specifically influencing the virulence of APEC. For instance, if a phage could bind to a cell wall protein such as the protease encoded by *ompT* (Grodberg & Dunn, 1988), often associated with pathogenic strains of *E. coli* (Maluta *et al.*, 2014; Hejair *et al.*, 2017), these proteins could then be used by bacteriophages to adhere to the *E. coli* cells by their tail fibres, infect the cell and lyse the cell during phage replication. This again highlights the importance of identification and understanding of virulence genes in the host, in this case APEC, to distinguish between potential pathogens and the normal microbiota.

1.4.10 Bacteriophage treatment studies done on poultry:

In a study on immune interferences, it was found that the poultry gained some immunity to the bacteriophage after the first treatment, which decreased efficacy in secondary treatments. It is possible that the bacteriophage in question (designated SPR02) is highly immunogenic, and that other phages may elicit a lower immune response (Huff *et al.*, 2010). Nevertheless, follow-up treatments may use different bacteriophages to overcome this problem. Immunity to phages in poultry is unlikely to be a major problem in the broiler industry, as the broiler birds are at slaughter weight around 34 days of age. Treating an initial *E. coli* infection should therefore be enough to increase yields in poultry production by decreasing the incidence of disease with only one treatment.

Different case studies may lead to contradicting findings, depending on the phages used and the methodology followed. For instance, Oliveira *et al.* (2010) isolated bacteriophages from sewage samples in poultry houses in Portugal. When single-phage inoculums of bacteriophage were prepared and used to treat 8-day old chicks infected with APEC strain H839E in experimentally controlled rooms, it was observed that infection rates decreased by up to 43 %. The study concluded that the use of a bacteriophage therapy in their experiments were highly successful (Oliveira *et al.*, 2010).

In another study, Tsonos *et al.* (2014a) developed a bacteriophage cocktail consisting of four bacteriophages that were chosen based on their broad APEC host range, low cross-resistance and obligate lytic infection pathway. Chickens were infected with APEC strain CH2 capable of a 50 % mortality rate after seven days of incubation. Two hours post-infection, bacteriophages were administered intra-tracheally, intra-esophageally or by addition to the drinking water. No differences between the control and experimental groups were observed, even though the re-isolated APEC strain from the infected chickens were still sensitive to the phage cocktail.

While promising *in vitro* results can translate into promising *in vivo* results, as seen in the study by Oliveira *et al.* (2010), this is not always the case, as seen with the study by Tsonos *et al.* (2014a). This could be due to the *in vitro* environments of a complex growth medium that could be ideal for the proliferation of some bacteriophages, but will likely differ considerably from the conditions inside

a chicken circulatory system or organs. Specifically, immunogenic bacteriophages could also be neutralized by the host's immune system. The composition of the bacteriophage cocktails, the APEC strains that are targeted and the environmental conditions required for bacteriophages to proliferate in a host are therefore potential major setbacks for the development of effective APEC bacteriophage therapies.

1.5 Endolysins

As it has been established above, one of the chief challenges of phage therapy is the extreme specificity of the phage for their host. Thus, choosing a broader alternative such as heterologously expressed bacteriophage enzymes may be pursued.

In the final stages of bacteriophage replication in the lytic cycle, the new phages are assembled and packaged, and the new viral particles are ready to infect new host cells. At this stage, they must be released from their current host cells. This is usually achieved by enzymes mainly of bacteriophage origin that damage the bacterial peptidoglycan, either by targeting sugar bonds, peptides or amides in order to weaken the bacterial cell wall (Hermoso *et al.*, 2007). The enzymes responsible for this degradation are known as lysozymes, endopeptidases or amidases respectively (Fischetti, 2005). These types of enzymes are collectively referred to as endolysins, lysins or virolysins (see also A.2. in Appendix A). Despite variations in shape, size, mode of action and origin, they all recognise bacterial cell wall components and cleave specific bonds, resulting in a weakened cell wall that leads to cell lysis (Nelson *et al.*, 2012).

These enzymes are favourable candidates for use as treatments because they can be heterologously expressed, potentially be applied externally, and in some cases, show an ability to affect different bacterial cell walls (broader specificity). They also offer the potential advantage of pharmacokinetic linearity absent from entire phages, as these enzymes can be quantitatively administered and do not self-replicate. The specificity of lysins toward specific cell walls has been addressed by various research groups (Payne & Hatfull, 2012; Proença *et al.*, 2012; Keary *et al.*, 2013; Tišáková *et al.*, 2014). In order to understand lysins better and to develop potent lysins, chimeric lysins have been engineered, which can detect or lyse different bonds. This can lead to increased lytic activity and broader target spectrums regarding different bacteria (Roach & Donovan, 2015). The key to potential application of lysins lies within such genetic recombination, to tailor enzymes toward different needs and targets without incurring known resistance, as well as in their abilities to penetrate biofilms (Viertel *et al.*, 2014).

A product is available on the market, Staphefekt™ (Micreos), which is the active enzyme in products sold under Gladskin for the treatment of conditions such as eczema, rosacea, acne and skin irritation, which infect the intact skin (https://www.staphefekt.com/en/). The enzyme is able to affect

Staphyloccocus aureus infections, including methicillin-resistant *S. aureus* (MRSA), without any known resistance.

Another research avenue regarding endolysins is attempts to overcome the lipopolysaccharide (LPS) layer barrier during application of lysins to Gram-negative bacteria (Roach & Donovan, 2015). Although Gram-positive bacteria have the cell walls several micrometres thicker than Gram-negative bacteria, they lack the protective outer LPS layer (Silhavy *et al.*, 2010). The LPS layer consists of lipid A, oligossaccharides and the polysaccharide O antigen. This layer is also responsible for stimulating the immune system of hosts along with endotoxins, which also adds an incentive to developing endolysin usage against Gram-negative bacteria (Briers *et al.*, 2014). A potential drawback of endolysin use in Gram-negative bacteria however is that fragmented LPS layers may continue to be immunostimulatory.

Some Gram-negative endolysins have been fused with LPS layer destabilizing peptides such as a polycationic peptide, a hydrophobic pentapeptide, Parasin and lycotoxin (Roach *et al.*, 2008). These additional peptides have improved lysins that can be used for treatment from the exterior of the cells, and allows LPS layer penetration in the absence of additional chemicals (Briers *et al.*, 2014; Roach & Donovan, 2015). Different studies have established the use of endolysins against both or either Gram-negative and Gram-positive bacteria (Nelson *et al.*, 2001; Loeffler & Fischetti, 2006; Pastagia *et al.*, 2013; Schmelcher & Loessner, 2016). One such study is that of Dong and co-workers (2015), whose endolysin derived from *Stenotrophomonas maltophilia* has a high amino acid sequence homology to lambda phage gpR. The gene was overexpressed in *E. coli* and was demonstrated to have *in vitro* effects against strains such as *Bacillus subtilis*, *B. cereus*, *Staphylococcus aureus*, *Klebsiella mobilis* and *Shigella flexneri*, among others.

1.6 Other alternative treatment options in a post-antibiotic era

As stated previously, even as antibiotic monitoring continues in order to prolong the effectivity of current antibiotics there remains a need to find alternative or supplementary agents. The possibility of bacteriophage therapy and bacteriophage-origin solutions (such as the lytic enzymes) have been mentioned and discussed. It is important to note that this is not the only potential solution against antibiotic resistance. Here we mention some of the viable options that other researchers are investigating.

Other natural sources of potential antimicrobial agents under investigation include plant extracts (Elisha *et al.,* 2016), essential oils (Oh *et al.*, 2017), marine sources such as algae (de Jesus Raposo *et al.*, 2016; Shannon & Abu-Ghannam, 2016) and bacteriocins (Al Atya *et al.*, 2016). Another strategy is passive immunization or antibody therapy, in which antibodies (historically convalescent sera) specific for a pathogen are directly administered to an infected individual. IgY from chicken egg

yolk is an example (Chalghoumi *et al.*, 2009; Diraviyam *et al.*, 2014). Obtaining large amounts of IgY antibodies from chicken eggs for large scale application is, however, impractical. The rise of market for monoclonal antibody production for human use, including the development of more efficient production methods such as recombinant antibody production using microbial cultures (Ecker *et al.*, 2015), may offer opportunities for more feasible application in the poultry industry.

In conjunction with treatment strategies, it is important to strictly maintain effective biosecurity practices, as a first line of prevention against the spread of infections in poultry environments (Taylor *et al.*, 2016). Effective disinfectants and their correct methods of application need to be identified, and established disinfection practices need to be strictly adhered to maintain biosecurity, as resistance to disinfectants can also develop (Bragg *et al.*, 2014).

1.7 Conclusions

The rapidly increasing bacterial resistance to antibiotics is leading us ever closer to a frightening reality of catastrophic consequences for human health and food security, including the poultry industry. This review aimed to discuss some of the potential strategies to overcome antibiotic resistance, while also highlighting the need for more research towards realising feasible application of these strategies. Concerted efforts therefore need to be invested into such strategies to ensure that we are prepared for the possible eventuality of complete ineffectiveness of antibiotics. As more knowledge is gained with regards to the virulence genes and their mechanisms in APEC, novel methods of control and/or prevention can be developed to ensure continuous and economically feasible poultry production in the future. Alternative methods are becoming much more prominent and essential to ensure the health of flocks. Vaccination represents a good control measure, provided that effective vaccines are developed and are then effectively administered. Further development of recombinant subunit vaccines, particularly against bacterial infections, could play a crucial role in this process.

In the event of vaccine or vaccination failure however, treatment options for infected birds will be necessary. Various sources of antimicrobial compounds are receiving attention toward this end. Regarding bacteriophage therapy, the most realistic current approach to this may be based on the administration of a bacteriophage-cocktail, although this would require some preliminary evaluation of causative strains involved.

The world is becoming more aware of the effects of antibiotic resistance and how this affects us on the human, veterinary and scientific research platforms. Preserving the antibiotics that are currently resistance-free, discovering novel antibiotics, revisiting older antibiotics and expanding the world of antimicrobials are among the most relevant methods of prevention of a post-antibiotic era. An area of alternative treatment against bacterial infection is bacteriophages and bacteriophage-based

products. Lytic bacteriophage use against avian colibacillosis has shown positive results *in vitro*, however mixed results *in vivo* show that there will be stumbling blocks during the development of effective lytic therapies. The problem of bacteriophage therapies having non-linear pharmacokinetics creates problems during product formulations and therefore registration of these products for veterinary use has yet to be solved.

Other bacteriophage-based treatments have shown an increasing viability as shown by their use in the food industry on products that are ready for consumption as well as on raw vegetables. The numerous phage-based products on the market in the food industry have paved the way forward for future development and potential use of phage in different settings. This can be beneficial for their use in animal husbandry, poultry and agriculture. One of the biggest incentives of research and development in this field is the room for growth and expansion. As the different areas affected by antibiotic resistance emerge the applications for bacteriophage-based products increase, allowing for more products to be developed. Coupled to this is the use of different facets of bacteriophages like their cell-wall degrading enzymes. Endolysins pose an opportune treatment as they can be heterologously expressed; manipulated into chimeric forms that may be tailored to target cells of interest; have not found to incur resistance in bacterial cells and are able to be effective even in the presence of some biofilms. They could also reduce the lack of pharmacokinetic linearity of whole phages, as they can be quantitatively applied without any self-replication.

The alternative measures that have been discussed in this article reflect the need for creative approaches to the challenge of antibiotic resistance. A combination of vaccination strategies and alternative therapies in conjunction with good biosecurity measures, as well as stewardship of current and future antibiotics, could ensure that the poultry industry will continue to produce good quality products and ensure food security.

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Appendix A: Additional information to Chapter 1: Literature review

A.1. Bacteriophage lambda (λ)–gene regulation and the bi-switch

Gene regulation is important for cells to function optimally according to their roles (Bell *et al.*, 2011; Cheadle *et al.*, 2005; Tessitore *et al.*, 2014). It refers to the switching 'on' and 'off' of genes to either induce or inhibit expression via a process of transcription and translation. By regulating the processes of RNA and protein production this gives rise to different pathways in a cell. Phage lambda has been studied since its discovery by Andre Lwoff, Francois Jacob and Jacques Monod particularly for its inherent ability to regulate genes both in the host organism as well as its own genome (Norrby, 2008).

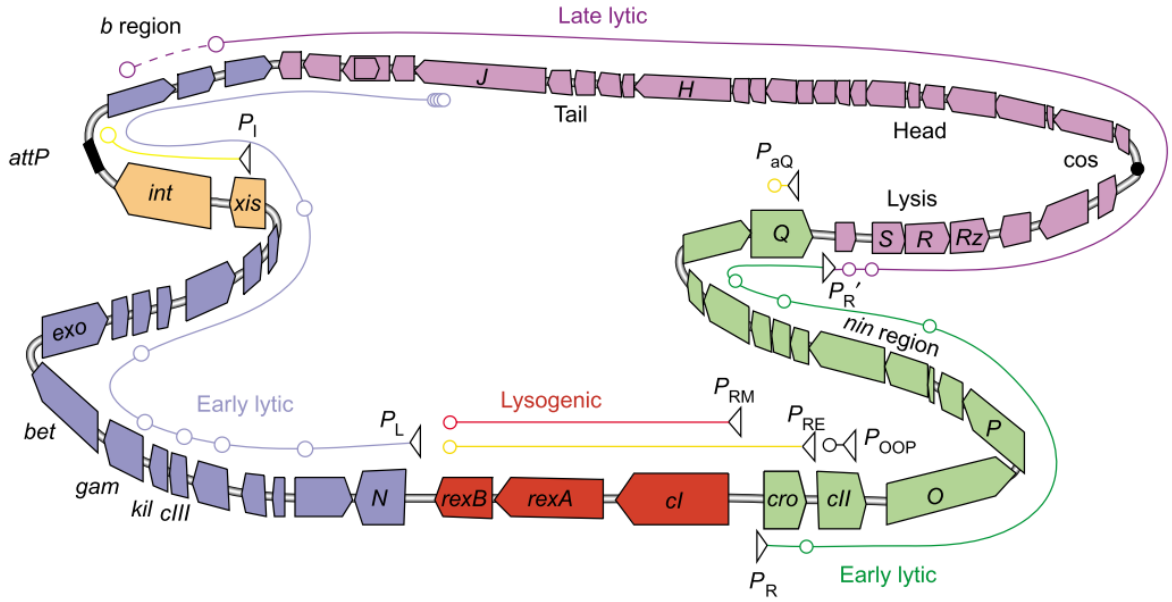


Figure A 1: An overview of the circular genome of bacteriophage λ. The lysogenic and early lytic gene groups that are the bi-switch are indicated in red and green boxes respectively. Several genes play a part in the role of confirming which direction the bi-switch will be transcribed, but for this study the focus is on the role of *cro* and *c*I. The proximity and adjacent placement of the genes display the duality of the gene functions in the bi-switch. At the end of bacteriophage virion production and maturation the genes involved in lysis of the cell (here in purple: S, R and Rz genes) are activated and lead to the production of holin, endolysin and cross-linking proteins respectively. (Adapted from Dodd *et al*., 2005).

Lysogeny is the phage lifecycle during which the lytic lifecycle is not induced and the viral nucleic acid is instead integrated into the host genome (Fortier & Sekulovic, 2013; Wittebole *et al*., 2013). The integrated phage is known as the prophage, while the prophage-containing host cell is referred to as the lysogen. Other forms of lysogeny include circular phage genome preserved within the host (Gill & Hyman, 2010). Temperate phages differ from virulent phages as they maintain lysogeny within the host more readily. In lambda (λ) phage there is a genetic bi-switch which is encoded by the *c*I/*cro*

genes which are responsible for determining lysogeny or lysis of the cell after it is infected (Dodd *et al*., 2005). This bi-switch is affected by a myriad of related enzymes / proteins that determine which lifecycle the phage will choose.

If physiological conditions are favourable, the temperate phage will revert to lysogeny and integrate its genome into that of the host cell or, in some cases, remain as circular replicons within the host (Weinbauer, 2004). They remain in this state indefinitely where they are maintained as part of the host cell. Lysogeny is affected once there is extensive host genomic DNA damage (Ptashne, 1987). In the case of *E. coli*, upon such damage the host's SOS repair system is activated and the phage genome is de-repressed, which are both achieved through the action of an enzyme RecA (Ptashne, 2011). When single-stranded DNA occurs due to damage, RecA binds to these lesions with the help of single stranded DNA binding proteins (SSB), and repairs the gaps (Cox, 2007). LexA is cleaved in the presence of RecA that is bound to DNA, resulting in declining LexA levels that in turn results in the de-repression of the SOS regulon, which is comprised of more than 40 genes in *E. coli* (Sanchez-Alberola *et al*., 2012). This allows expression of proteins involved in DNA repair and recovery (Little, 1993). Once the DNA repair is complete, RecA unbinds from the DNA and the levels of LexA rise to homeostatic levels and represses the SOS regulon genes again. RecA also cleaves the Cro protein from lambda prophage (Ptashne, 2011). The Cro protein from lambda is similar to LexA in that they are both present in their dimeric form which represses regulons, they are auto-regulatory and they are both cleaved by the DNA-bound RecA through autoproteolysis (Dodd *et al*., 2005). Upon the cleavage of dimeric forms to monomers, the repression is relieved and the proteins of the regulon are expressed (Brent & Ptashne, 1980). In this case it results in the conversion from the lysogenic state to the lytic, with replication of the phage genome and expression of proteins essential for virions to be packaged and lysis of the host cell for release into the environment.

Temperate phages often contribute to bacterial genetic fitness and regulation of bacterial behaviour, in such cases benefiting the host (Argov *et al*., 2017). Lysogenic integration into the host genome allows for gene transfer to and from the host. In this way temperate phages, upon becoming prophages, are able to alter the bacterial genome making them potentially more pathogenic (Gill & Hyman, 2010). Host specificity is determined by several factors: host membrane protein composition for phage attachment, host nucleic acid replicating machinery and phage mutation or variance (Brock, 1964; Stuttard, 1982).

Due to lack of lytic ability, as well as the transfer of notably pathogenicity-related genes to the host (Brüssow *et al*., 2004), make temperate phages impractical for use as therapeutic agents. Also, the host range may be too narrow and defined for temperate phage to be used for treatment of bacterial infections. Virulent phages on the other hand, can remain lytic and have a broader host range.

A.2. Endolysins and holins

Viral particles that are assembled and packaged are ready to infect new host cells following release from the initial host cells. This is usually achieved by bacteriophage enzymes that damage the bacterial peptidoglycan during the late stages of viral replication. These enzymes either target sugar bonds, peptides or amides in order to weaken the bacterial cell wall. The enzymes that are responsible for this degradation are lysozymes, endopeptidases or amidases respectively, which are usually coded for by dsDNA phages (Fischetti, 2005). These are collectively referred to as endolysins or virolysins. In lambda phage, this enzyme is coded by gene *R* for the R protein also known as endolysin or lysin (Bienkowska-Szewczyk *et al*., 1981). Endolysins are classified into different groups based on their site of catalysis on the peptidoglycan structure (Roach & Donovan, 2015). Glycosidases cleave between glycan residues; amidases cleave between N-Acetylmuramic acid (MurNAc) and the first L-alanine residue, which is conserved. Endopeptidases cleave between amino acid residues (Nelson *et al.*, 2012). Glycosidases can either cleave between MurNAc and N-acetylglucosamine (GlcNAc) (muramidases) or between GlcNac and MurNAc (glucosaminidases) (Roach & Donovan, 2015). Endolysins from Gram-positive infecting phages naturally differ from those of the Gram-negative infective phages. Typically endolysins from the former group have an enzymatically active domain (EAD) on the N-terminal and a cell wall binding domain (CBD) at the C-terminal. The Gram-negative phage derived endolysins are generally globular and do not have CBDs (Walmagh & Boczkowska, 2013; Walmagh *et al*., 2012). The endolysin encoded by the *R* gene in λ is a transglycosylase, not a murein hydrolase, which attacks the glycosidic linkages in the peptidoglycan resulting in a cyclic 1,6-disaccharide in the form of a terminal 1,6-anhydromuramoyl product (Bienkowska-Szewczyk *et al*., 1981). The R protein is a soluble endolysin predicted to be comprised of 158 residues, which accumulates intracellularly as it lacks a secretory signal sequence (Young *et al*., 2000). The *R* genes are in a cassette alongside *S* genes encoding holins, which assist in release from host cells. The efficiency of endolysins have been shown to have a binding affinity like that of an IgG antibody molecule, which suggests that they are likely needed in several molecules to weaken the cell wall effectively, as observed via a *Listeria* phage enzyme by Loessner and co-workers (2002). The *Rz*1 and *Rz*2 genes code for small proteins that cleave the cross-links in the peptidoglycan and those between the peptidoglycan and the outer membrane (Young, 1992).

While the mechanism of breaking through the cell wall belongs to the peptidoglycan-degrading endolysins, the timing and propulsion of lysis is regulated by holins. As the endolysins cannot permeate the cytoplasmic cell membrane barrier to reach the cell wall, it relies on holin proteins to create pores in the membrane through which endolysin can escape. Lambda S holins were found to usually occur in dimeric forms in a study that tracked the insertion of holin particles into the cell membrane by tagging the holin with green fluorescent protein (GFP) (White *et al*., 2011). As the dimers accumulate in the membrane they form a ‘raft’ (Figure A 2) which continues to expand (White *et al*., 2011). The lambda holin falls under class 1 holins, for which it is the prototype, and are usually

90 residues or more in length with two transmembrane domains (Smith *et al*., 1998) with the N-terminal domain in the periplasm and the C-terminal in the cytoplasm (Graschopf & Bläsi, 1999). Although there is a case for a three-transmembrane domain structure for class 1 holins (Gründling *et al*., 2000), it has been found that the λ holin more likely has two (Reddy & Saier, 2013; Saier & Reddy, 2015).

As important as its function, is the timekeeping role of these proteins, with these proteins often referred to as 'molecular clocks' due to their function during cell lysis (Wang *et al*., 2000). Endolysins accumulate intracellularly until there are enough holin-formed holes in the cell membrane (Krupovič & Bamford, 2008). The nature of the holin controls the rate of pore formation, and the survival of the lytic phage depends on this critical timing, as demonstrated by mutagenic studies on holin (Wang *et al*., 2000). Correct timing ensures that the intracellularly forming virions are not released prematurely; thereby ultimately guaranteeing the proliferation of phage. It has also been observed that the intracellular accumulation of holin proteins may lead to bacterial cells with decreased viability.

The *S* gene has two gene products, S105 (holin) and S107 (antiholin), denoted by the number of residues that they consist of (Chang *et al*., 1995). The two different length products are due the "dual-start motif", consisting of two start codons from which transcription can initiate, with S105 being translated from the third codon and S107 from the first codon of the gene (Smith *et al*., 1998). The codon shift causes a positively charged residue at position 2 in S107 as opposed to S105, resulting in an inactive holin. This was demonstrated by replacing this residue with a non-basic residue, which restored holin activity (Graschopf & Bläsi, 1999). Antiholin prevents pore formation until the appropriate time for lysis, at which point it contributes to pore formation with holin (Bläsi *et al*., 1990).

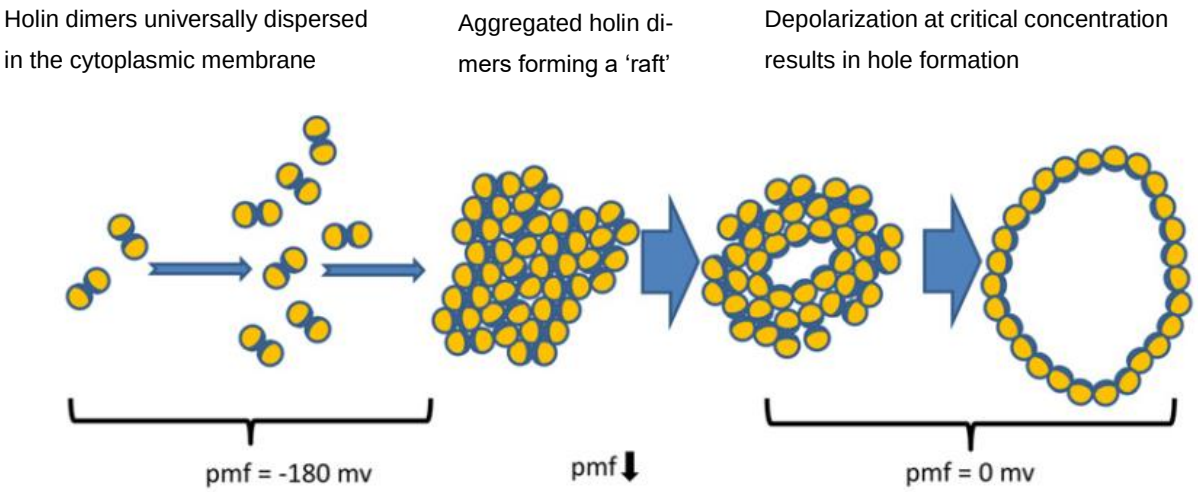


Figure A 2: Aggregation and holin timing for hole formation in the cell membrane. This top-down view shows the holin dimers aggregating in the cytoplasmic cell membrane. Hydrophilic faces of the holins are shaded in blue and are positioned inwardly in the raft formation. The raft is formed with hydrophilic faces positioned inwards and hydrophobic surfaces outwards. At the critical concentration of holin depolarization will begin and the raft releases the proton motive force of the cell membrane in the aggregated areas and a hole will form. (Adapted from White *et al*., 2011)).

The Rz proteins are also important for leakage of outermembrane permeability and lead to the formation of circular cells that have lost their rigid structure. However, it is the presence of holins and endolysin that accounts for complete cell lysis.

Chapter 2 Introduction to the present study

The escalating trend in antibiotic-resistance has been established over a short period of time and continues to rise causing surveillance programmes and bans to be established by different countries (Maragakis *et al.*, 2008). The potential of unmanageable global bacterial infections may lead to a post-antibiotic era or state which mimics that of the pre-antibiotic era with masses of people dying from untreatable infections (Alanis, 2005). In the past there have been differing opinions about whether the banned use of antibiotics as growth promoters in the animal food industry and surveillance programmes contributes to decreased antibiotic resistance in medically relevant bacteria (Casewell *et al.*, 2003; Phillips, 2007). However, some have reported that Denmark's approach to reducing antibiotic use in food animals since banning avoparcin in 1995 has resulted in decreased antibiotic usage without losses in the pig industry of Denmark (Levy, 2014). Through antibiotic resistance surveillance programmes (Goff *et al.*, 2017), education of the public (Enani, 2015), preservation of existing antibiotics and discovery and development of new antibiotics (Braine, 2011; Roberts *et al.*, 2012) there is an effort to extend the use of antibiotics. While keeping antibiotics relevant is necessary, there is a need for alternative treatments of bacterial infections to ensure that infection by pathogenic bacteria remains treatable and controllable. There are several different approaches to alternative treatments such as phytochemicals, essential oils, plant proteins and peptides, vaccines against pathogenic bacteria and phage therapy (Bull *et al.*, 2002; Cowan, 1999; Diarra & Malouin, 2014; Parisien *et al.*, 2008).

Phage therapy has a range of facets from whole lytic phage preparations to peptides of phages (Goodridge, 2010). For phage therapy, whole virulent bacteriophages that are obligately lytic are used because they are unable to enter lysogeny and thus result in the bacterial cells being lysed or nonviable (Loc-Carrillo & Abedon, 2011). Typically, bacteriophages specific for different bacterial strains are mixed in various 'phage cocktails' to broaden the spectrum of treatment (Chan *et al.*, 2013). These phage cocktails are a mixture of individual bacteriophages which are then tested to determine whether they are effective against a variety of pathogenic bacteria; this method is repeated as necessary for different bacterial infections (Örmälä & Jalasvuori, 2013). Temperate phages are not useful in phage therapy as they are not obligately lytic and do not always result in lysis of the bacterial cell. They can also contribute adverse or virulent factors to the bacterial genome as they integrate and exchange genetic elements e.g. structural genes for the cholera toxin (CT) are encoded by a filamentous bacteriophage CTXϕ, which is related to coliphage M13 (Waldor & Mekalanos, 1996). Yet with an estimated 50 % of environmentally isolated bacteriophages being temperate (Weber-Dabrowska *et al.*, 2016), there are many phages that are able to enter lysogeny and may be overlooked for use as phage therapeutics.

Another aspect of bacteriophage therapy that has been garnering attention over the last two decades and increasing in applications is bacteriophage cell wall hydrolases or bacteriophage endolysin

treatment (Callewaert *et al.*, 2011). With a wide variety of hydrolysing mechanisms and different types of cell-wall hydrolysis available (Hermoso *et al*., 2007), bacteriophage endolysins are choice elements because they can be heterologously expressed, tested against different bacteria and chimeric forms created to offer the most virulent or effective endolysins (Pires *et al.*, 2016; Walmagh *et al.*, 2012). Although resistance against some bacterial cell wall hydrolases, such as lysozymes, have been detected there has not yet been any resistance against bacteriophage endolysins that have been reported (Callewaert *et al.*, 2011). This does not exclude the possibility of resistance against bacteriophage endolysins being detected in the future, but currently adds to their list of benefits. Bacteriophage endolysins that are active against Gram-positive bacteria have been effective against such medically relevant bacteria such as methicillin- and vancomycin-resistant *Staphyolococcus aureus* (MRSA and VRSA), vancomycin-resistant *Enterococcus faecalis* and *Enterococcus faecium* (VRE), and penicillin-resistant *Streptococcus pneumoniae* (Loeffler *et al.*, 2001; Nelson *et al.*, 2001; Pastagia *et al.*, 2013). As Gram-negative bacteria have an outer membrane consisting of the lipopolysaccharide layer (LPS), there is a challenge to finding an endolysin that is effective from outside of the cell because the LPS layer creates a permeability barrier which prevents larger molecules to cross it freely (Nikaido, 2003).

Different approaches to helping the LPS layer lose its stability are available. Theoretically the LPS layer has a net negative charge due to its phosphate groups and sugars (Vaara, 1992). To stabilize this negative charge, divalent cations such as Mg^{2+} and Ca^{2+} ionically interact with the phosphate groups (Oliveira *et al.*, 2014). Chemical compounds such as polymyxin, and aminoglycosides compete with the divalent cations and displace them while trying to bond with the LPS layer (Briers & Lavigne, 2015). Other chemicals such as EDTA are chelators that bind with the divalent cations and remove them from the LPS layer (Briers *et al.*, 2011). Physical permeabilising methods such as high hydrostatic pressure (HHP) used in the food industry increased sensitivity of bacteria to bacteriocins, but only during the application of HHP and not afterwards which reduces its application (Masschalck *et al.*, 2001). There are also reports of endolysins which were able to elicit outer membrane permeabilising effects without an added permeabiliser (Briers & Lavigne, 2015; Dong *et al.*, 2015). The most recent development in this area are the Artilysins which are biological peptide-endolysin fusions that distort the LPS layer and result in auto-induced uptake of the endolysin (Briers *et al.*, 2014; Briers & Lavigne, 2015).

2.1 Aim of the project

The aim of this project is to investigate the induction of lambda prophage as well as produce bacteriophage lambda endolysin as a potential source of treatment against bacteria.

The objectives that are addressed are:

1. Use PCR screening for identification of lambda prophages followed by various prophage inducing methods for the lytic conversion of prophages in potential *E. coli* lysogens.
2. Extract bacteriophage genomic material and manipulate it for the removal of lysogenic genes.
3. Heterologous expression of bacteriophage lambda endolysin and holin in bacterial and yeast vectors.
4. Test expressed endolysins and holins against bacteria, with and without the presence of permeabilisers, to determine the potential for treatment.

Chapter 3 Temperate phage induction for use as a host-pathogen-specific treatment

3.1 Introduction

Broad-spectrum antibiotics that are generally used to treat bacterial infections have the side-effect of disturbing the microbiome of the colon, which is important for health (Dethlefsen *et al*., 2008). Once this ecology is disturbed, there is an increased chance of opportunistic pathogens colonizing the gut, which in turn prevents the beneficial microbiota from reforming a protective layer to reinforce host-bacterial commensalism. There is also the concern of antibiotic pressure present in the gut leading to acquired resistance in the bacteria, which can be passed onto subsequent generations or other bacteria via horizontal gene transfer (Jakobsson *et al*., 2010). It would therefore be beneficial for treatments to specifically target only the pathogenic bacteria responsible for infection, thereby aiding the establishment and persistence of bacterial commensals.

Bacteriophage genomes have regions of high genetic plasticity and are known to be one of the major contributors for genetic element transfer in bacteria along with plasmids and transposons (Tinsley *et al*., 2006). They contribute to genetic diversity and transfer of genes via lysogenic conversion genes known as morons (Brüssow *et al*., 2004). Aside from this, there is an organisation of structural and assembly genes that are conserved (Hatfull, 2008) which is essential for the bacteriophage to functionally maintain its genome. Bacteriophage lambda (λ) has been comprehensively studied and it is a model organism for molecular science (Casjens & Hendrix, 2015). Lambda was therefore chosen as the reference bacteriophage for this study; because the genome sequence has been well researched and is readily available, and its lifestyle mechanisms are well understood. The mechanism of lysogenic-lytic lifecycles of bacteriophages has been described by studying the phage λ bi-switch (Gottesman & Weisberg, 2004). The c*ro* (repressor) and *c*I (antirepressor) genes have been found to be the core genes in the 'decision-making' pathway. The CI promoter, P_{RM} (Promoter of repressor maintenance), and the Cro promoter P_R are orientated in opposing directions; with expression of the former favouring lysogeny, and expression of the latter favouring lysis (Schmeissner *et al*., 1980; Ptashne, 1987; Lewis *et al*., 2016), as seen in Figure 3-1.

The *cro* gene is about 200 bp in length and located between 38041–38241 bp and codes for the anti-repressor Cro (control of repressor and other genes). This region is adjacent to the gene *c*I which encodes the CI repressor protein, with each gene under the control of its own promoter. The CI promoter P_{RM} points polymerases 3′-5′ in the direction for transcription of CI and thus maintenance of lysogenic state, while the Cro promoter P_R points polymerases 5′-3′ in the opposite direction for transcription of Cro in favour of lysis (Schmeissner *et al*., 1980; Ptashne, 1987; Lewis *et al*., 2011) as seen in (Figure 3-1). Between the P_{RM} and P_R are the three operator sites that are composed of O_R1, O_R2 and O_R3 positioned adjacent to each other (Ptashne, 1987). Both CI and Cro function

exclusively in their dimeric forms, and are able to bind to these regions with opposite effects and both sets of proteins. The CI dimers have an inherent affinity for the operator sites in the format $O_R1 > O_R2 = O_R3$ (Ptashne, 1992), however since binding to O_R1 and O_R2 is cooperative, they are always simultaneously occupied by CI dimers. This occupation simultaneously ensures repression of Cro expression and lysis, and promotes P_{RM} for CI production. Excessive CI however results in CI dimer binding to O_R3, which leads to the inhibiton of further production of CI by blocking P_{RM} through a mechanism not covered in this review (further details available in Lewis *et al*., 2016; Lewis *et al*., 2011).

Repressor molecules are attracted to repressors that are already bound to an operator site and thus fill up the sites in a preferential sequence. This is a self-serving control mechanism which makes the presence of dimers maintain lysogeny. When the sites O_R1 and O_R2 are filled with repressor dimers they prevent Cro from binding and blocking RNA polymerase from transcription in that direction, directing it instead to transcribe in the opposite direction to create repressor RNA (Michalowski & Little, 2013). The opposite is true for Cro, as the operator site O_R3 has a higher affinity for Cro than the other two sites; essentially $O_R3 > O_R1 = O_R2$ (Deb *et al*., 2000). When Cro binds to O_R3 it directs RNA transcription in favour of itself and as more Cro binds, it ensures that the lysogenic state is repressed and the lytic cycle induced.

In a lysogen the CI dimers bind continually to the operator sites, and dimers that become unbound are replaced with others. The lysogenic state is maintained indefinitely unless SOS-response of the cell is initiated, e.g. through DNA-damaging shortwave ultraviolet (UV) light, which leads to phage induction. This SOS response alters bacterial RecA, a protein which is used to catalyse the recombination of DNA, to recognise and cleave a range of repressors that also includes the monomeric lambda repressor CI (Little, 1993). Cleaved CI repressors are unable to form dimers and consequently are unable to bind to the operator sites.

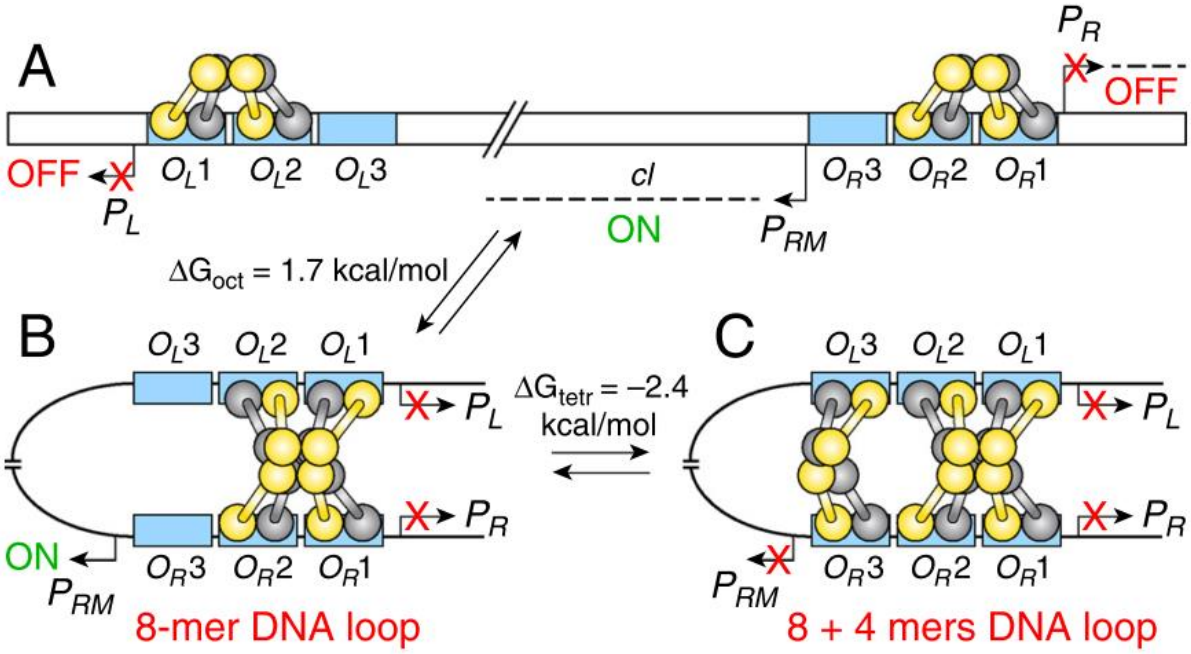


Figure 3-1: Regulation of PR and PL by CI repressor. (A) Promoters (PR, PL, and PRM); operators OL (OL1, OL2, OL3) and OR (OR1, OR2, OR3) are in blue rectangles; CI dimers (one monomer is shown in yellow, the other is in gray). The bent arrows show the transcription start points of promoters OL and OR they promote the transcription of more CI proteins. The cI gene is transcribed from PRM. (B) The CI dimers interact with each other until they reach octamer formation (8-mer) by CI tetramer binding to OL 1-OL 2 interacts with that at OR1-OR2 and creates DNA looping. C) Once the octamer and tetramer (forming a dodecamer) formation of CI binding to OL and OR have reached saturation the promoter is turned off (Red x at PRM). (Adapted from Lewis *et al*., 2011)

Thus, repressors fall away from the operator sites allowing for Cro molecules to bind. Coincidentally, when the repressors do not occupy O_R1 and O_R2, transcription of *c*I is stopped and repressors are no longer expressed. As soon as O_R1 and O_R2 sites are free, RNA polymerase binds and begins transcription of the *cro* gene at P_R together with the early lytic genes situated close to the *cro* gene (Schmeissner *et al*., 1980). The repressor is thus a negative regulator for lysis, but both a positive and negative regulator for lysogeny and transcription of *c*I. However, Cro is only a negative regulator and binds to the operator site with a preference for O_R3 and once this site is filled it is followed by O_R1 and O_R2 without further preference. Binding of Cro to O_R3 inhibits P_{RM}, and once all three sites are occupied, transcription of *cro* and the early lytic genes are stopped as RNA polymerase is blocked from recognising the promoter region P_R. This system is managed by the switch being flipped, once the levels of repressors decrease five-fold and unintentional induction is thus avoided. During intentional induction, the system is reversed and Cro is removed from the operator sites to allow production of early lysis genes.

Temperate bacteriophages are specific to their hosts as they specifically integrate into regions of the host genome or are maintained as free-standing intracellular circular nucleic acids (Argov *et al*., 2017). The focus of this chapter is to harness this host specificity to have bacteriophages that are

highly specific for pathogenic bacteria without disturbing the normal microbiota. The aim is to induce lysogens to promote lytic conversion of the phage. Once it has been established that the phage is inducible from lysogenic to the lytic phase, it is to be genetically manipulated to keep the host specificity of the temperate phage while promoting lytic activity.

Screening for *cro* (repressor), *c*I (anti-repressor) and *int* (integrase) genes via PCR as targets, might allow identification of bacterial lysogens harbouring potential inducible prophages. There are, of course, many other genes that can be targeted for this purpose as the mechanism of excision is not limited to these two genes. However, these were selected as they are the core genes that determine whether the bi-switch can be flipped from lysogenic to lytic phase.

3.2 Materials and Methods

3.2.1 Cro, CI and Int screening for lysogens containing λ prophage

Cro, CI and Int PCR screening tests were developed using Enterobacteria phage lambda, complete genome (NCBI Reference Sequence: NC_001416.1) as the template DNA sequence for designing primers.

Table 3-1: List of primers used for screening of prophage in *E. coli* lysogens

Primer name	Sequence (5`- 3`)	Binding site	Melting Temp. (°C)	Amplicon size (bp)
FWD1Cro	ATGTACTAAGGAGGTTGTATG	*cro* start	53.2	293
RV1Cro	TAGGTGTGGTTTAATTTGATG	*cro* end	55.6	
λ CI RV long	CACTGACTAGCGATAACTTTCCCCACAACG	*c*I start	61.9	713
λ CI FWD long	GTTCCATACAACCTCCTTAGTACATGCAACCA	*c*I end	61.2	
λ Int FWD	AGGTCACTATCAGTCAAAATAAAATCA	*int* start	52.8	1070
λ Int RV	GTGGCCTTTTGAAGAGGATCAGAA	*int* end	57.2	

PCR amplification was performed using *Taq* DNA polymerase with Thermopol Buffer (New England Biolabs) according to the manufacturer's specifications.

3.2.2 Death Curve for UV induction

The death curve of *E. coli* K12 cells under UV stress was determined as a reference for the APEC cultures to establish the effect of UV on *E. coli* cells under specific laboratory conditions. *Escherichia coli* K12 cells were grown from an overnight broth culture in tryptic soy broth (TSB) at 37 °C until an $OD_{600\ nm}$ = 0.8 ± 0.05 was obtained. Five (5) mℓ of culture was centrifuged and the cell pellet was resuspended in the same volume of fresh TSB. This was transferred to sterile disposable petri dishes without lid and placed in a CaptAir PCR workstation under sterilising UV light at ~254 nm (Philips TUV T8 UV bulb) in a sterile PCR cabinet 16 cm away from the light source. Samples were

taken at different time points: 0 min, 0.5 min, 1 min, 2 min, 3 min, 4 min, 5 min and 20 mins. $OD_{600\ nm}$ readings for the samples were determined in parallel to those of 10-fold dilutions of each sample, the latter being plated by spread plate method on plate count agar (PCA). For the spread plate method 0.1 mℓ of sample was pipetted from a dilution onto the agar surface and spread around using a sterile glass rod. Once the plates dried they were incubated at 37 °C overnight, followed by plate counts the following day. Agar plates of the dilutions that had between 30 and 300 colonies were chosen and counted. These plate counts were multiplied by the dilution factor and converted to colony forming units (cfu). This was done in triplicate.

3.2.3 UV induction of Avian Pathogenic *E. coli* (APEC)

The following APEC strains were induced in a UV light chamber using sterilizing short UV wavelength on overnight cells and 7-day old cells: K12, 1080, 1304, 31P, B841 and B1634.

All of these bacterial isolates were cultivated from Microbank™ beads except 1304, which comes from a 15 % glycerol stock. These isolates were selected based on a positive screen with the Cro PCR (section 3.2.1). Microbank™ beads were inoculated in 10 mℓ TSB and incubated at 37 °C overnight as a pre-inoculum. One (1) mℓ of pre-inoculum was added to 9 mℓ TSB and the tubes were incubated for 4 hours at 37 °C on a shaker at 150 rpm to $OD_{600\ nm}$ = 0.6-0.8. The cultures were transferred to 15 mℓ Falcon tubes and centrifuged at 25 °C at 4000 x *g* for 15 mins. Pelleted cells were resuspended in 5 mℓ of 0.1M KH_2PO_4 buffer and transferred to a sterile plastic petri dish.

The petri dishes were placed in the CaptAir PCR workbench without lids for 1 min 10 seconds under UV irradiation to allow for prophage-inducing cell damage to occur. Fresh TSB (5 mℓ) was added to each petri dish to increase the volume of the sample. The petri dishes were swirled to homogenously mix the samples with the added broth, sealed with parafilm and incubated at 37 °C on a shaker at 50 rpm for 3 hours. The contents of the petri dishes were transferred to new 15 mℓ Falcon tubes and centrifuged at 7000 x *g* for 5 mins. The supernatants were used in the agar overlay method (described later in section 3.2.7) to observe for the presence of plaque formation.

A second UV induction experiment was performed as described above on cells which were grown until stationary stage for 18-24 hours at 37 °C; $OD_{600\ nm}$ = 1.8-4.0, compared to cells grown to $OD_{600\ nm}$ = 0.6-0.8 at 37 °C on a shaker at 150 rpm. The induced cells were centrifuged and the supernatant of irradiated cells plated out for plaque formation (as described in section 3.2.7). The pelleted cells were resuspended in new TSB and incubated at 4 °C in the dark for 36 hours before being plated out with agar overlay in the same manner. All plaques used in subsequent passages were cut out using a pipette tip with the tip cut to allow ample diameter for the plaques.

3.2.4 Heat induction of temperate prophages

Bacterial isolates were cultivated from Microbank™ beads inoculated in 5 mℓ TSB in a test tube and grown overnight to $OD_{600\ nm}$ = 0.6-0.8 at 37 °C. Two (2) mℓ of these cultures were transferred to sterile 2 mℓ microcentrifuge tubes and placed at 42 °C in a heating block or water bath for 15 mins. Thereafter, the cells were incubated at 37 °C for 2-3 hours. A few drops of chloroform were added to lyse the remaining cells. The cellular debris was centrifuged out at 7000 x *g* for 10 mins and the supernatants were collected for the released phage particles. Similar heat induction experiments were performed at 44 °C, 46 °C, 48 °C, 50 °C and 55 °C, followed by the determination of the presence or absence of plaque formation by plaque overlay (section 3.2.7).

3.2.5 Mitomycin C minimum inhibitory concentration (MIC) determination

Bacterial isolates to be tested were grown in TSB to $OD_{600\ nm}$ ~0.6–0.8. Cells were grown from pre-culture for 4 hours and diluted to 10^4–10^5 CFU/mℓ and 5 µℓ of bacterial culture was inoculated into wells 1-11 of each row in a 96-well microtiter plate. Mitomycin C was prepared to a concentration of 256 µg/mℓ, to cover a wide range of concentrations, filter sterilized and stored at -20 °C. In the first column of wells, 100 µℓ of TSB medium was added to 100 µℓ of the prepared antibiotic solution, resulting in an antibiotic concentration of 128 µg/mℓ. The antibiotic was then serially diluted further in columns 2–10 in TSB, while wells 11 and 12 were used as the positive and negative controls, respectively. Plates were incubated at 37 °C overnight (12-14 hours) and the MIC was visually determined as the highest antibiotic dilution that still inhibited bacterial cell growth.

Induction was performed according to Raya and Hébert (2009). The strains were grown to $OD_{600\ nm}$ of 0.6-0.8 at 37 °C on a rotary shaker at 150 rpm. The induction assay was performed by adding the appropriate amount of mitomycin C to the cells and incubated for 1 hour at 37 °C to induce endogenous prophages (McDonald *et al*., 2010). After induction experiments plaque formation was visualised using the plaque overlay method (section 3.2.7). Mitomycin C induction was also tested in conjunction with pH ranges of 6–8.5 in pH value increments of 0.5.

3.2.6 Resuspension of mitomycin C treated cells

Blocks of the agar from exponential and stationary phases of cultures of the K12 and 1080 strains affected by 2.5 µg/mℓ, 3 µg/mℓ and 4 µg/mℓ mitomycin C, were cut out and placed into 15 mℓ sterile falcon tubes containing 5 mℓ SM buffer, and incubated at room temperature for 1 hour with agitation every 10 mins to allow resuspension of cells and cell lysate/debris in the SM buffer. Afterwards 1.5 mℓ of resuspended cell lysate containing debris was centrifuged at 4000 x *g* for 10 mins to sediment cells and debris. The supernatant was filter-sterilized using 0.45 µm cellulose syringe filters. 1 mℓ of filter-sterilised supernatant was inoculated with 1 mℓ *of E. coli* to 9 mℓ agar overlay. 1.5 µg/mℓ mitomycin C was added to this mixture, mixed and placed onto TSB agar. The plates were

allowed to solidify and incubated at 37 °C overnight, prior to observation of plaque formation (section 3.2.7).

3.2.7 Plaque overlay method for plaque formation

Ten (10) mℓ of molten agar was used per sample of phage to be plated out. The molten agar was cooled to an appropriate temperature before phage-containing solution (0.1 mℓ) and bacterial host culture (1 mℓ) were inoculated into the molten agar. The tubes were mixed before the contents were transferred to a petri dish. The petri dish was gently agitated to ensure homogenous mixing, and the mixture was allowed to set before incubation at 37 °C overnight, followed by observation of plaque formation.

3.2.8 Phage DNA extraction

Phage particles either from agar plates (plaques) or broth (bacterial cells incubated and lysed with bacteriophages) were used for virion precipitation and DNA extraction.

Phage plaques from agar plates were resuspended in SM buffer for 30 mins at room temperature before 1 mℓ was collected, while 1 mℓ of the lysed and centrifuged cell solution from broth was used for precipitation.

The phage-containing solution was filter sterilized using a 0.2 µm sterile cellulose acetate membrane syringe filter (GVS filter technology, U.S.A). To this solution 2 µg/mℓ each of RNase and DNase were added before incubating at 37 °C for an hour to remove host nucleic acids. Then 100 mℓ of each 20 % PEG6000 and 1M NaCl were added to the sample and incubated on ice for 2 hours or at 4 °C overnight. The solution was centrifuged at 20 000 x *g* at 4 °C for 30 mins. The supernatant was decanted carefully and the precipitate resuspended in 1 mℓ SM buffer. Afterwards, 100 µℓ of each 10 % SDS and 0.5 M EDTA were added and mixed by inversion. This mixture was incubated at 68 °C for 30 mins, followed by addition of 100 µg/mℓ of Proteinase K and further incubation at 56 °C for 30 mins.

3.2.9 Genomic DNA amplification using Phi29 Polymerase

Stock λ DNA (Thermo Scientific) was diluted to 1 ng/µℓ, 20 ng/µℓ and 100 ng/µℓ from a 0.3 µg/µℓ stock solution. These concentrations were run on a 1 % agarose gel to determine a suitable concentration for PCR template.

The PCR set up and cycles using the Phi29 polymerase are as listed in Table 3-2 and Table 3-3. The Phi29 reactions were amplified by an initial incubation at 30 °C for 12 hours, followed by inactivation of Phi29 polymerase at 65 °C for 10 mins and storage at 4 °C.The phages induced from *E. coli* strains (section 3.2.8) were used as template for amplification using Phi29 polymerase.

Table 3-2: PCR reaction set up for DNA Amplification by Phi29 polymerase

Reagent	Quantity per reaction
Template DNA	1-40 ng
Phi29 Random hexamer primer	1 µl
2x annealing buffer	2.5 µl
Deionized water (80 mM Tris-HCl pH 8.0, 20 mM $MgCl_2$)	Up to 5 µl

Hexamer used: Transcriptor High Fidelity (DNA Synthesis Kit)

Table 3-3: Denaturation reaction set up and conditions of Template for Phi29 polymerase amplification

Heat 3 min at 94 ℃	
Cool down	
Combine with premixed:	
Phi29 10x buffer	2 µl
Phi29 DNA Polymerase	5-7 units
dNTPs 2mM	2 µl
dH_2O	Up to 15 µl
Total	20 µl

3.3 Results and Discussion

3.3.1 Cro, CI and Int PCR screening for lysogens containing λ prophage

Lambda was chosen as the reference bacteriophage as the genome is well researched, genome sequence readily available and mechanisms understood. While it has served as a model for molecular biology for decades, it is still relevant in the present time, with recent research on it highlighted in a recent review (Casjens and Hendrix, 2015). The screening of prophages present in the bacterial cells was based on the bacteriophage λ sequence on NCBI. The phage sequence revealed a 48502 bp linear DNA sequence of the complete Enterobacteria phage lambda (NC_001416). In particular, the bi-switch of lambda is important as it is the site where the lysogen may be maintained or converted to lysis. The PCR screening targeted the *cro* (gene ID on NCBI: 2703467), *c*I (gene ID on NCBI: 3827059) and *int* (gene ID on NCBI:2703470). The *cro* gene is 293 bp in length (38023-38315), with the designed forward and reverse primer amplifying a target gene of 278 bp (38022-38300). The *c*I gene is 714 bp in length (37227-37940), with the designed forward and reverse primer amplifying a target gene of 780 bp (37258-38038). The *int* gene is 1070 bp in length (27812-28882), for which the whole sequence should be amplified using the designed primers. The screening reactions were performed according to manufacturer's instructions using primers as listed in Table 3-1.

Only 31P appeared to contain cro and cI (Figure3-2) while about one third of screened strains tested positive for the int gene (Figure 3-3). This makes it likely that this strain harbours an intact lambda phage that is potentially inducible from lysogeny into the lytic pathway. An interesting observation is seen in Figure 3-3 (a) where *E. coli* strains B771, APEC 1323, B98, 76, 0142/0125, 01517:H7, 104P and CosR (Figure 3-3) have *int* genes in their genomes although they do not carry the other screened genes.

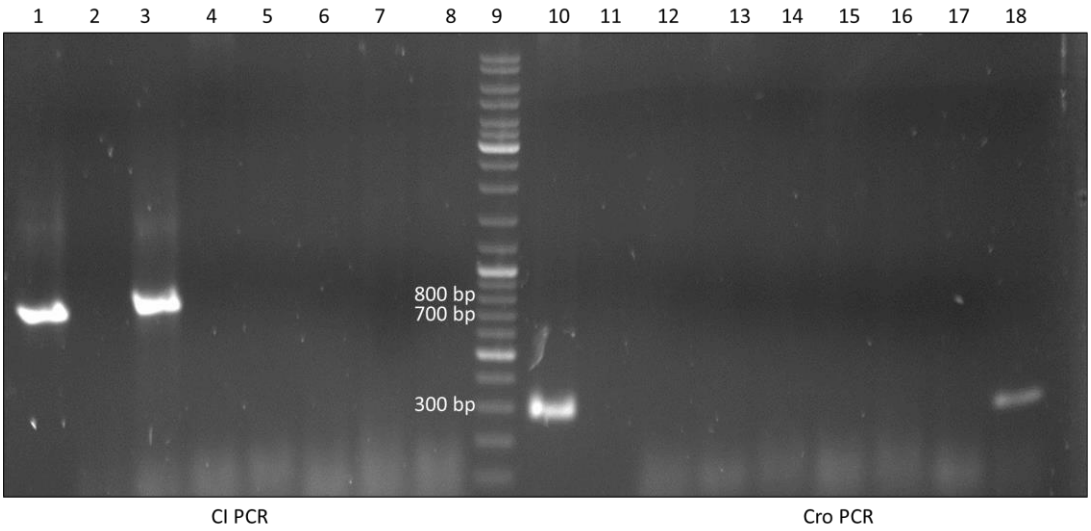


Figure 3-2: Screening PCR for E. coli lysogens containing λ cI and cro genes. Lanes 1–8 are CI PCRs: lane 1–Positive control (λ DNA), lane 2–Negative control (no template), lane 3–31P, lane 4–87, lane 5–1304, lane 6–WCD2, lane 7–CosR, lane 8–172, lane 9–O'Generuler DNA ladder mix. Lanes 10–18 are Cro PCRs: 10–Positive control (λ DNA), lane 11–Negative control (no template), lane 12–87, lane 13–87 (repeat), lane 14–1304, lane 15–WCD2, lane 16–CosR, lane 17–172, lane 18–31P. The expected band sizes of CI and Cro amplicons are 713 bp and 293 bp respectively.

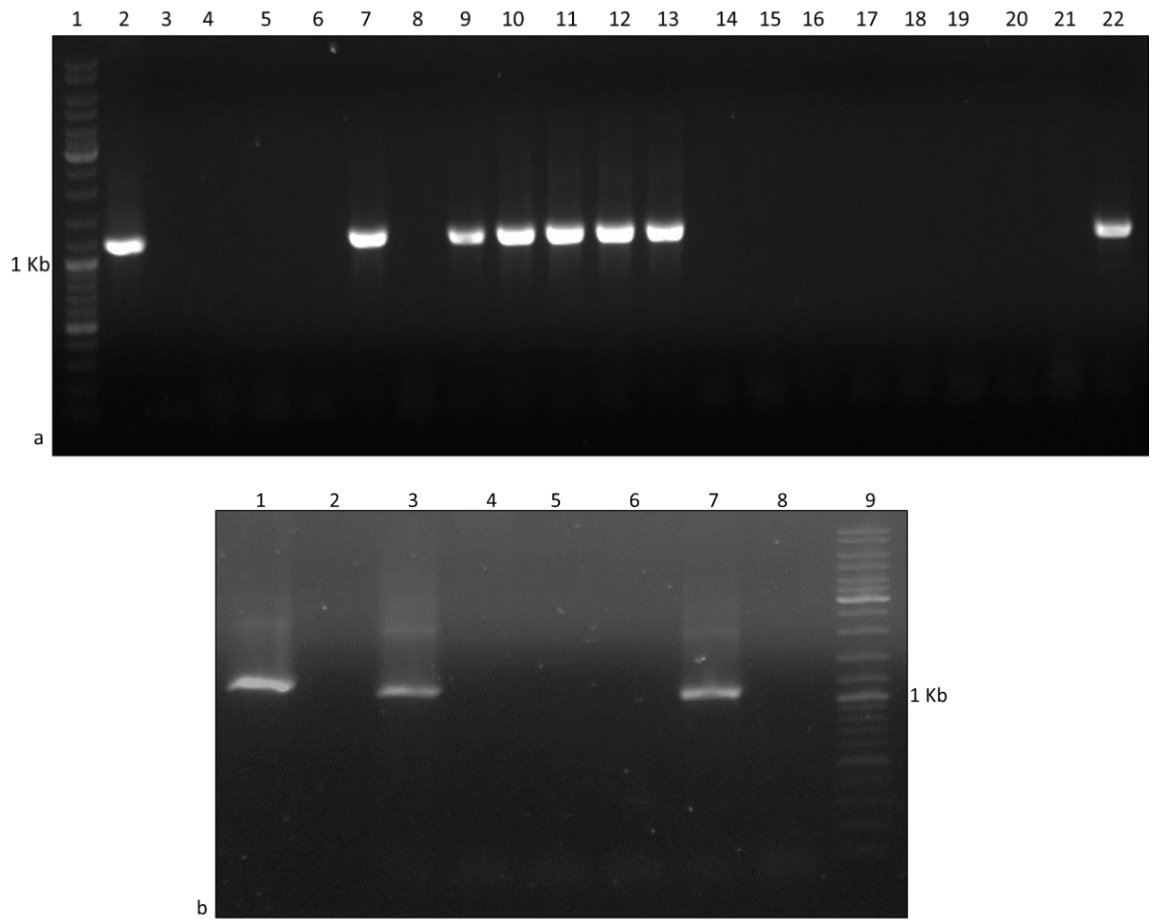


Figure 3-3: Screening PCR for *E. coli* lysogens containing λ *int* gene. (a) First batch, lane 1–O'GeneRuler DNA ladder mix, lane 2–Positive control (λ DNA), lane 3–Negative control (nH2O), lane 4–25922, lane 5–B1634, lane 6–018, lane 7–B771, lane 8–WCD1, lane 9–APEC 1323, lane 10–B98, lane 11–76, lane 12–0142/0125, lane 13–01517:H7, lane 14–1080P, lane 15–WCD1, lane 16–ATCC, lane 17–WCD3, lane 18–214, lane 19–163, lane 20–Exegg, lane 21–104N, lane 22–104P. (b) Second batch, lane 1–Positive control (λ DNA), lane 2–Negative control (nH2O), lane 3–31P, lane 4–87, lane 5–1304, lane 6–WCD2, lane 7–CosR, lane 8–172. The expected band size of int amplicon is 1070 bp.

These *int* genes may have been transferred horizontally from other strains or may be remnants from previous infection with lambdoid phages, which remained at site-specific locations in the bacterial host genome (Campbell *et al*., 1992). The phage integrases allow homologous recombination between phage and bacterial DNA at attachment sites *attP* and *attB* respectively (Rajagopala *et al*., 2011). This site specific attribute of phage integrase insertion differentiates it from retroviral integrases as well as transposases, which exhibit their random integration (Groth *et al*., 2000). Some speculations for phage integrase genes being retained by the bacterial genome are due to increasing the fitness of the host cell. In some cases having one or multiple integrase genes inserted adjacent to one another 'cures' the bacterial cell from infection by certain phages (Hacker & Carniel, 2001). This is however only heteroimmunity, as a new phage may still infect a lysogen that has immunity towards phage infection. The phage genes may also be retained, depending on what is retained with the integrase, due to an improved bacterial phenotype by conferring enhanced virulence through

phage-encoded toxins such as those of cholera and diphtheria toxins (Davies *et al*., 2016; Reidi & Klose, 2002).

3.3.2 Induction of phages

Induction of phage is founded in the function of prophage mechanism of escaping the host during DNA damage. The principle of phage induction relies on several different factors which include, but are not limited to: the presence of the complete prophage, the genetic bi-switch and a functional host SOS response. The bacterial strains testing negative using PCR, were still induced in case some other prophages could be present that may have been lissed using the PCR screening based on phage lambda. This is based on research indicating that many bacteria tested in laboratories contained intact and functional prophages (Figueroa-Bossi & Bossi, 1999; McClelland *et al.*, 2001; Popp *et al*., 2000; Schmieger & Schicklmaier, 1999). The objective was to induce potential prophages into the lytic cycle. The nature of prophage induction is intertwined with the host's own DNA damage recovery mechanism; the SOS system. This means that induction of prophage relies on DNA damage to the point where the SOS system must be employed to recover, repair or maintain the integrity of DNA within the cell. Therefore, UV irradiation, heat and mitomycin C induction were employed in parallel as each of these damage the cell's DNA.

3.3.3 Death Curve for UV induction

In order to determine the exposure time allowing sufficient stress induction in the cells for lysogeny induction, but without killing the cells, a death curve was constructed based on UV-irradiation.

The rapid decline of viable bacterial cells after the first 5 mins of UV irradiation (Figure 3-4) are indicative of the power of UV sterilisation and the effect it can have with induction of lysogens. For the purposes of this study a UV irradiation time of 1 min was chosen as an effective induction time.

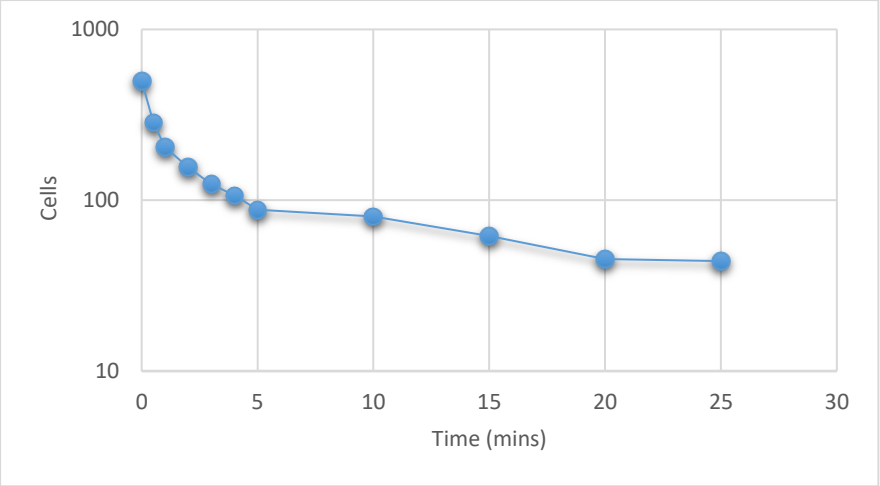


Figure 3-4: Curve of cell decline after *E. coli* K12 cells have been affected by UV illumination. The decline is shown over a time period of 25 mins where samples are collected in time intervals of 30 sec, 1 min, 2 min, 3 min, 4 min, 5 min, 10 min, 15 min, 20 min and 25 min.

3.3.4 UV induction of Avian Pathogenic *E. coli* (APEC) strain 31P

Table 3-4: Number of plaques generated by UV induction of *E. coli* strains

	1st passage	**2nd passage**	**3rd passage**
***E. coli* B1634**	No plaque		
***E. coli* 1080**	<10 plaques	3 plaques	No plaque
***E. coli* K12**	<10 plaques	5 plaques	No plaque
***E. coli* B841**	<10 plaques	7 plaques	No plaque
***E. coli* 31P**	<10 plaques	No plaque	

Reduced plaque formation was observed with increased passages of UV induction as seen in Table 3-4. Thus, the UV induction was repeated with varying days of cell recovery and cell age. An experiment was performed involving the bacterial strains of *E. coli* 1080, K12 and B841, in which UV induction of exponential phase cells (fresh cells) was compared to induction of cells in stationary phase (old cells). Furthermore, the plaque overlays were done in two ways, either using the supernatant of the irradiated culture on the same day of irradiation, or after three days of post-irradiation incubation in the dark at 4 °C (Table 3-5).

Table 3-5: Comparison of plaques observed from UV induced *E. coli* cells with agar overlays performed on the same day as irradiation as well as after incubation post-induction

	Plaque observed	
***E. coli* strain**	Same-day overlay	Post-irradiation incubation before overlay
1080 fresh cells	No	
1080 old cells	No	
1080 fresh cells	No	No
1080 old cells	No	No
K12 fresh cells	No	
K12 old cells	No	
K12 fresh cells		No
K12 old cells		Yes, 17 plaques
B841 fresh cells	No bacterial lawn	
B841 old cells	Yes, one plaque	
B841 fresh cells		No
B841 old cells		No bacterial lawn

The promising treatment was observed when stationary phase cells of strain K12 were incubated post-irradiation. It was unclear why the fresh cells used the same day for overlay did not give plaques as before.

3.3.5 Heat induction of temperate prophage at 42 °C

Similar results were obtained for induction using temperatures of 44 °C, 46 °C, 48 °C, 50 °C and 55 °C compared to those of the heat induction of temperate prophage at 42 °C (Table 3-6).

Table 3-6: Plaques produced after heat induction of *E. coli* strains

	1st passage	**2nd passage**
***E. coli* B1634**	No plaque	No plaque
***E. coli* 1080**	No plaque	No plaque
***E. coli* K12**	1 plaque	No plaque
***E. coli* B841**	4 plaque	No plaque
***E. coli* 31P**	No plaque	No plaque

The heat induction of the *E. coli* strains at 42 °C resulted in either no visible plaques for most of the strains (*E. coli* B1634, *E. coli* 1080 and *E. coli* 31P) or very few plaques (*E. coli* K12 and *E. coli* B841) as seen in Table 3-6. Similarly, the heat induction at temperatures of 44 °C, 46 °C, 48 °C, 50 °C and 55 °C were unsuccessful at producing plaques. The induction of prophage by heat treatment is caused by direct denaturation of a mutant, temperature-sensitive repressor (CI) (Rokney *et al*., 2008; Svenningsen *et al*., 2005). Thus where wild-type CI are found, the lysogen will remain uninducible using heat as an inducer.

3.3.6 Mitomycin C induction

The MICs of mitomycin C for *E. coli* strains K12 and 1080 were determined to be 4 µg/mℓ and 2 µg/mℓ, respectively. Based on these values, the two strains were induced using mitomycin C concentrations of 2.5 µg/mℓ, 3 µg/mℓ and 4 µg/mℓ to test the effect of induction with sub-MIC concentrations (results in Table 3-7).

Table 3-7: Old and fresh cultures of K12 induced under different concentrations of mitomycin C for plaque induction.

[MitoC]	Culture	Plaque
2.5 µg/mℓ	K12 old	No
	K12 fresh	No
	1080 old	Yes
	1080 fresh	No
3 µg/mℓ	K12 old	No
	K12 fresh	No
	1080 old	Yes
	1080 fresh	Yes
4 µg/mℓ	K12 old	No bacterial lawn
	K12 fresh	No bacterial lawn
	1080 old	No bacterial lawn
	1080 fresh	No bacterial lawn

There were some instances of plaque formation using different concentrations of mitomycin C as can be seen in Table 3-7 especially with *E. coli* 1080 at both 2.5 µg/mℓ and 3 µg/mℓ. It was seen that 4 µg/mℓ was too high a concentration as the bacterial lawn did not form.

It has been shown that pH has an effect on mitomycin C efficacy (Jauhiainen *et al*.,1983). As can be seen in Figure 3-5, there is no clear difference between the different pHs used in conjunction with mitomycin C. Thus, the neutral pH of 7.0 was used during the induction experiments.

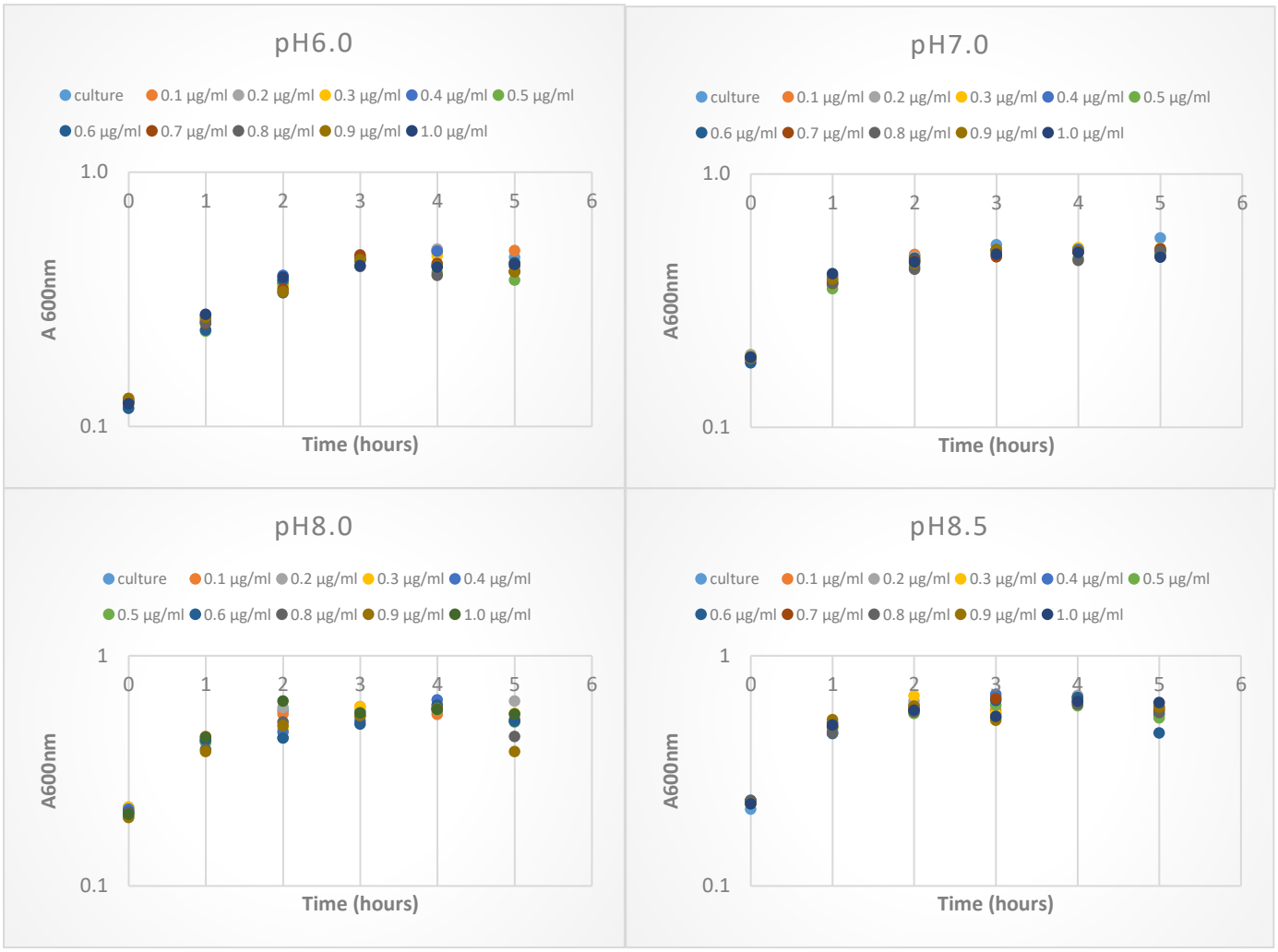


Figure 3-5: Effect of pH on different mitomycin C concentrations on *E. coli* 31P. Concentrations of mitomycin C ranged from 0.1 to 1.0 µg/mℓ, and the pH ranged from 6 to 8.5. Mitomycin C was tested in different pH in order to optimize its efficiency.

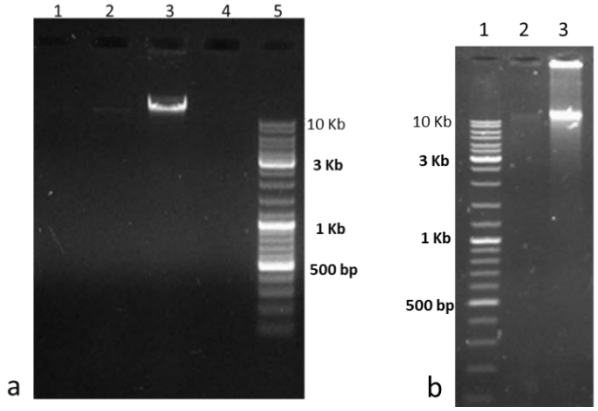
3.3.7 Genomic DNA amplification using Phi29 Polymerase

Part of the challenge of this project was to ensure that the phage escaping the host cell can infect and reproduce in new cells in order to extract enough phage DNA to use for further applications. This problem was worsened by fewer plaques being observed with each subsequent passage, resulting in insufficient DNA being extracted. Therefore, the amplification of released DNA was attempted using Phi29 polymerase in rolling circle amplification (RCA).

Phi29 polymerase was discovered in *Bacillus subtilis* phage phi29 (φ29) and can perform rolling circle amplification by means of strand displacement and 3´→5´ proofreading exonuclease activity. This enzyme has been used to amplify large DNA fragments and even genomes. It amplifies at sites where random primers are annealed to the DNA and this is made even more efficient using random hexamer primers which can bind at multiple places. Multiply-primed RCA is the annealing of multiple primers to the circular template DNA creating several replication forks which are elongated and resulting in a double-stranded product as it rolls off of the parent strand (Dean *et al*., 2001).

Different concentrations of lambda DNA were run on an agarose gel for visualization (Figure 3.6a) From these results, 1 ng/µℓ λ DNA was selected for amplification using Phi29 polymerase (Figure 3-6 b).

Figure 3-6: (a) Different concentrations of λ DNA to determine which starting template concentration



to use for the Phi29 PCR and (b) Phi29 PCR amplification performed on 1 ng/µℓ λ DNA. Lanes for gel (a) 1–1 ng/µℓ λ DNA, 2–20 ng/µℓ λ DNA; 3–100 ng/µℓ λ DNA, 4–nH2O and 5–MWM (O'Generuler DNA ladder mix). Lanes for gel (b) 1–MWM (O'Generuler DNA ladder mix); 2–1 ng/µℓ λ DNA and 3–Phi29 amplified DNA from 1 ng/µℓ λ DNA.

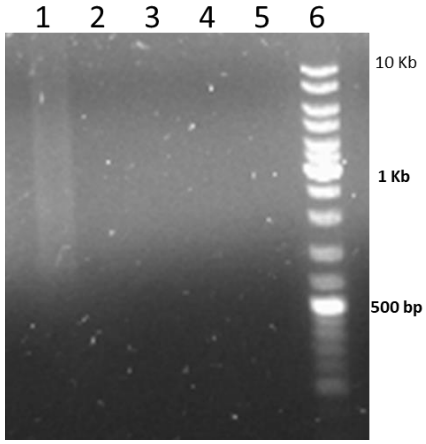


Figure 3-7: Phi29 PCR amplification performed on extracted gDNA of *E. coli* strains. Lanes for gel 1–1080, 2–31P; 3–1323, 4–B98 and 5–B841. A smear was observed in the first lane, which is *E. coli* 1080 gDNA extraction.

The Phi29 amplification of the induced prophage genomic DNA was unsuccessful (Figure 3-7) when compared to standard lambda DNA (Figure 3-6 b). This may be due to the extracted genomic DNA not being of sufficient quality or quantity, while the effect of phenol in the extraction could also have inhibited the polymerases as it is a protein denaturant (Schrader *et al*., 2012).

3.4 Concluding remarks

PCR screening of strains for three of the many genes that contribute to conversion to lytic phase, based on bacteriophage lambda, surprisingly only yielded one strain that contained all three genes.

It was expected that lambda could be present as has routinely been isolated from *E. coli*, but other phages may of course be present as the concurrent presence of more than one prophage is common (Chen *et al*., 2006). Furthermore, the modular or mosaic element of bacteriophage genomes may contribute to the difficulty experienced with prophage induction. This modular or mosaicism of bacteriophage genomes refers to the readiness with which bacteriophage genomes transfer genetic information horizontally within the host bacterial genome (Juhala *et al*., 2000). This may contribute to an increased fitness of the host cell which encourages the host-prophage interaction leading to lysogeny. However, it may also be a reason for the challenge of induction for some prophages as they may only be remnants and are therefore uninducible (Juhala *et al.*, 2000). Furthermore, Svenningsen and Semsey (2014) found that the multiple copies of λ chromosomes were required before late lytic regulator Q mediated the switch from lysogeny to lysis (Svenningsen & Semsey, 2014). Which leads to the conclusion that there is difficulty when it comes to induction and that not all prophages are inducible (Brüssow *et al*., 2004).

Successful induction of phages was observed, especially when induced with mitomycin C. However, insufficient DNA could be retrieved for further applications, seemingly including for amplification using Phi29 polymerase. It was therefore concluded that this was not a feasible approach for production of a specific lytic phage. This was compounded by the fact that virulent phages were already quite specific for their hosts, therefore further efforts in this direction seemed unwarranted.

Chapter 4 Production of λ phage-derived enzymes

4.1 Introduction

The λ endolysin and holin proteins are a paired system of phage-encoded enzymes for breaking open the cell from the inside to release the fully assembled virions into the environment after packaging (Young *et al*., 2000; Wang *et al*., 2000). This system relies on holin proteins assembling as dimers that aggregate and form a 'raft'-like structure, which integrates into the inner cell membrane. This creates a hole or pore that is large enough for λ endolysin to access the cell wall from within the cells (Krupovič & Bamford, 2008). The nature of the dimers is such that the hydrophobic portions face the cell membrane while the hydrophilic portions interact with each other without causing any harm to the cell membrane (Gründling *et al*., 2001). The formation of the raft decreases the proton motive force of the cell membrane; which in turn causes a conformational change in the holin raft so that a 'hole' is formed in the cell membrane where it is inserted (White *et al*., 2011). These holes can be non-uniformly greater than 300 nm (White *et al*., 2011). The holin is responsible for timing the lytic event, which is likely due to the rate of monomers forming dimers, which ultimately integrate into the inner cell membrane. There are other genes which are part of the lambda lysis cassette, namely *Rz* and *Rz1*, which codes for outer membrane associated proteins with supplementary lytic effects of cleaving between the peptidoglylcan and the outer membrane lipoproteins (Wang, *et al.*, 2000). However, these factors are not necessary for *in vitro* lysis of bacteria (Wang *et al*., 2000) and were therefore not included in this study. The objective of this chapter was to express lambda (λ) phage-derived holin and endolysin in order to evaluate the bactericidal activities of endolysin.

Protein expression in eukaryotic systems present advantages for the heterologous expression of eukaryotic genes, which requires various modifications during transcription and translation, and also often require post-translational modifications (Struhl, 1999). However, eukaryotic expression can also be beneficial when compared to prokaryotic expression due to the general safety of certain eukaryotic cells versus bacterial cells. Bacteria generally tend to be pathogenic or at least opportunistic pathogens. Certain eukaryotic cells on the other hand have been denoted generally regarded as safe (GRAS) status, with yeasts such as *Saccharomyces cerevisiae* and *Yarrowia lipolytica* being among these (Branduardi *et al*., 2008; Müller *et al*., 1998). Furthermore, many yeast culture processes are well developed and understood, allowing for convenient production of recombinant proteins (Bokulich & Bamforth, 2013; Ejiofor *et al*., 1996). Furthermore, yeasts are able to secrete proteins due to native secretion systems, which may allow for easier recovery of biologically active products e.g. enzymes (Zhang *et al*., 1996). *Saccharomyces cerevisiae* has been used as a host organism to express enzymes for a long time, but this yeast has certain limitations such as low product yields, plasmid instability, hyperglycosylation, and

unsuccessful secretion of proteins. Therefore, alternative hosts for yeast expression systems have been considered (Böer *et al*., 2009; Müller *et al*., 1998). *Yarrowia lipolytica* is a yeast known to naturally secrete several native enzymes such as proteases, lipases, esterases and RNase (Barth & Gaillardin, 1997), and has also been demonstrated as a superior host for heterologous protein production to *S. cerevisiae* for active enzymes (Müller *et al*.,1998). Over 130 proteins have been heterologously expressed in this yeast, including viral proteins (Madzak, 2015; Madzak *et al*., 2004). This yeast grows at temperatures of up to 32 °C, with an optimum of 28 °C and is a strict aerobe, which adds to its GRAS status (Barth & Gaillardin, 1997; Fickers *et al*., 2005).

The *Y. lipolytica* strain used in this study, Po1h, is an uracil auxotrophic null mutant (*matA*, *ura3- 302*, *xpr2-322*, *axp1-2*), which was constructed by the deletion of the URA3 gene by replacing it with the *S. cerevisiae* SUC2 gene, resulting in a Ura^- and Suc^+ phenotype. The use of auxotrophy is a practical means to determine successful transformation of the yeast cells with introduced foreign genes carrying marker genes that supplement nutritional requirements (Pronk, 2002).

The pINA 1317 vector used in this study (Figure 4-12) contains an expression cassette consisting of the semi-constitutive hp4d promoter, and the terminator and secretion sequences of alkaline extracellular protease (*XPR2*). In addition, the vector contains zeta integration sites, a uracil prototrophic marker (ura3d1) and a bacterial backbone carrying the kanamycin (Kan) resistance gene (kan^R) (Nicaud *et al*., 2002). The bacterial backbone can be liberated by *Not*I digestion to isolate the yeast cassette to be integrated into the yeast genome. In addition, the pINA 1317–YlCWP110 variant also contains the C-terminal anchor domain of *YlCWP1* to allow cell surface display of recombinant proteins via a glycosylphosphatidylinositol (GPI) anchor (Yue *et al*., 2008).

Zeta sites are long terminal repeats (LTR) derived from Ylt1 retrotransposon, which act as sites of homologous recombination events in certain strains of *Y. lipolytica* which contain chromosomal counterparts of these sequences. These zeta sites have also been shown to allow random integration into the genomes of *Y. lipolytica* strains devoid of corresponding zeta sequences (Fickers *et al*., 2003). The hp4d promoter is a synthetically developed promoter consisting of four repeats of the upstream activator sequence 1 (*UAS1*) of the *XPR2* promoter and a minimal *LEU2* promoter. It allows semi-constitutive expression comparable to the *XPR2* promoter, but with less complicated regulation (Madzak *et al*., 2000).

This chapter describes the bacterial expression followed by yeast protein expression of the bacteriophage lambda proteins, as the two expression systems were used in different experiments.

4.2 Materials and Methods

Part A: Expression in *E. coli*

4.2.1 Bacterial strains and plasmids used in this study

The pGEM®-T Easy vector system (Promega) was used as a sub-cloning vector to introduce amplified DNA targets into the destination vector via a plasmid-propagation strain. Both the pET28b(+) and pETDuet-1 expression vectors (New England Biolabs) used in this study allow expression controlled by the T7 promoter / lac operator system. pET28b(+) contains a kanamycin-resistance marker, and allows fusion of 5'- and /or 3'- sequences encoding hexa-histidine tags. pETDuet-1 contains two separate multiple cloning sites for co-expression of genes, and contains an ampicillin (Amp)-resistance marker (Amp^R). NEB® 10-β competent *E. coli* (High Efficiency) cells (New England Biolabs), genotype Δ*(ara-leu) 7697 araD139 fhuA* Δ*lacX74 galK16 galE15 e14- ϕ80*d*lacZ*Δ*M15 recA1 relA1 endA1 nupG rpsL* (Str^R) *rph spoT1* Δ*(mrr-hsdRMS-mcrBC)* were used as the propagation strain while *E. coli* BL21 (DE3) competent cells (New England Biolabs), genotype: *fhuA2* [*lon*] *ompT gal* (λ DE3) [*dcm*] ∆*hsdS λ DE3 = λ sBamHIo* ∆*EcoRI-B int::*(*lacI::PlacUV*5::T7 gene1) i21 ∆*nin5,* were used as the expression strain*.*

The pETDuet-1 plasmid was chosen as vector to concurrently express both the holin and endolysin proteins in BL21 (DE3) competent *E. coli* cells (New England BioLabs). This plasmid has two multiple cloning sites, MCS1 and MCS2, for co-expression of proteins. The BL21 (DE3) is an ideal model strain for protein expression as it lacks *lon* (producing an ATP-dependent protease) and OmpT (aspartyl protease) proteases that cleave proteins. The pET28b vector was used to clone the lysin gene into BL21 (DE3) competent cells, as it allows the optional incorporation of N- and C-terminal hexa-histidine (His) tags to the endolysin when expressed. The His-tags are used for purification using nickel column affinity purification. All *in silico* work was performed using Geneious Pro R9 (http://www.geneious.com, Kearse *et al*., 2012) for the prediction and analysis of primers, alignment, insertion of target genes into vectors and sequence analysis. The vectors utilized for the cloning in bacterial expression section of the work are described in Table 4-1.

Table 4-1: Cloning and expression vectors utilised for bacterial protein expression

Vector name	Selective marker	Size (bp)	Features
pGEM®-T Easy	*amp*^{r}	3015	A sub-cloning vector that has lac operator which allows blue-white selection of negative/positive colonies.
pETDuet™-1	*amp*^{r}	5420	An expression vector with two multiple cloning sites for the coexpression of two target genes. Selection on ampicillin-containing medium.
pET28b	*kan*^{r}	5368	Optional fusion to N-and/or C-terminal polyhistidine tags. Selection on kanamycin-containing medium.

4.2.2 Cloning and sequencing of genes

Standard molecular biological techniques were used in this research, as described by Sambrook and Russel (2001). All DNA modification enzymes were used according to the specifications of the manufacturer.

Primers were designed using the IDT oligoanalyzer tool (https://eu.idtdna.com/calc/analyzer) for the amplification of the holin and lysin genes, and were supplied by Whitehead Scientific (Table 4-2).

Table 4-2: Primers used in this study to amplify genes intended for bacterial protein expression

Primer name	Gene target (bp[1])	Primer sequence	Tm (°C)	Primer length (bp)	Restriction site
Primers for amplification of holin and lysin for cloning					
HolpDuetF	45191-45508	5`- CAAGCTTTTGATTTCTACCATCTTCTACTCCG-3`	60.2	31	*Hin*dIII A AGCTT
HolpDuetR		5`- GGATCCGCCAGAAAAACATGACCTG-3`	58.8	25	*Bam*HI G GATCC
LyspDuetF	45481-45969	5`- GCCATATGGTAGAAATCAATAATCAACGTAAGG CG-3`	60.3	35	*Nde*I CA TATG
LyspDuetR		5`- CTCGAGTCATACATCAATCTCTCTGACC-3`	57,8	29	*Xho*I C TCGAG

Primer name	Gene target	Primer sequence	Tm (°C)	Primer length (bp)
Primers for confirming insertion into expression vectors				
pETUpstream	**pETDuet MCS1**	5`- ATGCGTCCGGCGTAGA-3`	56.7	16
DuetDOWN		5`- GATTATGCGGCCGTGTACAA-3`	55.5	20
pETUP2	**pETDuet MCS2**	5`- TTGTACACGGCCGCATAATC-3`	55.5	20
T7 terminator		5`- GCTAGTTATTGCTCAGCGG-3`	53.4	19
pETUpstream	**pET28 MCS**	5`- ATGCGTCCGGCGTAGA-3`	56.7	16
T7 terminator		5`- GCTAGTTATTGCTCAGCGG-3`	53.4	19

[1]Regions according to Enterobacteria phage lambda (λ), complete genome (NC_0014161)

*The target gene size of holin is 317 bp and for lysin 488 bp.

The primer pair HolpDuet F and R for holin gene amplification included *Hin*dIII and *Bam*HI restriction sites in the forward and reverse primers, respectively. Likewise, the primer pair LyspDeut F and LyspDeut R for lysin gene amplification included *Nde*I and *Xho*I restriction sites in the forward and reverse primers, respectively. These sites were included for cloning into pET28b(+) and pETDuet-1. Holin and lysin were amplified using *Taq* DNA polymerase with thermopol *Taq* buffer (NEB) according to manufacturer's specifications, using thermocycling conditions as listed in Table 4-3

Table 4-3: Thermocycling conditions for general PCR

	Temperature (°C)	**Time**	**No. of cycles**
Heated lid	110		
Initial denaturation	94	30 s	1
Denaturation	94	30 s	25-35
Annealing	51-57*	30 s	
Amplification	72	1 min**	
Final extension	72	5 min	1
Storage	4	∞	

* The range of annealing temperatures used, as temperatures differed, were determined according to (Table 4-2).

**An amplification time of 1 minute per kb of DNA to be amplified was used as an index and was adjusted according to the length of the target gene.

Colony PCR was performed by using 5 µℓ of overnight LB-broth-grown culture as template in a 50 µℓ PCR reaction. The initial denaturation was extended to 6 min at 94 °C to lyse cells and release the DNA template.

The holin PCR product was digested with *Hin*dIII and *Bam*HI restriction enzymes, while the lysin PCR product was digested with *Nde*I and *Xho*I (all supplied by Thermo Scientific) according to the manufacturer's instructions.The digestions were electrophoresed at 90V for 35 min on a 1 % agarose gel containing ethidium bromide (EtBr). The correlating bands for the holin and lysin were excised from the gel and extracted using the Promega Wizard® SV Gel and PCR Clean-up System according to the manufacturer's recommendations.

DNA Sanger sequencing using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Scientific) was performed using the primers listed in Table 4-2. This was performed on Applied Biosystems HITACHI 3130*xl* Genetic Analyzer (Molecular lab, Department Microbial, Biochemical and Food Biotechnology, UFS) after EDTA/Ethanol postreaction cleanup. All methods followed the manufacturer's protocols.

Cloning was performed according to the NEB 10-beta competent *E. coli* (High Efficiency) high efficiency transformation protocol, and transformed cells were plated on LB agar plates containing either 100 µg/mℓ ampicillin (pETDuet-1), 50 µg/mℓ kanamycin (pET28b(+)) or 25 µg/mℓ chloramphenicol (pRARE). Five to ten colonies from each transformation were selected for screening by restriction analysis on extracted (miniprep) DNA or by colony PCR, for presence of the desired insert. Colonies containing the insert were stored as 15 % glycerol stocks at -80 °C and with Microbank™ beads at -20 °C.

Correct recombinant plasmids were extracted using the PureYield™ Plasmid Miniprep System (Promega), prior to use for transformation into *E. coli* BL21 (DE3) competent cells for protein expression.

Table 4-4: List of bacterial transformants with vector details and gene insert names

Transformant name	Vector(s)	Gene insert	Antibiotic resistance
Duet control	pETDuet™-1	No gene insert	Amp
Duet lysin	pETDuet™-1	Lysin	Amp
Duet holin	pETDuet™-1	Holin	Amp
Duet holin and lysin	pETDuet™-1	Holin and Lysin	Amp
pRare control	pETDuet™-1 & pRARE™	No gene insert	Amp & Cam
pRare lysin	pETDuet™-1 & pRARE ™	Lysin	Amp & Cam
pRare holin	pETDuet™-1 & pRARE ™	Holin	Amp & Cam
pRare holin and lysin	pETDuet™-1 & pRARE ™	Holin and Lysin	Amp & Cam
Duet mutant holin	pETDuet™-1	Mutant holin	Amp
Duet mutant holin and lysin	pETDuet™-1	Mutant holin and Lysin	Amp
pET28 lysin	pET28	Lysin	KanR

4.2.3 Correcting for codon bias in expression

The *R* and *S* genes were tested using the GenScript OptimumGene™ codon optimization tool to determine whether codon bias was a factor to consider during expression.

The pETDuet™-1 constructs containing the holin, lysin and combination of both genes (Table 4-4) were transformed into BL21 (DE3) competent cells already containing the pRare™ plasmid. The transformants were grown on either plate count agar (PCA) or TSB containing final concentrations of 100 µg/mℓ ampicillin and 25 µg/mℓ chloramphenicol (Cam) for the pETDuet™-1 and pRare™ plasmids, respectively. The transformants were also grown at 37 °C in 5 mℓ TSB containing the same final concentrations of antibiotics, by inoculation with 50 µℓ of pre-culture ($OD_{600\ nm}$ of 0.8 ± 0.05).

When the optical density of the culture reached $OD_{600\ nm}$ of 0.6-1.0 after 4 hours of incubation post-inoculation the culture was induced with 1 mM IPTG and incubated for a further 3 hours at 28-30 °C. Thereafter, the cells were centrifuged at 4000 x *g* and supernatant was discarded. The cells were re-suspended in 1 % phosphate buffered saline (PBS), pH 7.4 to a concentration of 0.1 $g_{wcw}.m\ell^{-1}$ (grams of wet cell weight per litre) before cell disruption at 30 kPa ± 0.5 kPa using the One-shot cell disruptor (Constant Systems). The resultant cell debris was pelleted by centrifugation at 6000 x *g*, the supernatant was mixed with Laemmli sample buffer, boiled at 95 °C for 5 min, and loaded onto a 12 % SDS-PAGE for electrophoresis.

4.2.4 Autolysis

4.2.4.1 Freeze/thaw assisted autolysis

Cell cultures of control (containing only pET-Duet) and test transformants (pET-Duet lysin and pET-Duet holin and lysin) were grown in LB broth containing 100 µg/mℓ ampicillin to an $OD_{600\ nm}$ of 0.4–0.6, before induction with 1 mM IPTG. The cultures were pelleted by centrifugation at 5000 x *g* for 5 min at room temperature. The cell pellet was re-suspended in 20 mM Tris/HCl, pH 7.5 and frozen at -20 °C for 30 min, followed by thawing at 37 °C for 4 hours.

4.2.4.2 Non-assisted autolysis

The control and test transformants were inoculated as mentioned previously in 50 mℓ of TSB and induced with 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) at $OD_{600\ nm}$ 0.4-0.8 and incubated at 30 °C on a shaker. The flasks were removed from the shaker after 4 hours and left at room temperature on a bench without shaking. The effects of autolysis in the flasks of holin/lysin transformant were observed consistently by comparing the flasks to plasmid control flask. This experiment was conducted eight times.

The non-assisted autolysis experiment was also repeated with the inclusion of a transformant containing a mutant version of holin (Johnson-Boaz *et al*., 1994; Wang *et al*., 2000) carrying the A52G mutation that results in earlier permeabilization of the inner cell membrane than the native holin. The transformants were inoculated, induced and incubated as described above, with samples taken regularly. After incubation at room temperature without shaking, these samples were centrifuged at 20 000 x *g* in microcentrifuge tubes to remove whole cells and cell debris. The supernatants were prepared for 12 % SDS-PAGE by adding β-mercaptoethanol to a final concentration of 5 % and Laemmli sample buffer, followed by boiling for 5 min.

4.2.5 Protein purification of endolysin

The pET28 lysin transformant was grown from a pre-inoculum in 50 mℓ of TSB containing a final concentration of 50 µg/mℓ of kanamycin at 37 °C, 150 rpm with shaking for 2-3 hours until an $OD_{600\ nm}$ of ~0.4-0.6 was reached. Thereafter, the culture was induced with a final concentration of

1 mM IPTG and incubated at 30 °C overnight with shaking. The culture was centrifuged at 7100 *x g* for 5 min and the supernatant discarded. The pellet was re-suspended in binding buffer (50 mM Tris, 20 mM imidazole, 0.5 M NaCl, pH 7.4) at 100 g_{wcw}/ℓ before cell disruption at 30 kPa ± 0.5 kPa using the One-shot cell disruptor. The cell debris and unbroken cells were centrifuged out at 7195 *x g* for 5 min. The supernatant was ultracentrifuged to remove remaining cells and debris using an SW 32Ti rotor in a (Beckman Coulter Optima™ L-100 XP Ultracentrifuge) at 30 000 rpm (110330 x *g*) for 90 min at 4 °C. The resulting supernatant was loaded onto a purification column for either gravity-flow protein purification or fast protein liquid chromatography (FPLC). For gravity-flow purification, either 1 mℓ B-PER® 6xHis Fusion Protein Purification Kit columns (Pierce, USA) or 1 mℓ HisPur™ Cobalt Resin Spin Columns (Pierce, USA) were used. The protein purification columns were prepared according to the manufacturer's protocols, except for the cobalt gravity-flow columns, which were run using the following buffers: His-trap binding buffer (50 mM Tris, 500 mM NaCl, 20mM Imidazole, pH 7.4) and elution buffer (50 mM Tris, 500 mM NaCl and 500 mM Imidazole at pH 7.4).

For FPLC the supernatant was loaded onto a 1 mℓ HisTrap™ HP column (GE Healthcare) using a peristaltic pump to load the protein sample at an even rate. The proteins purified with the ÄKTA FPLC UPC-900 were bound using binding buffer (50 mM Tris, 500 mM NaCl, 20 mM Imidazole, pH 7.4) and eluted with elution buffer (50 mM Tris, 500 mM NaCl and 500 mM Imidazole at pH 7.4) in 1 mℓ fractions.

Both fractions collected with gravity-flow and FPLC were prepared and run on a 12 % SDS-PAGE at 120 V for 60-70 min in 1X Biorad TGS buffer (25 mM Tris, 192 mM Glycine, 0.1 % w/v SDS, pH 8.3). Thereafter, the gels were stained according to the method described by (Fairbanks *et al*., 1971). The gels were then placed on a 30 rpm shaker overnight for complete destaining. The purified protein concentration was determined using the Pierce™ BCA Protein Assay Kit (Thermo Scientific) according to the manufacturer's protocols.

4.2.6 Dialysis to remove imidazole

The imidazole-eluted protein samples were dialysed against 0.9 % NaCl buffer using the Slide-A-Lyzer® Dialysis Cassette (Extra Strength) 10 000 MWCO 0.5–3.0 mℓ capacity (Pierce) to remove the imidazole. This was done by carefully injecting the protein sample into the Slide-A-Lyzer® cassette using a gauge 19 needle and 5 mℓ syringe. The filled cassette was placed in a litre of 0.9 % NaCl buffer and placed at 4 °C overnight. The buffer was replaced twice thereafter for a period of 2 hours each at room temperature.

4.2.7 Holin extraction from cell membranes

Three methods were attempted for extraction of holin from cell membranes. The first method was

based on the method by Chang and co-workers (1995) which was used to quantitate induced S protein from inner membrane protein (Chang *et al*., 1995). Transformant cells were grown in TSB and induced at $OD_{600\ nm}$ = 0.5–0.8 with 1 mM IPTG. All three methods used the same growth and induction conditions, after which the cells were used in the different methods.

Cells were harvested by centrifugation and resuspended in 3 mℓ FP buffer (0.1 M sodium phosphate, pH 7.0, 0.1 M KCl, 5 mM EDTA, 1 mM dithiothreitol, Roche cOmplete™, EDTA-free protease inhibitor cocktail) and disrupted using the One Shot cell disruptor (Constant Systems) at 30±0.5 kPa. This cell disruptor homogenizes cell suspensions using high pressure, known as the French-Press method. The cell membranes were collected by ultracentrifugation at 30 000 rpm (114304 x *g*) for 90 min at 4 °C using a SW 32.1Ti rotor in a Beckman Coulter Optima™ L-100 XP Ultracentrifuge. The pellet containing the cell membrane was re-suspended in $1/100^{th}$ the original culture volume of ME buffer (10 mM Tris-HCl, pH 8.0, 35 mM $MgCl_2$, 1 % Triton X-100) and mixed on a shaker for 2 hours at 25 °C. The Triton-insoluble substances were pelleted out via ultracentrifugation at 30 000 rpm (114304 x *g*) for 30 min at 4 °C. The membrane extract was mixed with Laemmli sample buffer and boiled at 95–100 °C for 5 min, before being loaded onto a 16 % Glycine-SDS PAGE.

The second method used to purify holin proteins involved the use of carbonate buffer to extract bacterial cell membrane proteins (Molloy *et al*., 2000). This method also consists of a series of cell harvesting and resuspension steps, before the disruption of a 100 g/ℓ suspension using the One Shot cell disruptor (Constant Systems) at 30 ± 0.5 kPa. The buffer used for washing and resuspension of cells was 50 mM Tris-HCl, pH 7.5 (wash solution). Unbroken cells were pelleted by centrifugation at 2,500 x *g* for 8 min, and the supernatant was retained for carbonate extraction. The supernatant was added to 50 mℓ of ice-cold 100 mM sodium carbonate (carbonate extraction solution), and rotated slowly at 4 °C for an hour. This was followed by ultracentrifugation at 30 000 rpm (114304 x *g*) for 90 min at 4 °C using a SW 32.1Ti rotor in a Beckman Coulter Optima™ L-100 XP. Afterwards the supernatant was discarded and the pellet was washed with 2 mℓ of wash solution. The cell membranes were collected using ultracentrifugation at the same parameters as described above. After resuspension in 50 mM Tris/HCl containing 1 % Triton-X 100 the sample was mixed with Laemmli sample buffer and boiled for 5 mins, before being loaded onto a 16 % SDS-PAGE as described above.

In the third method HEPES buffer was used for extraction (Carlone *et al*., 1986). A 10 mℓ sample of the bacterial suspension adjusted to $OD_{600\ nm}$ of 0.5–0.8 was centrifuged at 7179 *x g* for 10 min at 4 °C. The supernatant was decanted and the pellet re-suspended in cold 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer, pH 7.4 to $100g_{wcw}/ℓ$ cell suspension. This was homogenised using the One Shot cell disruptor as described above. The unbroken cells and debris were removed by centrifugation at 14 000 x *g* for 2 min, 4 °C. The supernatant fluid was transferred to a 1.5 mℓ centrifuge tube, and the cell membranes were harvested from the supernatant by

centrifugation at 14 000 x *g* for 30 min, 4°C. The supernatant fluid was decanted, and the cell membrane pellets were thoroughly resuspended in 0.5 mℓ of 10 mM HEPES buffer by repeated pipetting, followed by centrifugation at 14 000 x *g* for 2 min, 4°C. The supernatant was decanted and the membranes were solubilized by addition of 100 µℓ of 10 mM HEPES buffer, pH 7.4 containing 1 % Triton-X 100. This sample was prepared for 16 % SDS-PAGE as described above.

4.2.8 Western blotting targeting His-tag of holin

Cells from 5 mℓ LB were pelleted and resuspended in 5 mℓ of 50mM Tris-HCl, pH 7.2 and lysed using freeze-thaw with lysozyme (100 µℓ of 50mg/mℓ in 5 mℓ cells); frozen at -20 °C, thawed at 42 °C and lysates collected. The lysates were loaded and run on 12 % SDS-PAGE using Bio-Rad fast-cast and the run performed at 220 V for ~35 min. The gel was transferred to membrane (Bio-Rad Nitrocellulose/Filter paper sandwiches 0,45um) using a Bio-Rad Trans-Blot® Turbo™ transfer system at 1.3 A, 25 V for 7 min. Western blots were performed according to a modified Bio-Rad Bulletin 6376 Rev A (http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6376.pdf). Briefly, after transfer, the gel was rinsed with water and stained using Ponceau solution to confirm protein transfer. The membrane was rinsed with PBS-Tween (PBST) until all the stain was removed. The membrane was blocked in 3 % BSA in PBST at RT for 1 hour with gentle rocking. The blocking solution was poured off and incubated in fresh blocking solution containing monoclonal Anti-Histidine antibodies (Sigma-Aldrich A7058-1VL) conjugated with horse radish peroxidase (HRP) at 1:2000 dilution. This was incubated for 2 hours at RT with gentle rocking and the membrane rinsed with PBST 5 times for 5 min each. The membrane was incubated for 5 min in Bio-Rad Clarity™ Western ECL Substrate according to manufacturer and viewed using a Bio-Rad ChemiDoc™ MP Imaging System.

4.2.9 Protein identification via LC Tandem Mass Spectrometry (MS/MS)

The protein bands were identified and confirmed using Liquid-chromatography Tandem Mass Spectrometry (LC-MS/MS). The protocol followed was based on the work done by Shevchenko and co-workers (2006). Bands of interest were excised from 12 % SDS-PAGE using methanol wiped surgical blades, followed by washing of excised bands several times with nanopure water. Washed bands were placed in clean microcentrifuge tubes and cut into small fragments (~1 mm^3). The smaller pieces were washed twice by alternating equal volumes of water and 50 % acetonitrile for 15 min each. Next the gel pieces were shrunk in 100 % acetonitrile, followed by swelling in 10 mM dithiothreitol in 100 mM NH_4HCO_3 at 56 °C for 45 min. The solution was replaced with 55 mM iodoacetamide in 0.1 M NH_4HCO_3 and incubated in the dark at room temperature. Thereafter, the gel pieces were washed with alternating equal volumes of water and 50 % acetonitrile. The acetonitrile was removed and the shrunken white gel pieces were dried. The dried gel pieces were re-hydrated on ice with digestion solution containing 100 mM NH_4HCO_3 with 2 µℓ trypsin (Promega) and incubated at 37 °C overnight. The digested sample is transferred into new microcentrifuge tubes

and the remaining gel pieces were covered with 5 % of formic acid, vortexed briefly and incubated for 15 min at room temperature. Acetonitrile (100 %) was added at a 1:1 ratio to the sample volume, followed by brief vortexing and incubation at room temperature for a further 15 min. The supernatant was removed and added to the digest solution supernatant. This process was repeated, followed by vacuum drying of the collective digest solution before being processed in the LC-MS/MS (Speedy Vac; Eppendorf Concentrator Plus) before being processed in the LC-MS/MS.

Part B: Heterologous expression of lambda lysin in *Y. lipolytica*

4.2.10 Cloning of the synthesised bacteriophage lambda lysin gene

The yeast expression plasmids pINA1317 and pINA1317-CWP were obtained from Dr Catherine Madzak (INRA, France).

The bacteriophage lambda lysin gene was optimized and synthesized by GeneArt® (Thermo Scientific), flanked by *Sfi*I (5') and *Hin*dIII (3') restriction enzymes sites, for expression in *Y. lipolytica.* The reference sequence was obtained from NCBI (Genbank accession number; NC_0014161). The gene was provided in a pMA-T vector (*ampR*, Col E1 origin). The lyophilised synthesized gene (5 µg plasmid DNA) upon arrival was reconstituted as described by the manufacturer.

High efficiency competent *E. coli* 10-beta cells (NEB) were used for plasmid propagation. *E. coli* clones were grown in 5 mℓ lysogeny broth (LB) containing 100 µg/mℓ ampicillin, before the plasmid was extracted using PureYield™ Plasmid Miniprep System (Promega) and digested with *SfiI* and *Hin*dIII enzyme. The insert was excised from an agarose gel and purified using the Promega gel extraction and purification kit. The purified DNA insert was ligated to the pINA 1317–YlCWP110 vector digested with the same enzymes. This ligation reaction was used to transform High efficiency competent *E. coli* 10-beta cells (NEB) cells, using transformation methods as described by Sambrook and Russel (2001). The transformed cells were grown on LB agar (10 g/ℓ tryptone, 10 g/ℓ NaCl, 5 g/ℓ yeast extract, 15 g/ℓ bacteriological agar, pH 7) supplemented with 30 µg/mℓ kanamycin (LB-Kan) plates at 37 °C for approximately 16 hours. Single colonies were selected and inoculated into 5 mℓ LB containing 30 µg/mℓ kanamycin and incubated for 16 hours at 37 °C. Transformation reactions performed with empty plasmids served as negative controls.

Colony PCRs were performed to confirm that the inserted genes were present, using the primer pair Chen6560F (5'-GATCCGGCATGCACTGATC-3') and CM-terX (5'-GAACCTCGTCATTGATGGAC-3') obtained from Catherine Madzak, INRA, France. Positive clones were prepared for Sanger sequencing as described in section 4.2.2 using BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Scientific), according to the manufacturer's instructions. Sequences were aligned to *in silico* determined sequences using Geneious R9 (http://www.geneious.com, Kearse *et al*., 2012).

Plasmids were extracted from positive clones using PureYield™ Plasmid Miniprep System (Promega) and digested using *Not*I (Thermo Scientific). The yeast expression cassette was excised from an agarose gel and purified with Promega gel extraction kit according to the manufacturer's protocol.

4.2.11 Transformation of *Y. lipolytica*

The *Y. lipolytica* yeast strain used for transformations and expression, Po1h (MatA, Ura$^-$, ΔAEP, ΔAXP, Suc$^+$) was obtained from Dr Catherine Madzak (INRA, France). The yeast strain was grown in 50 mℓ of YPD [10 % (w/v) yeast extract, 2 % (w/v) bacto peptone, 2 % (w/v) glucose] medium in 250 mℓ flasks at 28 °C for 18 hrs prior to transformation.

The purified *Not*I digested yeast expression cassette was transformed into *Y. lipolytica* Po1h competent cells using the one-step lithium acetate transformation described by Chen and colleagues (1997). Yeast transformants were grown on selective YNB medium [0.17 % (w/v) yeast nitrogen base without amino acids and ammonium sulphate, 1 % (w/v) glucose and 0.5 % (w/v) ammonium sulphate] plates at 28 °C for approximately 3 days.

In a second transformation method, competent yeast cells were prepared by growing Po1h cells overnight at 22 °C on YPD agar. Sufficient cells were scraped from the agar plate and resuspended into 1 mℓ TE buffer in a sterile microcentrifuge tube. This was centrifuged for 1 minute at 7000 x *g* and the supernatant discarded. The cells were resuspended in 600 μl of 100 mM Lithium Acetate, pH 6.0 and incubated for an hour at 28 °C in a water bath without shaking. The mixture was centrifuged at 2000 x *g* for 2 min and the supernatant discarded. The cells were then slowly resuspended with 120 μl of 100 mM Lithium Acetate, pH 6.0 which allows for up to 3 transformations.

Transformation reactions contained 40 μl of competent cells, 2 μl of carrier DNA and 3 μl of transforming DNA added in an eppendorf tube and incubated for 15 min at 28 °C in a water bath. To this tube 250 μl of PEG 4000, 100 mM Lithium Acetate, pH 6.0 and 16 μl of 1 M DTT was added and the tube incubated for an hour at 28 °C in a water bath without shaking. Thereafter, 40 μl of DMSO was added and the cells were heat shocked for 10 min at 39 °C. Afterwards, 600 μl of 100 mM Lithium Acetate, pH 6.0 was added and 200 μl of the transformation reaction was spread per plate of YNB-N_{5000} agar. The plates were incubated at 28 °C for 2-3 days and colonies were selected and insertion of the correct gene was confirmed using colony PCR and sequencing.

Colony PCR was performed on clones that grew on YNB-N_{5000} agar plates and bands were visualised on an agarose gel. Correct bands were excised from the gel and extracted using Promega Wizard® SV Gel and PCR Clean-Up System. The DNA concentration was determined using the NanoDrop® Spectrophotometer ND-1000 and used for Sanger sequencing with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The sequences were analysed using Geneious Pro R9

(http://www.geneious.com, Kearse *et al*., 2012).

4.2.12 Cell preparation for Scanning Electron Microscopy and GPI-anchor cleavage

The transformed *Y. lipolytica* strains were grown in 5 mℓ YPD broth in test tubes as a pre-culture. A flask containing 50 mℓ of YPD broth was inoculated at $OD_{600\ nm}$ = 0.1–0.2 and grown at 28 °C for 3–5 days on a shaker at 120 rpm. The cells were then harvested for i) viewing under Scanning Electron Microscope, and ii) cleavage of expressed protein from GPI anchor. Overnight cultures of selected clones were also stored with glycerol (15 % final concentration) at -80 °C.

4.2.13 Confirmation of recombinant endolysin using scanning electron microscopy (SEM)

Y. lipolytica cells were prepared for SEM by fixation and dehydration, followed by sputter coating with gold for viewing. Cells were fixed by resuspension in 3 % (v/v) glutardialdehyde in sodium phosphate buffer at pH 6.8–7.2 for 5 hours. The samples were then rinsed with the sodium phosphate buffer for 10 min before being fixed with 2 % (w/v) osmium tetroxide (OsO_4) for 2 hours. The cells were rinsed twice for 10 min in the above mentioned in 3 % (v/v) glutardialdehyde in sodium phosphate buffer. Dehydration was done stepwise using 50 %, 70 % and 95 % ethanol gradient for 10-30 min in each step. Final dehydration was achieved by placing the sample in 100 % ethanol for 15-30 min and repeating this step twice. The samples were subjected to critical point drying using a critical point dryer, involving replacement of ethanol with pressurised liquid CO_2 at 31.5 °C. After the samples were dried they were mounted on metal stubs and sputter-coated with gold. All scanning electron microscopy was performed by the Centre for Microscopy, University of the Free State, Bloemfontein, South Africa; with a Shimadzu SSX-550 Superscan SEM.

4.3 Results and Discussion

In this chapter, the first objectives were to clone the λ holin (*S*) and lysin (*R*) genes into *E. coli* BL21 (DE3) competent cells and *Y. lipolytica* Po1h cells using various vectors described in Table 4-4 and confirm protein expression on 12 % SDS-PAGE and using LC MS-MS. This is divided into Part A regarding expression and confirmation in *E. coli* followed by Part B which is the expression in *Y. lipolytica*.

Part A: Expression in *E. coli*

4.3.1 PCR amplification and cloning of *R* and *S* genes into vectors

The amplification of the lysin and holin genes using the primers described in Table 4-2 was successful, giving the correct band sizes of ~500 bp and ~300 bp respectively (Figure 4-1). The PCR

products were cleaned using the Promega Wizard® SV Gel and PCR Clean-up System and sequenced via Sanger sequencing as described in section 4.2.2.

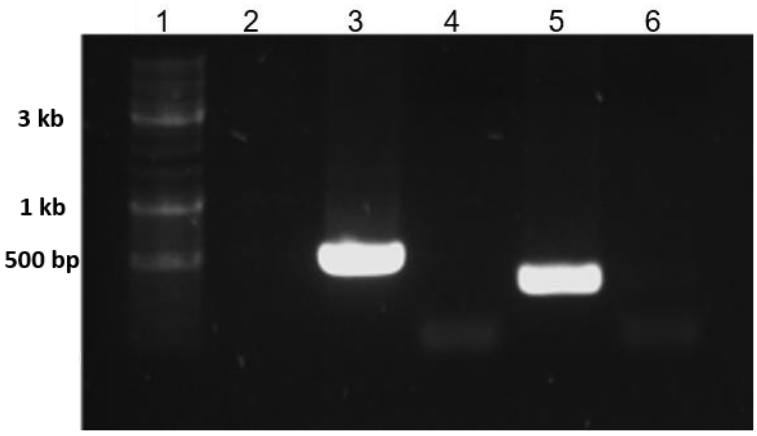


Figure 4-1: The lysin and holin PCR products used for cloning as run on a 1 % agarose gel stained with EtBr. Lane 1 is the DNA ladder, lane 2 is an open lane, lane 3 is amplification of lysin (~500 bp) using the LyspDuetF and LyspDuetF primer set, lane 5 is amplification of holin (~300 bp) using HolpDuetF and HolpDuetR primers, and lanes 4 and 6 are the no-template negative controls for the lysin and holin amplifications, respectively.

Purified PCR products were used for digestion with appropriate restriction digestion enzymes (section 4.2.2), followed by ligation into their respective vector systems and transformation of competent *E. coli* cells (Table 4-4). Clones were picked and grown overnight at 37 °C for colony PCR and mini-prep confirmation of the inserted genes. Colony PCR yielded expected band sizes (Figure 4-2) and the PCR products were purified and verified to be correct by sequencing.

Based on *in silico* model prepared using Geneious (Figure 4-3), the inserted genes are expected to be in-frame in the open reading frames (ORF). The clones carrying genes inserted into the pETDuet™-1 that were used for protein expression were confirmed using multiple cloning site specific primers which anneal outside the MCS regions and thereby amplify the inserted genes. The holin gene was confirmed to be inserted into the MCS1 region and lysin gene was confirmed to be inserted into the MCS2 region should contain lysin (Figure 4-4).

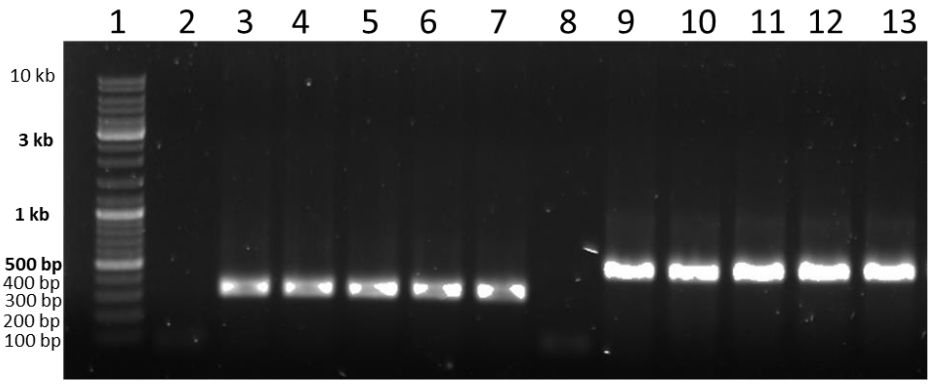


Figure 4-2: Colony PCR was performed for confirmation of correct inserts. Lane 1 is the DNA ladder, lane 2 is negative control for holin amplification using Duet control colony, lanes 3-7 are amplification of post-transformation colonies for confirmation of holin gene (~300 bp), lane 8 is negative control of lysin amplification using Duet control colony and lanes 9-13 are amplification of post-transformation colonies for confirmation of lysin gene (~500 bp).

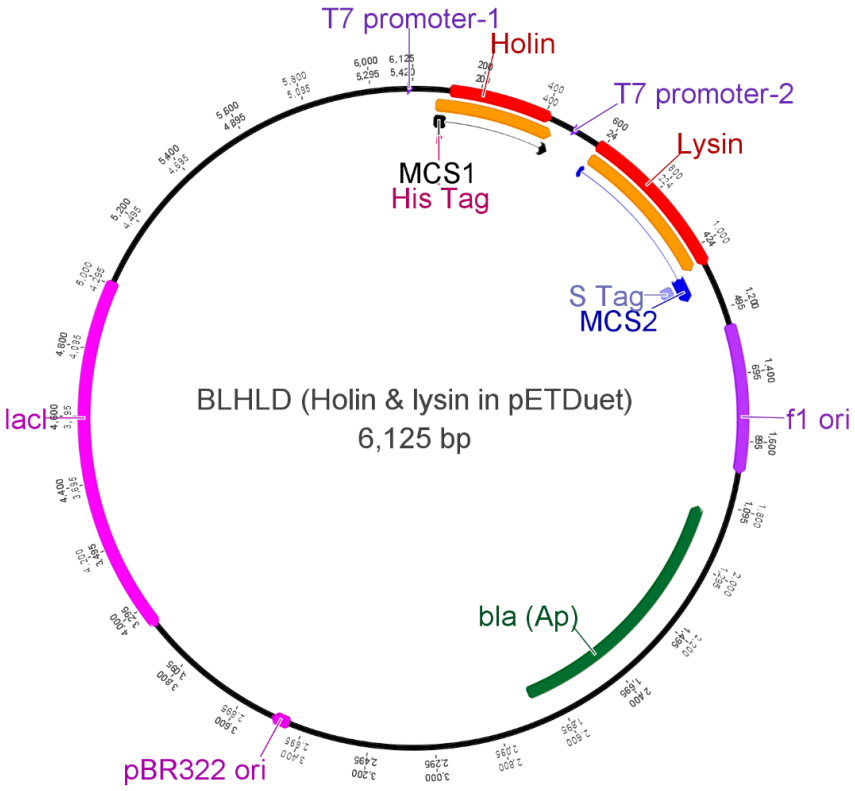


Figure 4-3: *In silico* prediction of pETDuet-1 with holin and lysin inserts. The holin gene will be inserted into MCS1 preceded by His-tag while the lysin gene will be inserted into MCS2. The orange tabs are open reading frames (ORFs) of the MCS1 and MCS2 regions. The available S tag in the MCS2 region was not included in the open reading frame as there was a stop codon at the 3′ terminal of the inserted gene.

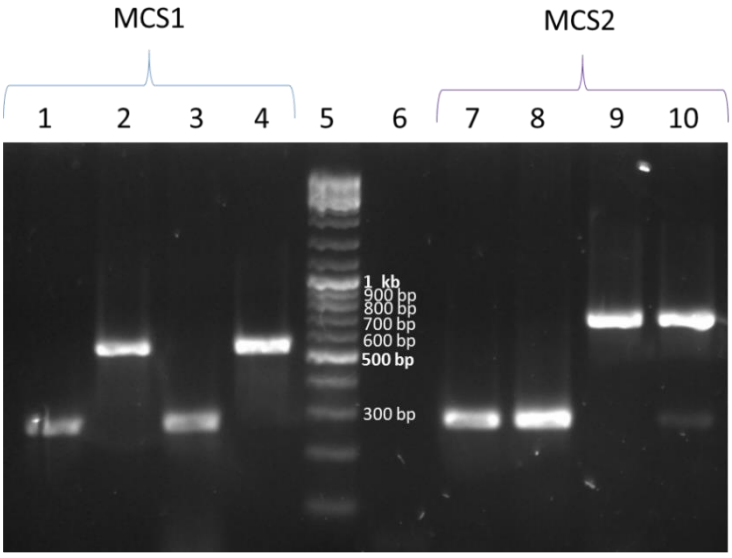


Figure 4-4: Colony PCR confirmation of holin and lysin using pETDuet MCS-1 (lanes 1-4) and MCS-2 (lanes 7-10) specific primers. In the absence of inserts, both the MCS1 and the MCS2 primers facilitate the amplification of a region of approximately 270 bp. In combination with holin, product sizes are approximately 600 bp, and in combination with lysin, product sizes are approximately 800 bp. Lanes 1 and 7 are Duet control; lanes 2 and 8 are Duet holin; lanes 3 and 9 are Duet lysin; lanes 4 and 10 are Duet holin and lysin; lane 5 is DNA ladder and lane 6 is no template negative control.

4.3.1.1 Codon bias optimisation

Codon bias refers to the preferential use of specific codons by an organism, which is linked to specific tRNA abundances, and often varies between organisms and may even vary within the organism itself for different operons (Gustafsson *et al*., 2004). Based on the algorithm that is used by OptimumGene™ (described in section 4.2.3) CAI values of 0.68 and 0.67 were obtained for expression of lysin and holin, respectively, in *E. coli.* A CAI value of 1 indicates very good expression, a CAI greater than 0.8 is acceptable expression while below 0.8 tending towards poor expression.

Therefore, the Duet control (pETDuet-1 without gene insert i.e. plasmid control); Duet lysin (pETDuet-1 with lysin gene insert); Duet holin (pETDuet-1 with holin gene insert) and Duet holin and lysin (pETDuet-1 with both lysin and holin gene inserts) were transformed into BL21 (DE3) competent cells either containing pRARE or not, to observe for effects on the expression of the proteins. Growth and induction of the different pRARE containing transformants was performed as described above (4.2.3), except for the addition of 50 μg/mℓ chloramphenicol for the selective marker of pRARE.

While comparing the protein expression between pRARE and non-pRARE transformants (Figure 4-5) it was found that the former had slower growth when inoculated with similar inoculum ($OD_{600\ nm}$ = 0.8±0.05), which may be due to the additional antibiotic for selective pressure and the maintenance of the pRARE plasmid that imposes additional metabolic burden on the cells. Thus, the

lower amount of produced endolysin in pRARE cells compared to the non-pRARE transformants after the same time post-induction was attributed to lower biomass, and it was concluded that the proteins being produced did not significantly differ between the two strains. Therefore, the rest of the experiments were performed using the non-pRARE transformants as more protein was produced in the same amount of time, which is beneficial for recombinant protein production.

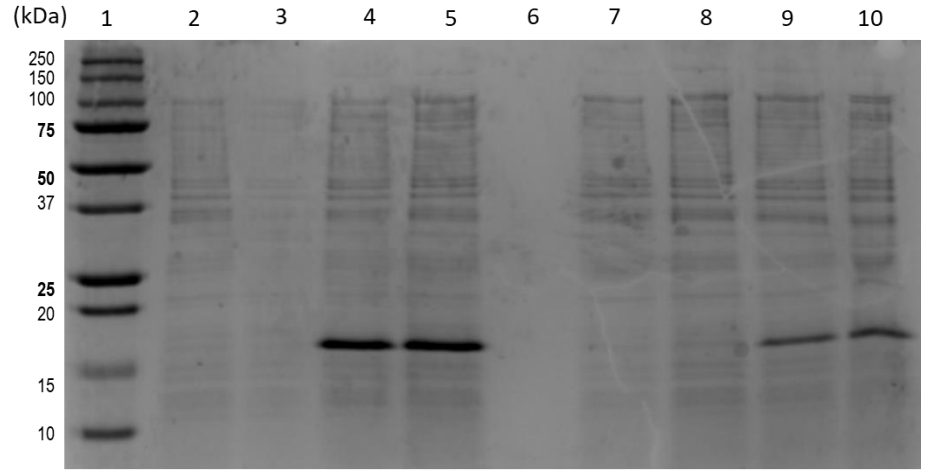


Figure 4-5: Comparison of expression between non-pRARE transformants and pRARE transformants. Lane 1–Bio-Rad Precision Plus Protein™ Prestained Protein Standards, lane 2–Duet control, lane 3–Duet holin, lane 4–Duet lysin, lane 5–Duet holin and lysin, lane 6–empty lane, lane 7–pRARE control, lane 8–pRARE holin, lane 9–pRARE lysin and lane 10–pRARE holin and lysin. The non-pRARE transformants show a brighter band for lysin on the 12 % SDS-PAGE when compared to their pRARE counterparts. OD_{600} nm measurements at the time of sampling revealed that the pRARE transformants were consistently OD_{600} nm of ± 0.4 less than that of non-pRARE transformants. Endolysin MW 18.3 kDa, holin MW 11.3 kDa.

Aside from co-transforming an expression host with a plasmid that encodes rare codons, another solution to the codon bias challenge is to synthesize genes that are codon optimized to those that are used preferentially by the expression host. Different plasmids/vectors offer features that allow for simple, accurate, traceable insertion of the target gene to form a working construct and are able to propagate or express in certain competent cells. Plasmids that encode rare codons of the expression host are useful because they provide the tRNAs complementing rare codons and a non-native protein may be correctly expressed due to this availability of tRNAs (Rogulin *et al*., 2004). Novagen's pRARE is a chloramphenicol resistance-carrying plasmid that supplies the rare tRNAs for AGG and AGA (Arg), AUA (Ile), CUA (Leu), CCC (Pro), GGA (Gly) codons, aside from the CGA/CGG for Arg in *E. coli* (Novy *et al*., 2001). This helps to improve the low protein expression levels that result from codon bias in *E. coli* expression cells without the need for synthesis of codon optimized genes.

4.3.2 Holin expression and extraction from membranes

Due to the membrane-associating nature of holin, different methods were used to extract recombinant holin from the cell membrane (section 4.2.7). Extracted proteins were analysed using 16 % SDS-PAGE (Figure 4-6).

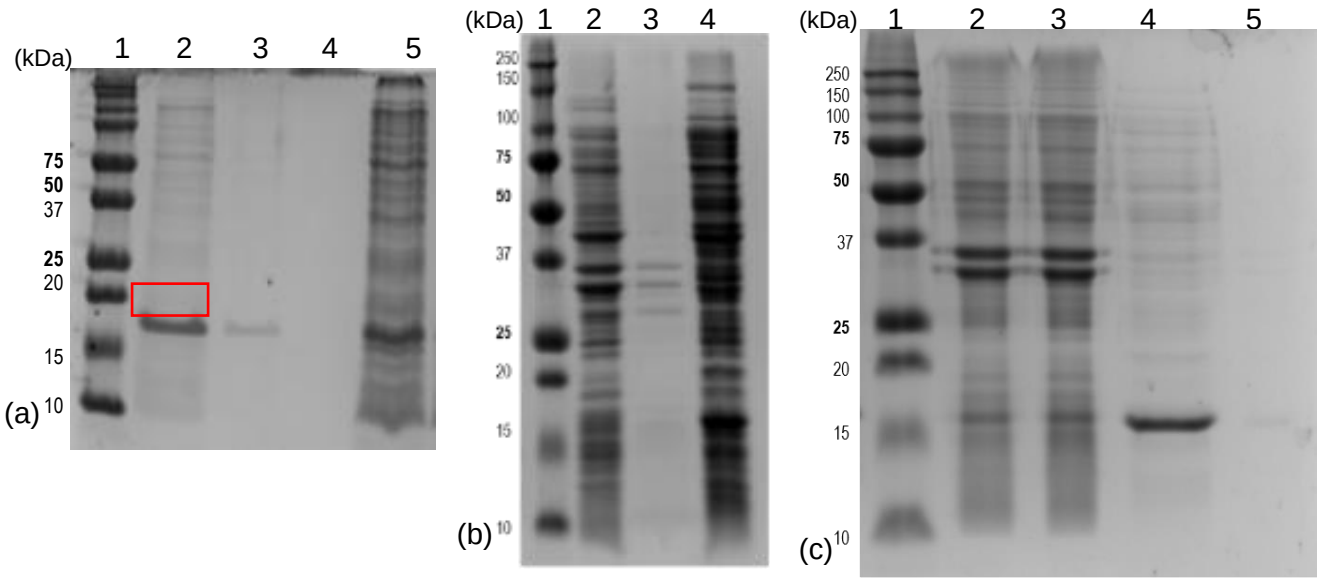


Figure 4-6: Comparison of different extraction methods of holin from cell membrane. (a) Holin extraction method by Chang and colleagues (1995) using ME buffer. Lane 1 is O'Generuler DNA ladder mix, lane 2 is holin lysate resuspended in ME buffer which was used for lanes 4 and 5, lane 3 is holin re-suspended in 0,1 % Triton X-100, lane 4 is flow through from ultrafiltration holin, lane 5 is concentrated holin using ultrafiltration.

(b) The carbonate extraction method (Molloy *et al*., 2000) compared to the HEPES buffer method, where 4 mℓ of cell culture was used to extract the holin proteins. Lane 1 is DNA ladder, lane 2 is holin extraction using HEPES buffer, lane 3 is holin extraction using carbonate buffer, lane 4 is cell-free extract.

(c) The use of the carbonate extraction method (Molloy *et al*., 2000) increased to 50 mℓ instead of the 4 mℓ used in (b). Lane 1 is DNA ladder, lanes 2 & 3 are holin extraction using carbonate buffer, lane 4 is supernatant from the carbonate buffer extraction and lane 5 is the wash after first ultracentrifugation step in the carbonate buffer extraction method.

In lane 2 of Figure4-6 (a), the very faint band between 10 & 15 kDa (indicated by the red box) may indicate holin, as the expected size was ~12kDa. Although the HEPES extraction showed more proteins in lane 1 there was no distinct protein between 10 & 15 kDa to indicate holin. Thus, the carbonate extraction method was chosen for extraction from a larger volume (50 mℓ), used as the cell sample for Figure4-6 (b). Although more proteins could be extracted using a larger volume, there were still no distinct bands between 10 and 15 kDa in lanes 2 & 3 to indicate holin.

4.3.3 Western blotting of holin His-tag

A Western blot was performed using anti-Histidine antibodies according to section 4.2.8 to confirm whether the His-tag demonstrate the extraction of holin (Figure 4-7). There is a band that consistently appears on the Western blot at about ~37 kDa when compared to the ladder. However, this band also appears in the control lanes (Figure 4-7, lanes 3 & 4) which are not transformed with the holin gene. This band was previously analysed using LC-MS/MS and was found to not correlate with lambda holin protein.

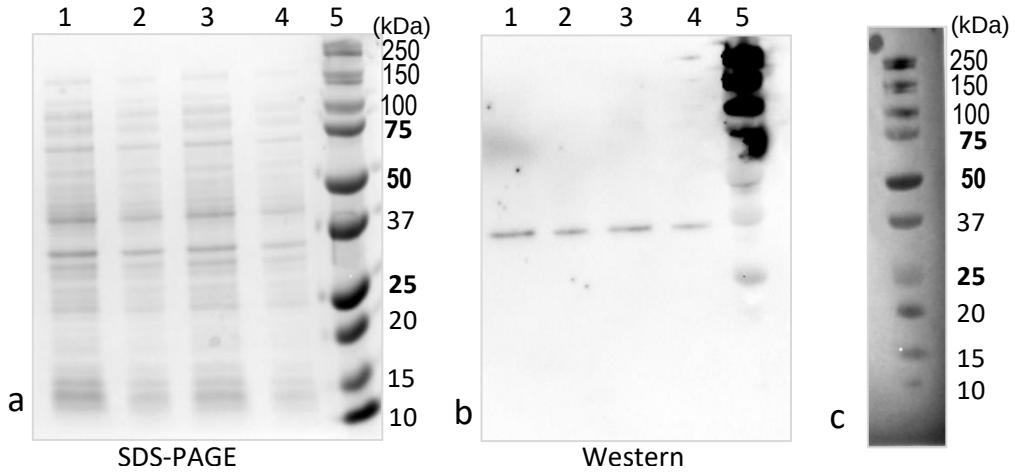


Figure 4-7: Duet holin and Duet control on (a) 12 % SDS-PAGE and (b) Western blotting with anti-His antibodies conjugated with HRP. (a) On 12 % SDS-PAGE; lane 1–15 µℓ Duet holin lysate, lane 2–7,5 µℓ Duet holin lysate, lane 3–15 µℓ Duet control lysate, lane 4–7,5 µℓ Duet control lysate, lane 5–Bio-Rad Precision Plus Protein™ Prestained Protein Standard. (b) Western blotting with Anti-Histidine antibodies; lane 1–15 µℓ Duet holin lysate, lane 2–7,5 µℓ Duet holin lysate, lane 3–15 µℓ Duet control lysate, lane 4–7,5 µℓ Duet control lysate, lane 5–Bio-Rad Precision Plus Protein™ Prestained Protein Standard. (c) Bio-Rad Precision Plus Protein™ Prestained Protein Standard run on 12 % SDS-PAGE as comparison to ladder on Western blot.

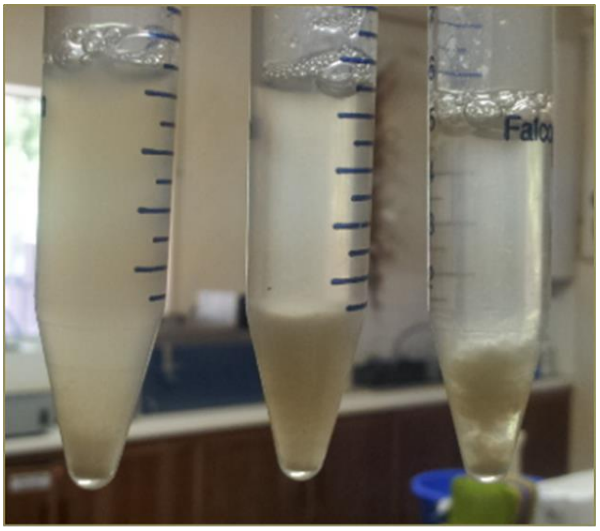
4.3.4 Evidence of correctly expressed endolysin and holin proteins

The transformed cells containing lysin and holin were investigated for expression of the endolysin and holin. Aside from the results obtained by SDS-PAGE and LC-MS/MS in 4.3.2–4.3.4, the transformant strains were also subject to autolysis using assisted and non-assisted autolysis of cells in 4.2.4.

4.3.4.1 Freeze-thaw showing lysis of bacterial cells

This experiment demonstrated the effect of the expressed endolysin and holin proteins *in vitro*. The freeze thaw experiment (Figure 4-8) proved that the recombinant endolysin is active because the comparison between Duet lysin and Duet control post-freeze thaw is markedly different. This means that the endolysin was able to access and degrade the cell wall intracellularly due to cell membrane damage from freeze thawing. The exaggerated cell lysis observable in the transformant expressing

holin and lysin simultaneously was also notable. As the cells have only gone through a single freeze-thaw cycle the decline in viable cells is expected to not be more than 40 %, as evidenced by other studies (Harrison, 1955; Sleight *et al*., 2006). Thus the obvious differences can be attributed to the correctly expressed endolysin and holin proteins.



Duet control Duet lysin Duet lysin and holin

Figure 4-8: Freeze thaw experiment demonstrating positive lysis in Duet lysin and Duet holin and lysin transformants compared to Duet control cells. Viable cells can be observed in suspension in the control, whereas the lysin-only transformant displays cellular debris sedimentation and the lysin and holin transformant displays cell breakage.

The freeze-thaw experiments were modified according to a study on the production of T7 lysozyme expressed in *E. coli* pBAD expression system, showing the effect of low-level production of T7 lysozyme in the lysis of producer *E. coli* cells (Wanarska *et al*., 2007). Freeze/thawing creates micro ice crystals which penetrate the cell membrane and allow for weakened areas which may lead to breakage. This process has been used as a means of cell lysis to release proteins after expression of T7 lysozyme (Studier, 1991; Wanarska *et al*., 2007).

4.3.4.2 Autolysis comparing native and mutant forms of holin

Further investigation into the expression and activity of the endolysin and holin was the autolysis experiment (described in 4.2.4). In Figure 4-9 the differences between lysed cells and unlysed cells are distinct. Lysed cells have an opaque collected mass surrounded by the media and cellular contents, while unlysed cells are in suspension even though some cells sediment at the bottom of the flask. There is no clear, translucent broth in the latter as the cells are suspended in the media.

Figure 4-9: Autolysis of Duet lysin, Duet holin and lysin and Duet mutant holin and lysin compared to Duet control, Duet holin and Duet mutant holin. The lysin and holin containing transformants (Duet holin and lysin and Duet mutant holin) show a sludgy mass indicative of cell breakage and DNA in the extracellular media. Duet control and holin only transformants (Duet holin and Duet mutant holin) show a culture still in suspension, indicative of unlysed cells.

A surprising observation was made, in which the Duet lysin transformant also showed autolytic activity. This is an unusual and unexpected result when compared to literature which states that holin is necessary for the endolysin to reach the cell wall intracellularly (White *et al*., 2011), yet this result was consistently observed.

Lysin only transformants (Duet lysin) may have holin or holin-like proteins which are being expressed and maintained by the cell and not introduced via cloning. This is plausible because it is known that programmed cell death in bacterial cells have parallels with apoptosis in eukaryotic cells (Pang *et al*., 2011). Both systems have holin-like proteins and antiholins which work together to regulate cell death as well as an autolytic enzyme which is similar to endolysin (Saier & Reddy, 2015). In the case of bacterial programmed cell death, it is possible that the mechanisms are linked to phage holin, antiholin and endolysin as phages are known for their ability to move genetic material around during infection and integration events (Bayles, 2014).

In some cases the SOS regulon responding to DNA damage may regulate this apoptotic or programmed cell death response as in the case of *Caulobacter crescentus* (da Rocha *et al*., 2007), which may potentially be a reason for the unexpected autolytic event occurring in Duet lysin. Most of these transgenetic elements are beneficial either for the host, the phage, or the survival of both; which promotes the acquisition of such genes (Jassim & Limoges, 2014). Thus, an explanation for the autolysis as observed by the lysin-only transformant (Figure 4-7) may have some grounding in the presence of holin or holin-like proteins, which allow the expressed endolysin to cross the inner membrane barrier (Bayles, 2014). Holins are naturally found in both Gram-negative and -positive bacteria (Saier & Reddy, 2015) and may be genomic or phage-related genes. If this is the reason for

holin to be present in the transformant in spite of not being transformed with plasmid containing the holin gene, it is still unknown what would trigger the transcription and translation of the gene. In an unprobable scenario, if naturally occurring holin gene is induced in the presence of IPTG this may account for the unexpected lysis of the lysin-only transformant.

The A52G holin mutant was reported to shorten the time between when the holins are produced and hole formation in the cell membrane (Young, 1992). The wild type holin usually causes lysis to begin 45 min post-induction in a lysogen. However, the mutant holin which has a single mutation of A52G, induced lysis at about 20 min after induction (Johnson-Boaz *et al*., 1994). This mutant affects the particle burst size of phages because it prematurely lyses the host before there are sufficient virions packaged in the late lytic stage. As this study aimed to determine the ability of the expressed wild type holin to assist the endolysin in penetrating the LPS layer of Gram-negative bacteria externally; it was hypothesised that the mutant holin would perform better for these purposes.

To further test the efficiency of the mutant holin versus that of the wild type holin in conjunction with endolysin, supernatant samples were taken at various time points and analysed for protein content (section 4.2.4.2, Figure 4-10). This would indicate whether there was autolysis due to the holin-endolysin combination as well as whether the mutant holin facilitated faster lysis than its counterpart.

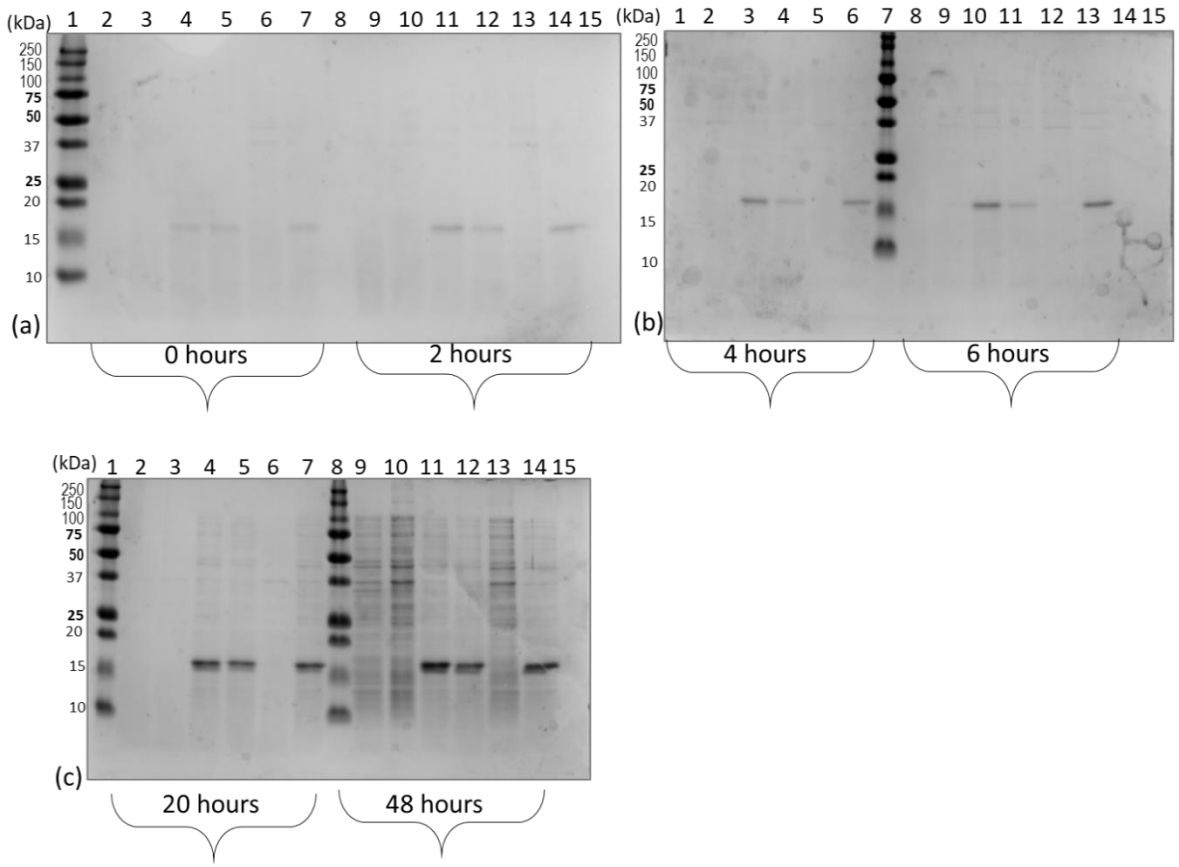


Figure 4-10: SDS-PAGE analysis of samples of IPTG induced transformants at various time points (0 hours, 2 hours, 4 hours, 6 hours, 20 hours and 48 hours respectively) and visualised on 12 % SDS-PAGE. Duet control samples are lanes: a2, a9, b1, b8, c2 and c9. Duet holin samples are lanes: a3, a10, b2, b9, c3 and c10. Duet lysin samples are lanes: a4, a11, b3, b10, c4 and c11. Duet holin and lysin are lanes: a5, a12, b4, b11, c5 and c12. Duet mutant holin samples are lanes: a6, a13, b5, b12, c6 and c13. Duet mutant holin and lysin samples are lanes: a7, a14, b6, b13, c7 and c14. DNA ladder are lanes: a1, b7, c1 and c8. Empty lanes are; a8, a15, b8, b15 and c15. As observed in Figure 4-9, the unexpected presence of lysin in the supernatant of Duet lysin indicating autolysis. Protein bands are observed between 15 kDa - 20 kDa which correlates to lambda endolysin ~18 kDa.

4.3.5 Mass spectrometry analysis for expressed protein identification

The LC-MS/MS results showed the queries matching several unique peptides with mass ranging between 17814–17883 Da. The high scores of 509–511 matched the peptides of proteins such as: gene 62 protein (Enterobacteria phage Sf6); endolysin (Enterobacteria phage lambda); lysin (Enterobacteria phage HK022); lysin (Enterobacteria phage HK97); Chain A, Bacteriophage lambda Lysozyme Complexed with A Chitohexasacharide; lysin (*Escherichia coli* UTI89) and lysozyme (Enterobacteria phage IME10) (http://cbio.ufs.ac.za/mascot/cgi/master_results.pl?file=../data/20160418/F009898.dat). These protein identifications share homologous identity with λ lysin which is a good indication that the protein band excised was λ lysin.

The ~18 kDa bands in lanes 4, 5, 9 and 10 of Figure 4-5 were excised from the SDS-PAGE for LC- MS/MS identification as described earlier. Of the results for this protein band, the masses of the

queries that were similar range between 17814–17883 Da and had several unique peptides as covered in section 4.3.4. The bands that were excised were intense, which reflected in the MS-MS data by the query hits being several of bacteriophage and endolysin or lysozyme origin. The proteins with the highest hit values identified with that of lambda endolysin and other closely related proteins.

The LC-MS/MS results were queried via MASCOT (www.matrixscience.com), which is an extensive search engine that queries mass spectrometry data against databases such as MSDB (Proteomics Department at the Hammersmith Campus of Imperial College London); NCBInr (NCBI's non- identical protein database); SwissProt (non-redundant searches and useful for peptide mass fingerprint searches) and dbEST (GenBank's Expressed Sequence Tag–searched if other databases do not provide a match) (Perkins *et al*., 1999). This search engine queries the data using three distinct criteria, which are: peptide mass fingerprint, sequence query and MS/MS Ion search. The first criterion refers to the peptide's mass, the second is the peptide mass together with the amino acid sequence and finally the third is searching MS-MS data as yielded without interpretation. The protein of interest is analysed after being digested by a proteolytic enzyme e.g. trypsin (section 4.2.3) by comparing the experimental mass values with the fragment ion mass values and searching for an identical or closely homologous entry in the databases. MASCOT gives multiple identification results of the protein that is queried and it is important to check the list of results and look for the mass cut off that is similar to the expected protein (Perkins *et al*., 1999). This is followed by searching for a unique peptide or unique peptides within the given results. A higher score value indicates a better match for the protein of interest and is concurrent with low expect values, which are indicative of random matches in the databases. Finally, the Exponentially Modified Protein Abundance Index (emPAI) values provide information on relative quantitation of the proteins in the run and the peptide matches that result from the database search (Ishihama *et al*., 2005; Shinoda *et al*., 2009).

Part B: Expression in *Y. lipolytica*

The aim of this section was to express the endolysin protein in *Y. lipolytica* Po1h using pINA 1317 – YlCWP110 with protein presented on the cell surface as well as using the pINA 1317 secretion vector for the secretion of the recombinant endolysin protein. The cell surface display vector pINA1317-YlCWP110 used in this study allows for the fusion of a protein of interest to the C- terminal anchor domain of *YlCWP1,* which is naturally GPI-anchored to the cell wall (Yue *et al.*, 2008). GPI anchors are post-translationally added to the C-terminus of various eukaryotic proteins and anchor proteins to the cell wall facing the exterior of the cell (Paulick & Bertozzi, 2008). The protein is attached to the glycan core of the GPI anchor through a phosphoethanolamine bond.

The use of pINA1317-YlCWP110 for displaying endolysin was considered especially potentially useful as a whole cell treatment for bacterial infections of poultry, as it would include the potential added benefits of nutritional value in the form of single cell protein for the poultry.

4.3.6 Cloning of lysin gene inserted into yeast vector pINA 1317–YlCWP110

The lysin gene of bacteriophage lambda was synthesized by GeneArt (Life Technologies™) with optimisation for expression by *Y. lipolytica* as well as to contain additional *Sfi*I and *Hin*dIII restriction sites. The synthesised gene was successfully removed through double digestion with *Sfi*I and *Hin*dIII restriction enzymes. The gene was successfully ligated into pINA 1317–YlCWP110 vector and propagated in *E. coli* cells. The re-digestion of the recombinant vector with the *Sfi*I and *Hin*dIII enzymes (Figure 4-11) were done experimentally to confirm the *in silico* insertion of the gene into the pINA 1317–YlCWP110 vector simulated using Geneious v.9.1.2 (Figure 4-12).

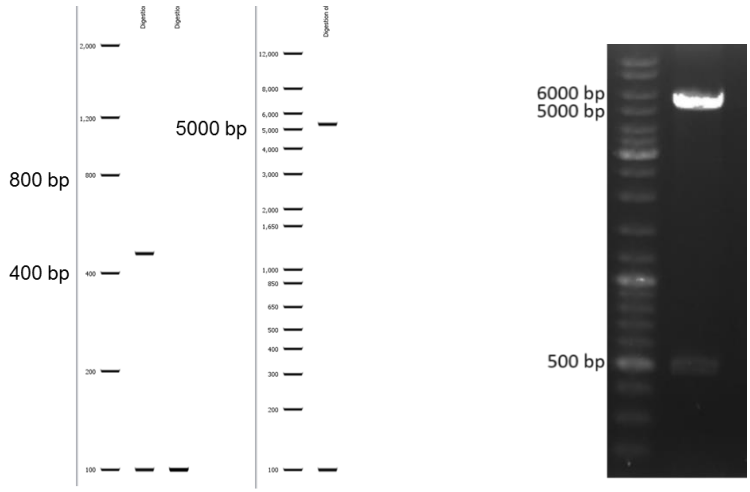


Figure 4-11: *In silico* digestion of pINA 1317 secretion vector with *Hin*dIII and *Sfi*I for confirmation of lysin gene insert (left) and the *in vitro* confirmation (right). The lysin gene excised is ~500 bp and the backbone of digested is expected to be ~5200 bp GeneRuler DNA Ladder Mix is used as DNA ladder in the *in vitro* gel. Similar results were obtained for pINA 1317–YlCWP110.

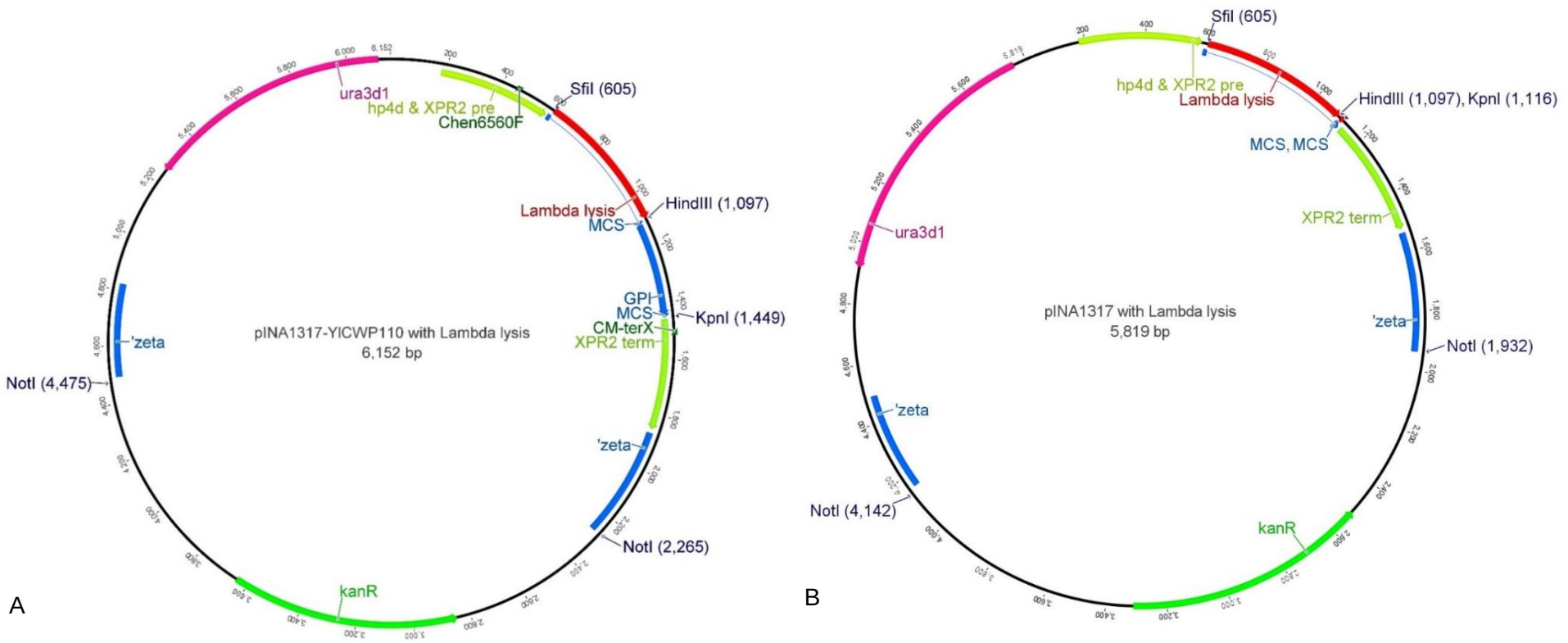


Figure 4-12: *In silico* plasmid map of lambda lysin gene (*R*') ligated into MCS of pINA 1317–YlCWP110 for surface display (A), and pINA 1317 for secreted recombinant proteins (B). Enzymes used to digest the gene and plasmid are *Sfi*I (605 bp) and *Hin*dIII (1097 bp). The hp4d promotor and the XPR2 secretion signal is indicated upstream of the lambda lysin and GPI-anchor sequence (in A only). The primer pair Chen6560F and CM-TerX are used to confirm the insertion of the gene and result in a PCR product of around 1060 bp. The bacterial backbone contains the kanamycin resistance gene for selection in *E. coli* and can be removed by digestion at *Not*I restriction sites at 2265 bp and 4475 bp. The Zeta-sites are for random integration of the yeast expression cassette into Po1h and the ura3d1 marker for selection in Po1h cells.

4.3.7 Yeast transformation and confirmation of gene insertion

Y. lipolytica Po1h transformants were selected from YNB-N_{5000} plates and confirmed via colony PCR to contain the lambda lysin gene. The resultant PCR products (Figure 4-13) were confirmed via Sanger sequencing and alignment (Figure 4-14).

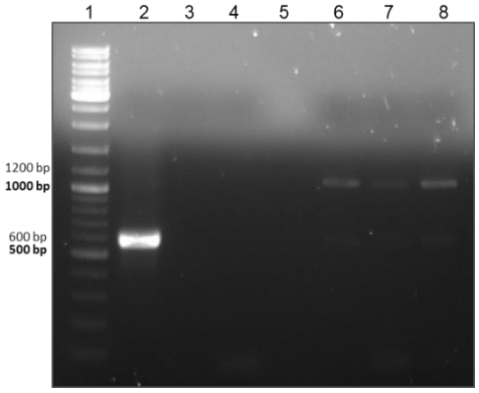


Figure 4-13: Confirmation of lysin gene-CWP integration into *Y. lipolytica* Po1h. Transformants were confirmed to contain the lysin gene by PCR using the Chen6560F and CM-TerX primers. Lane 1–GeneRuler DNA Ladder Mix, lane 2–PCR product of pINA-YlCWP110 without insert, lane 3–No template negative control, lanes 4-8–Clones 1-5. The correct PCR product size of ~1070 bp was observed in lanes 6-8. Similar results were obtained for pINA1317-lysin transformants (results not shown).

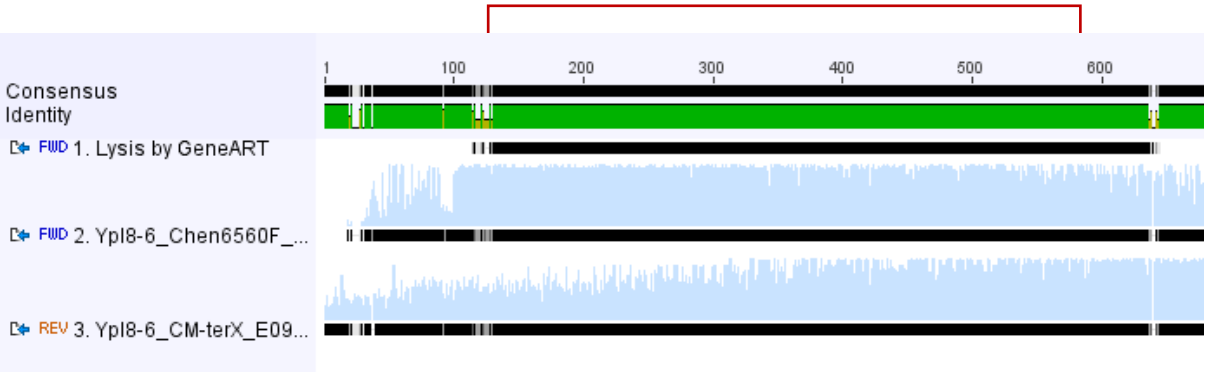


Figure 4-14: Alignment of lysin sequences amplified from transformants with the reference sequence provided by GeneArt. The red lines indicate the open reading frame (ORF, bases 605 to 1096) and the solid black bar between them indicates 100 % homology between the sequences. Similar alignments of sequences from pINA1317- lysin were also obtained.

The correct lysin gene sequence was thus shown to be integrated into the *Y. lipolytica* genome via both pINA 1317 and pINA 1317-YlCWP110 vectors.

4.3.8 Scanning electron micrographs of the cell surface of transformed yeast

The transformed yeast cells were subjected to scanning electron microscopy to observe whether there were differences between the cell surfaces of the control yeast (untransformed) and the transformants. As can be seen in Figure 4-15 the surfaces of control cells (a, b) appear smooth while some of the test cells (c, d) appear to have protrusions on the cell surface. Attempts to detect

secreted lysin and to remove displayed lysin by treatment with phospholipase C were however unsuccessful (results not shown).

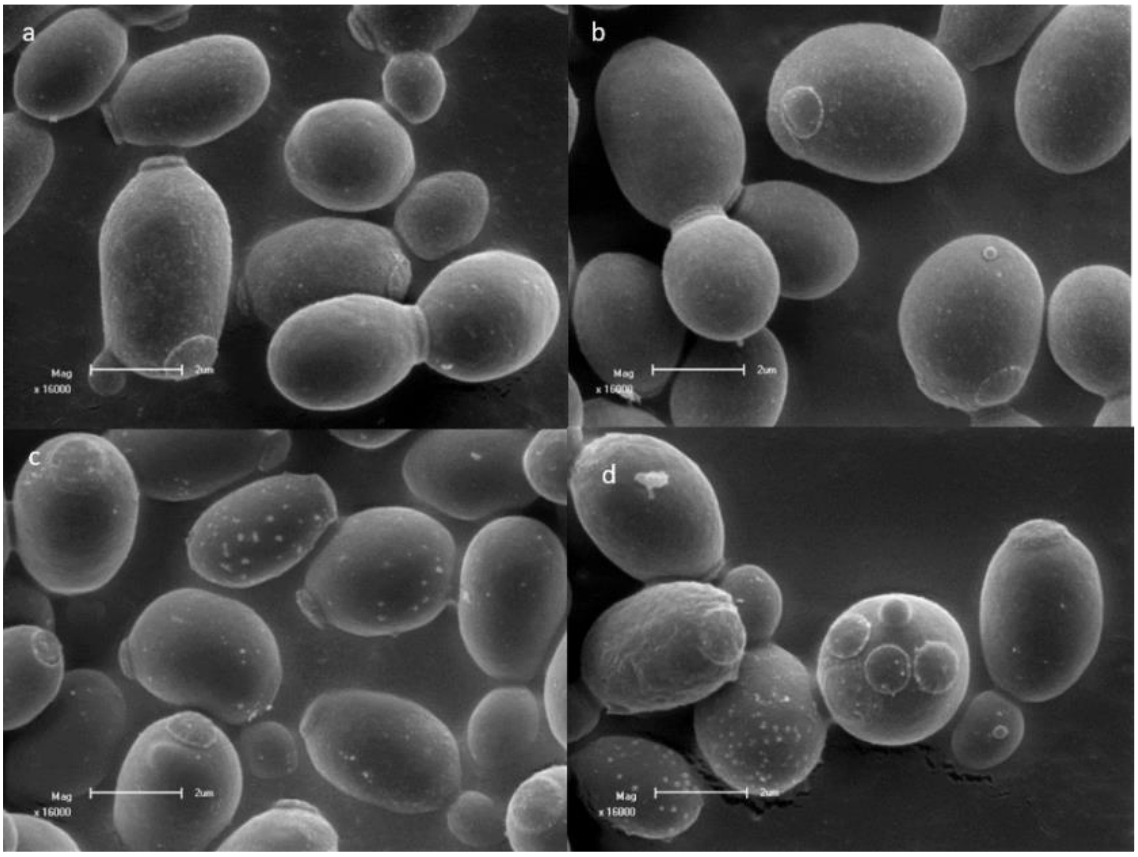


Figure 4-15: SEM micrographs comparing the negative control strain at 12 hours (a) and 24 hours (b) with the transformant cells at 12 hours (c) and 24 hours (d). Magnification is at 16 000 x and bar scale at 2 µm. Protrusions appear on cells from the transformants that are absent in control cells.

4.4 Concluding remarks

To summarize, the endolysin and holin proteins were correctly cloned into *E. coli* cells and both were correctly expressed as observed in the efficacy of autolysis and freeze-thaw experiments. While the endolysin was observed on SDS-PAGE and identified via LC-MS/MS, the expressed holin was unable to be detected via gel electrophoresis or to be purified for bacterial assays.

The inability to observe holin on 12 % SDS-PAGE was unexpected, as this protein has been clearly seen in other research papers (Bläsi *et al*., 1990; Chang *et al*., 1995). Various methods were used for removal of cell membrane bound or imbedded proteins. The buffers (HEPES, carbonate, $MgCl^{2+}$) are based on ionic strength which is able to solvate the outer membrane proteins that are detergent-insoluble (Carlone *et al*., 1986). Although there seemed to be a possible band in Figure 4-7(b) lane 2, when scaled-up from 4 mℓ to 50 mℓ cell sample the expected band of ~12 kDa was not observed. No holin could be observed on the SDS-PAGE even after His-tag purification. However, holin appeared to be expressed, as its effect was clearly demonstrated in Figure4-8 and Figure 4-9.

Expression of lysin by the yeast *Y. lipolytica* was attempted for the potential of using whole recombinant yeast cells for treatment of bacterial infections in poultry. This yeast has GRAS status and proven capabilities of protein production and secretion. The expression of the lysin gene in the Po1h strain of *Y. lipolytica* via pINA 1317-YlCWP110 cell surface vector appeared to cause protrusions on the cell surface (Figure 4-15 c & d) that were absent in the control cells (Figure 4-15 a & b). Yeast cells naturally display proteins on the cell surface, yet there is an observable difference between the micrographs of the transformed cells and their counterpart control cells with the transformed cells showing more protrusions, suggesting that post-transformation there were more proteins displayed on the cell surface when compared to the control cells.

The endolysin protein expressed in *E. coli* BL21 (DE3) and *Y. lipolytica* Po1h competent cells from this chapter were assayed against bacteria in chapter 5 to test the potential for bactericidal activity. The assays were done using bacterial cells that were permeabilised using various permeabilisers including the commercial products Biotronic® Top Line and Virukill®.

Chapter 5 Evaluation of bactericidal activities of λ phage endolysin

5.1 Introduction

Bacteriophages infect bacterial cells and direct the host replicating mechanisms to propagate their own genetic material and producing phage proteins (Marks & Sharp, 2000; Catalão *et al*., 2013). Once mature phages are assembled, they are ready to be released from the host cells (Catalão *et al*., 2013). Endolysin is a bacteriophage lysin that cleaves the β-1,4 linkage between N-acetyl-muramoyl and N-acetylglucosaminyl residues in peptidoglycan (Fischetti *et al*., 2006; Rodríguez-Rubio *et al*., 2013). Although often referred to as murein hydrolases they are technically murein transglycosylases as they produce a terminal 1,6-anhydromuramoyl by transferring glycosyl onto the C-6 hydroxyl group of muramoyl resulting in a non-reducing end (Bienkowska-Szewczyk *et al*., 1981). Endolysin, the product of the bacteriophage lambda *R* gene is a soluble protein that lacks a secretory signal, and therefore accumulates intracellularly in the absence of *S* gene product (holin), which normally allows endolysin access to the peptidoglycan layer. Heterologously expressed bacteriophage endolysins have been researched for use in the medical sector as well as the food industry (Loessner, 2005). Applications include accelerated cheese ripening by adding endolysins to the cheese culture, which saves production costs by decreasing ripening time (Tuler *et al*., 2002). The endolysin PlyP100 from a *Listeria* bacteriophage can be added to fresh cheese, and potentially other ready-to-eat food products, to prevent listerial contamination (Van Tassell *et al*., 2017). Endolysins are desirable as a treatment because they aid in the control of undesirable bacteria without affecting the microbiota (Loessner, 2005). Bacteriophage endolysins have been tested with various lipopolysaccharide (LPS) layer-degrading protein modifications as well as LPS-degrading agents for efficacy against Gram-negative bacteria (Dong *et al*., 2015; Roach & Donovan, 2015; Pastagia *et al*., 2013).

The commercial products used in this chapter as LPS-degrading agents are currently used in the avian industry, and have been proven to effectively decrease bacterial numbers. Biotronic® Top Line is a product which contains a mixture of organic acids and their salts, a phytochemical and the unique Biomin® Permeabilizing Complex. The latter helps the entry of the active ingredients i.e. the organic acids and the phytochemical into the Gram-negative bacteria by permeating the outermembrane (http://www.biomin.net/en/products/biotronic/). Virukill® is a broad-spectrum disinfectant, which uses Poly Dimethyl Ammonium Chloride (120 g/ℓ) as the active ingredient against viruses, bacteria, fungi, mycoplasma, yeast and algae (http://www.icaonline.co.za/Virukill.html).

This chapter evaluates the effect of the expressed endolysin proteins from chapter 4 on bacterial cells. The use of commercial LPS-degrading agents (used as permeabilisers in this work) has been included with the aim to allow them to be effective at lower concentrations with the

addition of expressed endolysin.

5.2 Materials and methods

Part A: *Y. lipolytica*-expressed endolysin

5.2.1 Inhibitory effect of *Y. lipolytica* expressed endolysin on permeabilised *E. coli* K12 cells

The *Y. lipolytica* cells transformed with the endolysin gene from bacteriophage lambda in chapter 4 were used to test the effect of the expressed endolysin on bacterial cells tested with permeabilising agents.

5.2.1.1 Permeabilisation of bacterial cells

E. coli K12 cells were grown in 10 mℓ TSB to an $OD_{600\ nm}$ of 0.6-0.8 and treated with LPS layer-permeabilising agents to allow access of the endolysin to the cell wall. The following permeabilising agents were used: (i) 0.1 M–2 M $MgCl_2$; (ii) 0.1–1 M Tris-EDTA, (iii) 0.1– 1 M Tris (all in 0.1 M increments), and (iv) 1 M PBS.

5.2.1.2 Treatment using whole recombinant yeast cells

Whole cells of recombinant *Y. lipolytica* cells expressing lambda endolysin were tested on *E. coli* K12. Permeabilised *E. coli* K12 cells (100 µℓ) were spread-plated onto TSA plates and left to dry. Thereafter, 10 µℓ of whole cells of recombinant *Y. lipolytica* Po1h cells were spotted onto the dried lawns of *E. coli* K12 cells and left to dry. The plates were incubated at 28 °C with observation over 72 hours.

5.2.1.3 Treatment using supernatants from recombinant cells

Permeabilised *E. coli* K12 cells (1 mℓ) were pelleted by centrifugation at 3000 x *g* for 5 min and resuspended in 900 µℓ TSB and treated with 100 µℓ of concentrated recombinant endolysin extract for 1 hour at 28 °C. The effect of the permeabilisers and the expressed endolysin on the *E. coli* K12 cells was observed by light microscopy using Gram-staining (Gregersen, 1978), and using SEM (section 4.2.13). Untreated *E. coli* K12 cells were used as controls.

5.2.2 Cleavage of GPI-anchored protein using phospholipase-C

The cleavage of endolysin from the GPI anchor on the cell wall surface of *Y. lipolytica* cells was done using 3.32 units of phospholipase-C from *Clostridium perfringens* (Merck, Sigma-Aldrich). Cells were treated in 50 mM Tris-HCl, pH 7.2, 1.0 % Triton X-100 and Complete Protease Inhibitor Cocktail (Roche) for an hour.

The cleaved proteins were concentrated using Amicon Ultra-4, 10 kDa molecular weight limit centrifugal filter unit (Merck-Millipore) by centrifugation at 7000 *x g* for 20 min. The concentrated proteins were resuspended in 1 x Laemmli sample buffer supplemented with 5 % β-mercaptoethanol, followed by boiling for 5 min. Samples were electrophoresed at 120 V for 70 min in a 12 % SDS- PAGE in Tris-HCl Glycine SDS (TGS) buffer. The gel was stained and destained using Fairbanks solutions A and D, respectively (Fairbanks *et al*., 1971). Protein gels were visualised using a Bio-Rad Gel Doc EZ system.

5.2.2.1 Protein precipitation and concentration

Three protein precipitation methods were used in this study, using either (i) trichloroacetic acid (TCA), (ii) ammonium sulphate, or (iii) acetone. Ultrafiltration columns were used to concentrate proteins from culture supernatant as well as precipitated proteins. Protein pellets were resuspended in 1 x sample buffer with fresh 5 % β-mercaptoethanol. The sample was vortexed briefly and incubated at 95 °C for 5 min before being loaded on a 12 % SDS-PAGE gel as described in 5.2.2.

5.2.2.2 Tri-chloro acetic acid (TCA) *precipitation*

TCA (100 %) was diluted to 10 % in the sample and inverted 8 times before being incubated at -20 °C overnight. After thawing, the sample was centrifuged at 7195 *x g* for 15 mins and the supernatant decanted. One (1) mℓ of milliQ water and 10 mℓ ice-cold 100 % acetone was added to the pellet, followed by vortexing for 30 secs every 20 min for an hour. The tubes were incubated at -20 °C overnight, followed by centrifugation at 7195 *x g* for 15 mins and decantation of the supernatant.

5.2.2.3 Ammonium sulphate [$(NH_4)_2SO_4$] *precipitation*

A saturated ammonium sulphate solution was made by dissolving 375 g of ammonium sulphate to 500 mℓ of distilled water in a Schott bottle. This was added to the protein extract at 30 % of the volume while stirring the solution for ~20 min and allowing precipitates to form while being careful not to form froth at the air interface. The mixture was centrifuged at 10 000 x *g* for 15 min and the precipitates collected by decanting the supernatant into another beaker. The saturated ammonium sulphate solution was added to the remaining supernatant at a 70 % volume and precipitates collected.

5.2.2.4 Acetone precipitation

Acetone (100 %) was added to 4 mℓ solution of sample culture to 10 % (v/v) and stored at -20 °C overnight for protein precipitation. The sample was thawed, centrifuged at 20 000 x *g* at 4 °C for 10 mins and the supernatant was decanted.

5.2.3 Concentration of proteins using ultrafiltration columns

Proteins both in solution or after precipitation were loaded onto Amicon Ultra-4 centrifugal filter unit with a 10 kDa molecular weight limit (Merck-Millipore), followed by centrifugation at 7000 x *g* for 20 min. The flow-through was discarded and the proteins washed on the filter column with 50 mM Tris-HCl buffer pH 7.4 and centrifuged at 7000 x *g* for 20 min. The proteins were resuspended in 100 µℓ 50 mM Tris-HCl buffer pH 7.4.

Part B: *E. coli* expressed endolysin

5.2.4 Inhibitory effect of *E. coli* expressed endolysin on permeabilised bacteria

As with the *Y. lipolytica*-expressed endolysin mentioned in sections 5.2.1–5.2.3 above, the bacteriophage lambda endolysin expressed by transformed *E. coli* cells (also from Chapter 4) was used to determine the effect of the endolysin in conjunction with bacterial cells permeabilised by the commercial products: Biotronic® Top Line and Virukill® as well as organic acids.

5.2.4.1 Bacterial assay on 96-well plate using His-tag purified endolysin

His-tag purified endolysin (section 4.2.5) was used externally to test against *E. coli* K12 (Veterinary Biotechnology collection), *Salmonella* Enterica Diarizonae (ATCC 12325; UFSBC 528) and *Lactococcus garvieae* (Veterinary Biotechnology collection) to test the effect of endolysin on bacterial cells. The strains to be tested were grown at $OD_{600\ nm}$ 0.6-0.8 and incubated at 37 °C, 150 rpm on a shaker. In a 96-well flat-bottomed titre plate 100 µℓ of TSB was added to the wells that were used for the assay. Then acid LPS-layer permeabilisers–0.05 % formic acid (columns 1 & 4, rows A-F), 0.05 % phosphoric acid (columns 2 & 5, rows A-F), and a combination of 0.025 % formic & 0.025 % phosphoric acids (columns 3 & 6, rows A-F)– were added for degradation in the LPS-layer. To these wells 100 µℓ of His-tag purified endolysin (section 4.2.5) was added and 10 µℓ of test strains were added as follows: *E. coli* (rows A 1-6; D 1-6), *L. garvieae* (rows B 1-6; E 1-6) and *S. aureus* strains (rows C1-6; F1-6). Positive controls for strains were *E. coli* in well A 8 & D 8, *L. garvieae* B 8 & E 8, and *S. aureus* strains C 8 & F 8. Negative controls of 100 µℓ broth were in wells: A 10-11, B 10-11 and C 10-11.

5.2.5 Bacterial assay using *E. coli* expressed endolysin on bacteria permeabilised with Biotronic® Top Line and Virukill®

Lysate was prepared as previously described (4.2.4.2) with the exception of protein purification. Bacterial test strains to be used as potential substrate in the bacterial assays were the following: *E. coli* K12 (Veterinary Biotechnology collection), *Salmonella* Enterica Diarizonae (ATCC 12325; UFSBC 528), *Pseudomonas aeruginosa* (UFSBC 13) and *Staphylococcus aureus* (ATCC 25923). These strains referred to as test strains were grown in TSB at 37 °C to an $OD_{600\ nm}$ of 0.6-0.8, cells

centrifuged and the cell pellet washed twice with 20 mM Tris-HCl, pH 7.0 before being re-suspended in the same buffer to an $OD_{600\ nm}$ of 0.5 ± 0.05 (Dong *et al*., 2015). To test the effect of selected permeabilising agents, bacterial lysates were prepared as previously described, except that the resuspension buffer of 20 mM Tris-HCl, pH 7.0 contained the permeabilising agent.

In these cases, cultures were incubated for 30 min to allow the cells to interact with the permeabilising agent. Thereafter, the cells were centrifuged and re-suspended in 20 mM Tris-HCl, pH 7.0 to an $OD_{600\ nm}$ of 0.5 ± 0.05. Both the treated and non-treated samples were added to the unpurified endolysin lysates in a 1:3 ratio. The control had buffer (20 mM Tris-HCl, pH 7.0) instead of the lysate. The reaction was incubated at 30 °C for 1 hour, before being diluted ten-fold and plated out in triplicate for plate counts.

The permeabilising agents used were Biotronic® Top Line and Virukill®. Minimum inhibitory concentrations (MICs) were established in triplicate for each test bacterial strain using each permeabiliser, according to EUCAST (European Committee for Antimicrobial Susceptibility Testing) standards of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID), (2003) for MIC determined by broth dilution. The MIC is defined as the highest dilution at which the growth of bacteria is still inhibited. Biotronic® Top Line and Virukill® were diluted to stock concentrations of 2 % and 1 % respectively prior to the MIC assays.

Student t-tests with unequal variances at 0.05 confidence level were used to determine the statistical significance of the test versus controls.

5.3 Results and Discussion

The aim was to determine whether the heterologously expressed λ holin and endolysin could have an effect on bacteria when applied externally in the presence of LPS permeabilising agents. The final objective was to use the expressed proteins for bacterial assays with and without the presence of LPS permeabilising agents to determine their effect on selected bacterial species.

Bacterial cell treatment with permeabilising agents allows access to the cell as it makes the outer membrane more penetrable. The LPS outer membrane of Gram-negative bacteria are robust and the lack of glycerophospholipids ensures that macromolecules cannot diffuse through to the cell wall (Vaara, 1992). Thus, treatment with a permeabilising agent will allow larger molecules to pass through the hydrophobic environment to access the cell wall beneath.

Tris binds to the lipopolysaccharide layer and replaces the Ca^{2+} and Mg^{2+} from their binding sites, effectively permeabilizing the outer membrane through destabilization (Vaara, 1992). The role of magnesium chloride is similar as it replaces the Mg^{2+} in the LPS layer (Hancock *et al*., 1981). EDTA is a known chelator of Mg^{2+} and allows the penetration of hydrophobic compounds through the LPS

layer (Egli, 2001). Increasing concentrations of phosphate buffer have also been demonstrated to increase permeability of *E. coli* cells (Gudiminchi & Smit, 2011).

Part A: *Y. lipolytica*-expressed endolysin

5.3.1 Scanning electron micrographs of *E. coli* cells treated with permeabilising agents

Cells were observed using SEM (Figure 5-1) to identify any structural alterations related to the treatment with permeabilising agents.

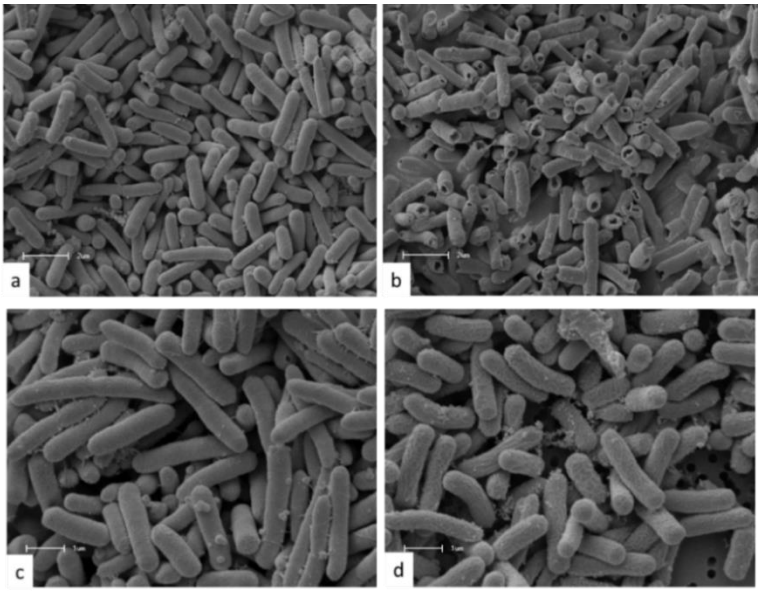


Figure 5-1: SEM micrographs comparing *E. coli* K12 cells grown for 12 hours without permeabilising agent treatment at (a) 12 000 x magnification and (c) 20 000 x magnification; cells treated with (b) 0.5 M EDTA at 12 000 x magnification and cells treated with (d) 1.5 M Tris at 20 000 x magnification.

The EDTA-treated cells (b) show cells that have holes in them when compared to the control cells (a). While the Tris-treated cells (d) have a ridged membrane appearance when compared to the control cells (c). The SEM results on bacterial cells treated only with 1 M Tris demonstrated that the effects observed in Gram-staining were probably only due to the 1 M Tris rather than the recombinant endolysin (results not shown).

The treatment of the *E. coli* cells with 1 M Tris apparently caused the cells to stain purple during Gram-staining (Figure 5-2). This was surprising because typically Gram-positive bacteria have a thicker layer of peptidoglycan which traps the crystal violet-iodine complex while the Gram-negative bacteria lose this complex because of a thin peptidoglycan layer as well as the ethanol removing the LPS layer and parts of the peptidoglycan layer allowing the crystal violet-iodine complex to be washed off.

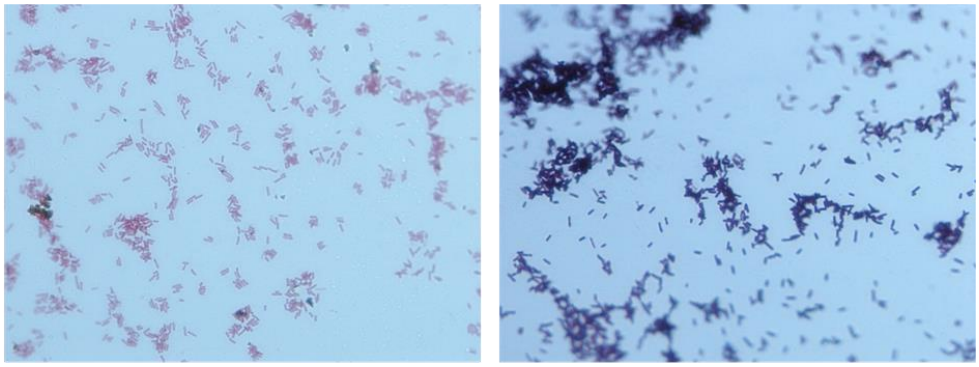


Figure 5-2: Light micrograph of Gram-stained untreated *E. coli* K12 cells (left) and cells treated with 1 M Tris and supernatant of *Y. lipolytica* Po1h transformed with endolysin (right). A change in Gram-staining properties was observed.

5.3.2 Inhibitory effect of *Y. lipolytica* expressed endolysin on permeabilised *E. coli* K12 cells

Based on the observed cell surface differences between *Y. lipolytica* negative control cells and transformant cells (Figure 4-15), *E. coli* K12 cells treated with permeabilising agents as mentioned in section 5.2.1 above were treated with whole *Y. lipolytica* transformants. However, significant differences due to the presence of the yeast cells could not be observed when compared to the *E. coli* K12 control cells that were treated only with permeabilisers. Subsequently, the following sections were aimed at obtaining the isolated protein and testing it on the bacterial cells.

5.3.3 Cleavage of GPI-anchored expressed endolysin

Since treatment with whole recombinant yeast cells did not yield satisfactory results, focus was shifted to isolating the recombinant endolysin for further testing.

The enzymatic removal of surface displayed proteins for protein isolation purposes has previously been reported (Bulani *et al.*, 2012). In that case, the recombinant protein of interest was removed from fusion surface displayed proteins using enterokinase. Since this approach was unsuccessful in previous work in our research group (van der Westhuizen, 2017), phospholipase C treatment was considered instead to cleave the GPI-anchor itself (Müller *et al.*, 2012).

The cleavage of the expected GPI-anchored endolysin using phospholipase C was however unsuccessful, as recombinant endolysin could not be detected even after different precipitation methods (results not shown).

Reasons for ineffective cleavage of GPI anchors may be due to availability of the GPI group in regard to the phospholipase C, as well as modifications on the GPI anchor itself. Phospholipase C may discriminate between GPI-anchors depending on e.g. whether the GPI has a further fatty acid moiety, like palmitic-acid, on the 2-hydroxyl on the inositol group which may lead to the anchor being resistant

to cleavage (Müller *et al*., 2012).

5.3.4 Protein precipitation of secretory and cleaved endolysin

Three protein precipitation methods were used namely trichloroacetic acid (TCA), acetone- and ammonium sulphate precipitation. These were used on extracts from both the original *Y. lipolytica* Po1h control cells as well as the transformant to observe recombinant endolysin at ~18 kDa.

At low concentrations, salts stabilize protein molecules, but beyond the point of maximum protein solubility salt removes water molecules from around the proteins and thereby precipitates the protein by salting-out (Wingfield, 2001). TCA also precipitates proteins by removing water molecules from around the protein molecules. Precipitation using acetone is based on the dielectric constant of the solution, as organic solvents with small dielectric constants, e.g. acetone, destabilize the dispersion of protein molecules in the solution unlike hydrophilic solvents with large dielectric constants (Mohsen-Nia *et al*., 2010). Of the methods used, the 10 % TCA was the least successful as no bands could be observed on 12 % SDS-PAGE (Figure 5-3). Faint bands were observed using ammonium sulphate precipitation in conjunction with ultrafiltration, while clearer bands were observed with acetone precipitation (Figure 5-3).

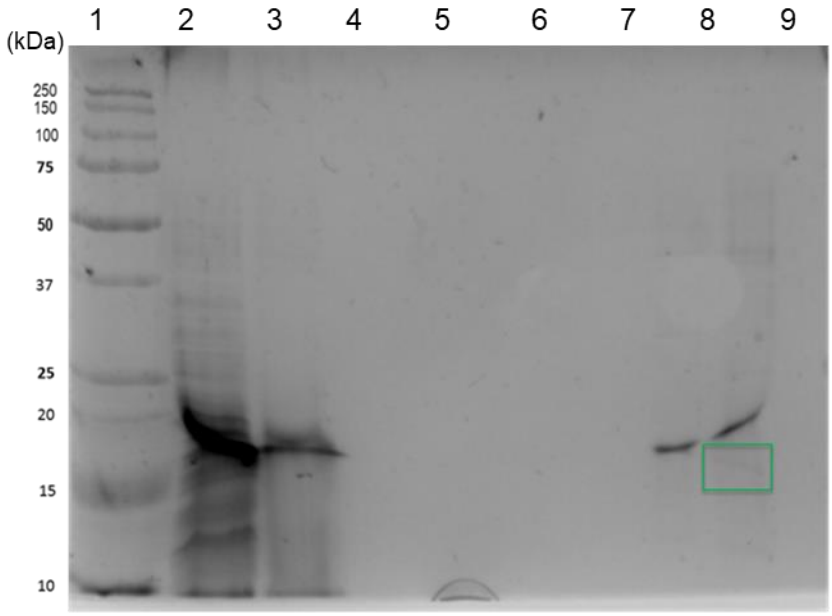


Figure 5-3: 12 % SDS-PAGE for comparison of the different protein precipitation methods used in this study. The gel shows the comparison of the proteins precipitated in the control and transformants using acetone (lanes 2 & 3), 10 % TCA (lanes 4 & 5), ammonium sulphate (lanes 6 & 7) and the concentrated ammonium sulphate precipitates run on an ultrafiltration column (lanes 8 & 9). The green block in lane 9 indicates where a very faint band is observed with the ammonium sulphate sample within the expected range of the endolysin.

However, neither case yielded specific bands in the test that were absent in the control, except potentially a faint band when ammonium sulphate precipitation was used. This band could however

not be detected using LC/MS-MS. This may be due to insufficient protein as the band is very faint, poor digestion or a combination of both. The general lack of proteins as observed from the concentration of the supernatants suggest that the proteins were either not expressed or poorly expressed.

Part B: *E. coli* expressed endolysin

5.3.5 Purified endolysin tested on bacterial cells

5.3.5.1 Endolysin purification using Fast Protein Liquid Chromatography

To test the effect of endolysin on bacterial cells, His-tagged recombinant endolysin was purified using gravity flow on a nickel-based IMAC column (section 4.2.5) from *E. coli* containing the pET28-lysin (Table 4-4). The fractions taken from the nickel column were tested on *E. coli*, *L. garvieae* and *S. aureus* (section 5.2.4.1). There appeared to be an effect on the *L. garvieae* as seen in rows C & F (Figure5-4) where the controls of cells and acid permeabilisers (C 1-3, F 1-3) show more growth than in C 4-6 and F 4-6 which have endolysin added**.**

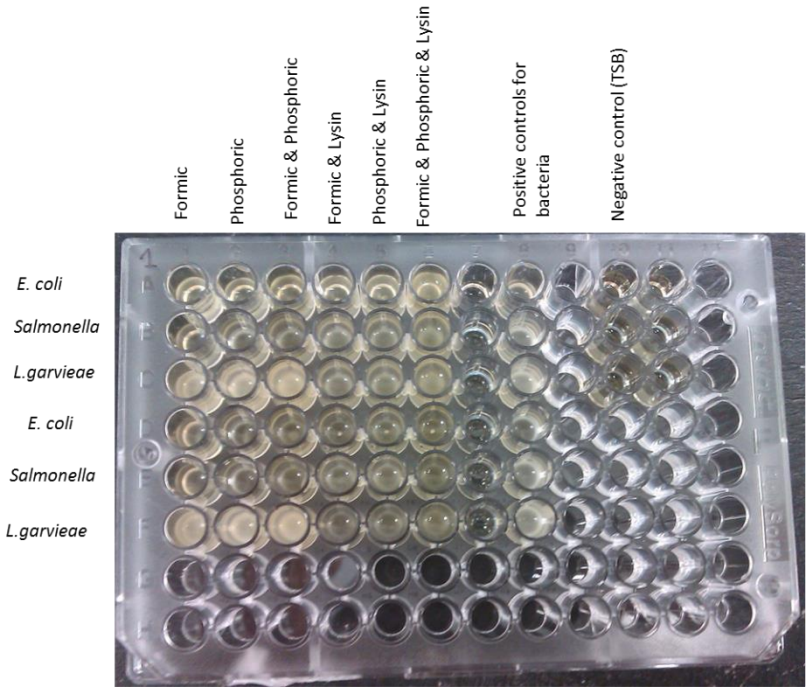


Figure 5-4: Treatment of *E. coli*, *Salmonella* and *L. garvieae* with acid permeabilisers and His-tag purified endolysin. *E. coli* (rows A 1-6; D 1-6), *L. garvieae* (rows B 1-6; E 1-6) and *Salmonella* strains (rows C1-6; F1-6). Positive controls for strains are *E. coli* in well A 8 & D 8, *Salmonella* B 8 & E 8, and *L. garvieae* strains C 8 & F 8. Negative controls of 100 µℓ broth in wells A 10-11, B 10-11 and C 10-11.

The collected fractions indicated good expression, but with a need for further fractionation for more stringent purification of the protein (Figure 5-5). The fractions 3-8 (lanes 6-11) however show good elution of the desired protein with a few contaminating proteins in the background.

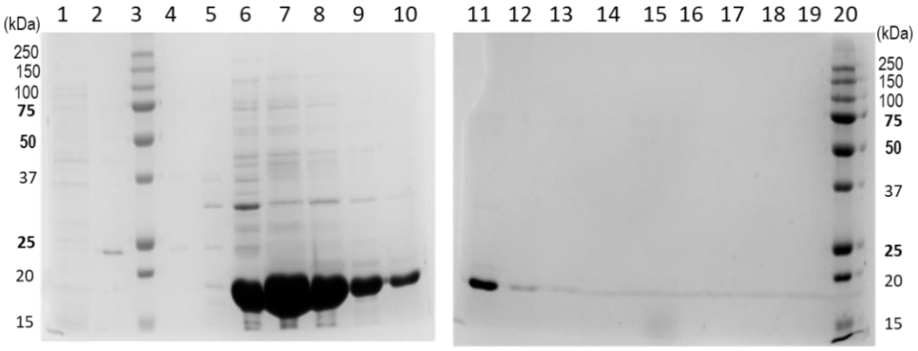


Figure 5-5: 12 % SDS-PAGE displaying fractions collected during purification of endolysin expressed in BL21 (DE3) pET28 from gravity flow Nickel column; Lane 1 is the flow through after loading the column, lane 2 is the wash, lane 3 is the protein ladder, lanes 4-19 are the 0.5 mℓ fractions (1-16) eluted from the column and lane 20 is the protein ladder. The fractions 3-8 (lanes 6-11) show good elution of the desired protein with a few contaminating proteins in the background.

The fractions collected from the nickel column gravity flow eluates were tested on *E. coli*. Endolysin was then purified using FPLC, and the resultant fractions were used for the bacterial assays as well. The FPLC fractions were dialysed prior to bacterial assays, which resulted in no effect on the test when compared to the controls. Thus, the previously observed effect of decreased cell growth could be attributed to the imidazole present in the fractions rather than effect of the endolysin.

The purification process was improved by purifying the proteins using FPLC (section 5.3.5.1) which gave more precise elution fractions, simplifying selection of fractions containing only endolysin. The BCA assay (section 4.2.5) gave the average protein concentration found in the pooled fractions of purified proteins eluted from gravity-flow columns as 5.7 ± 0.5 mg/mℓ and 16.85 mg/mℓ for FPLC purification. Dialysed (section 4.2.6) protein concentrations of FPLC samples showed a marked decrease in the protein concentration to 173.22 ± 13.9 µg/mℓ.

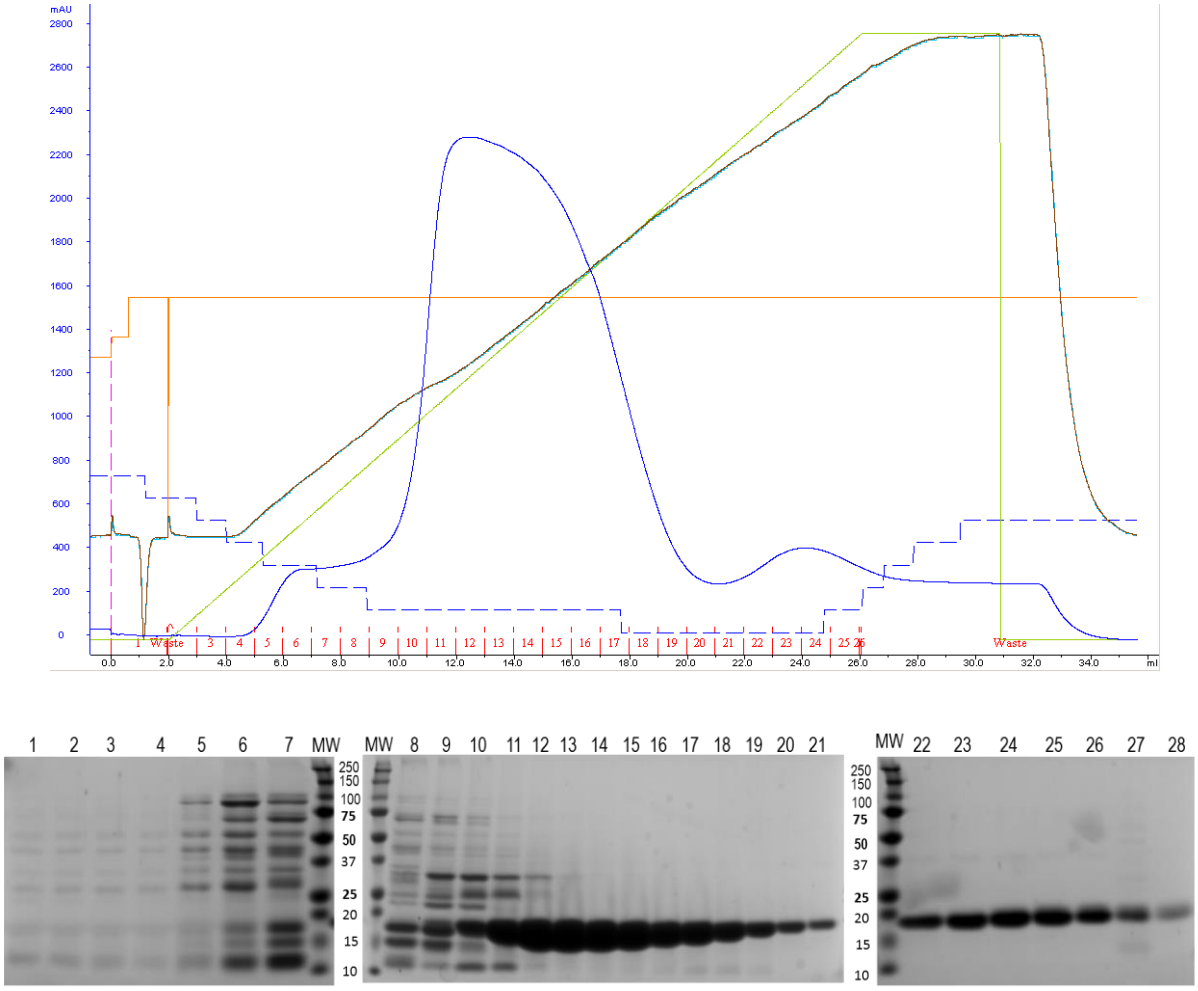


Figure 5-6: (a) ÄKTA FPLC UPC-900 elution graph of the expressed endolysin protein on 1 mℓ His Trap column (blue line indicates protein elution). (b) The SDS-PAGE gel of fractions eluted via the ÄKTA FPLC UPC-900. The profile of elution resembles earlier results (Figure 5-5) indicating purification of the enzyme from solution in fractions 13-28.The fractions 13-17 and 18-26 were pooled into two groups and dialysed against 0.9 % NaCl buffer using the Slide-A-Lyzer® Dialysis Cassette (Extra Strength) 10 000 MWCO 0.5–3.0 mℓ capacity (Pierce) to remove the imidazole.

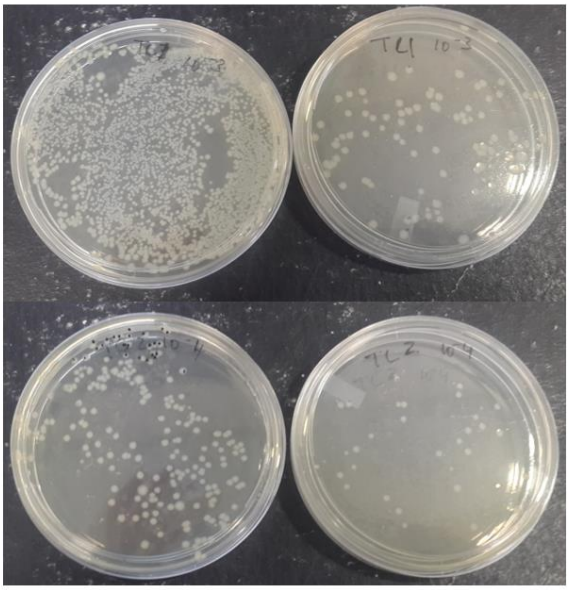
5.3.6 Bacterial assay using whole endolysin extract and permeabilisers

To test the effect of endolysin in combination with permeabilising agents, MIC values were first established for these agents. The MIC values for the test bacteria were as follows against Biotronic® Top Line (B) and Virukill® (V) respectively: *Escherichia coli* K12 (B = 0.476 %; V = 0.0074 %), *Salmonella enterica* Diarizonae (B = 0.238 %; V = 0.0074 %), *Pseudomonas aeruginosa* (B = 0.0554 %; V = 0.0148 %), *Staphylococcus aureus* (B = 0.238 %; V = 0.0037 %). The stock solutions of each permeabiliser was considered 100 % and the MIC hence represents the percent of the permeabilisers in the dilution.

This experiment was conducted and reported based on a modification of the method described by Dong and colleagues (2015) in section 5.3.6. The bacterial assay was tested against the *E. coli* K12 of the test bacterial strains, and initial results showed log count differences as shown in Figure 5-7

by direct comparison of examples of plates observed, providing strong indication that the enzyme lysate had an effect.

Figure 5-7: Initial bacterial assay test plates indicating (a) *E. coli* test cells at 10^{-3} dilution without



endolysin (control), (b) *E. coli* test cells at 10^{-3} dilution with endolysin, (c) *E. coli* test cells at 10^{-4} dilution without endolysin (control), and (d) *E. coli* test cells at 10^{-4} dilution with endolysin. The clear reduction in CFUs can be seen indicating effect of endolysin on the cells.

Following the initial bacterial assay plate results in Figure 5-7, the bacterial assay was extended to the complete range of test bacterial strains, and included the permeabilising agents described in section 5.2.5 (Figure 5-8). A distinct difference can be observed between the initial and final results, where the P-values of *E. coli* treated with endolysin only gave $p = 0.014$ and *E. coli* tested with Virukill® and endolysin gave $p = 0.001$. This means that these two treatments yield the only statistically significant results as $p < 0.05$ and therefore the null hypothesis was rejected. All the other bacterial assays using endolysin and permeabiliser combinations resulted in P-values greater than 0.05, accepting the null hypothesis and meaning there is no significant difference between the control and test samples.

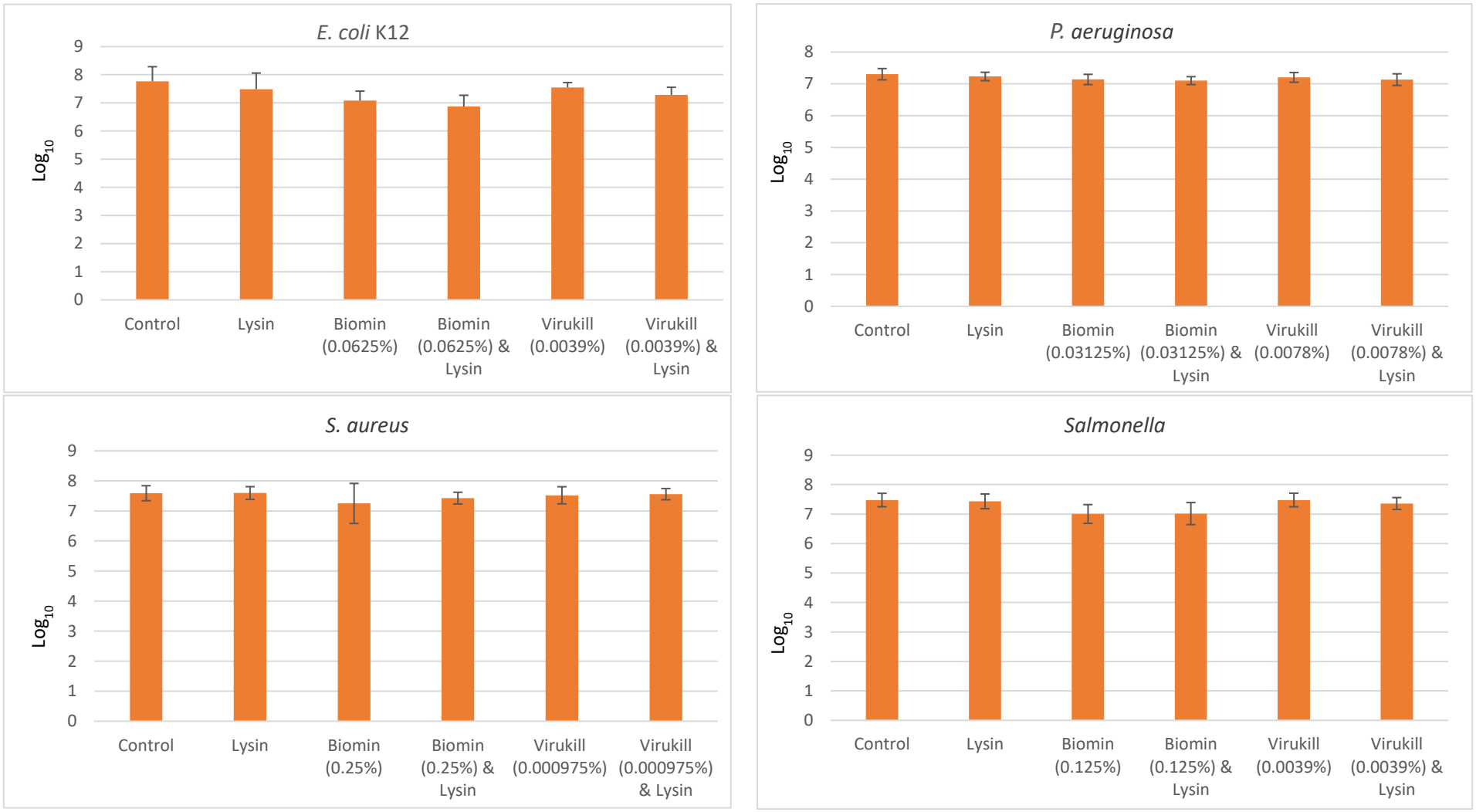


Figure 5-8: Final bacterial assay counts as compared between test with or without treatment with two different permeabilisers, compared to the relevant controls. The test was carried out as described in 5.2.5, however, contrary to initial CFU reductions as observed in Figure 5-7, significant differences between the controls and the test samples could not be observed. The statistical difference observed was in *E. coli* where the endolysin only treated test ($p=0.014$) and the Virukill® and endolysin treated tests ($p = 0.001$) were significantly different from the null hypothesis.

The bacterial assays using whole lysate and permeabilising agents (Figure 5-8) showed that the combination was not as effective as hypothesised. Though the initial results (Figure 5-7) indicated a good log reduction of the bacterial cells, the complete set of results showed lack of statistical support (Figure 5-8). The only two applications that showed T-test significance were the *E. coli* bacterial assays treated with endolysin only and endolysin and Virukill® treatment. Although these tests demonstrated a statistically significant difference between control and test, they still provided no substantial visible difference.

An interesting observation was that the endolysin-only treatment also had an effect, and this correlates to the observations of Dong and colleagues (2015) in the study that used EDTA as the permeabiliser. They observed reductions in bacterial counts regardless of whether EDTA was present, while the combined effect with the EDTA affected cells were more pronounced. The fact that the statistically relevant results were found with *E. coli*, the natural target of this endolysin, provided some encouragement that these results are significant enough to warrant further investigation.

It may be that the permeabilisers were used at too high a dilution to effectively permeate the LPS of the Gram-negative bacteria, although higher concentrations killed the cells without the presence of lysate. These specific permeabilisers, selected because they are already established products in the poultry industry, were intended to compliment endolysin activity, which is a beneficial role in terms of their efficacy being tested and actively used. The permeabilisers may however have a negative effect on the endolysin proteins, as the Biotronic® Top Line contains acids which may inactivate the endolysin proteins. In this case the permeabilisers would in fact be inhibiting the endolysin activity.

5.4 Concluding remarks

Although the cell-surface electron micrographs of *Y. lipolytica* transformants versus control cells (Figure 4-15 in chapter 4) showed a potential of proteins being expressed extracellularly, no observable antibacterial effects could be observed after treatment using the *Y. lipolytica* transformants. The whole recombinant yeast cells did not affect test bacteria beyond the effects of the associate permeabilising agents tested. Failure to identify the endolysin proteins through SDS-PAGE and subsequent LC-MS/MS leaves the expression of the lysin gene in *Y. lipolytica* inconclusive. One possibility for future research would be to screen more clones for their expression of endolysin proteins, as differences in protein expression can occur between clones depending on how many copies of the gene are inserted into the genome of the yeast (Flagfeldt *et al*., 2009).

The bacterially expressed endolysin showed initial log decreases between treated and untreated cells (Figure 5-7). The initial aim was to observe the effect of endolysin in conjunction with commercial permeabilisers i.e. Biotronic® Top Line and Virukill® to potentially add an advantage to

these products. However, the unpurified lysate was not effective against most of the tested bacteria, and only mildly effective against *E. coli* K12. Tests using the purified endolysin post-dialysis were also unsuccessful at lysing the bacteria. Future work could include the combination of the tested permeabilisers with concentrated, purified endolysin protein to see the effect of the enzyme in the presence and absence of these agents.

Chapter 6 General Discussion and Conclusions

The field of bacteriophage research for therapeutic use, including bacteriophage-based products, has broadened over the past three decades, with abundant information and discoveries now available. Yet, the interest in bacteriophage research as a treatment to bacterial infection persists, as is evidenced by the number of advances this field has garnered over the years and the number of new products that make their way to the various industries.

The initial direction of this study was to use the resource of prophages lodged within the bacterial genomes as a means to modify existing prophages and manipulate them into maintaining virulence in the lytic cycle. The idea was to tap into unused bacteriophage resources as bacteriophage therapy was based on using virulent bacteriophages; usually in mixtures of several different bacteriophage strains known as a 'cocktail' (Chan *et al*., 2013).

Taking advantage of the lytic-lysogenic bi-switch of bacteriophage lambda, the PCR screening was proposed as an easy mining system to identify hidden lambda prophages within *E. coli* hosts. As this approach did not yield many prophages, induction of potential prophages hidden in *E. coli* hosts was attempted, as prophages are known to be littered throughout bacterial genomes (Brüssow *et al*., 2004). Three different induction methods were used i.e. UV-, heat-, and mitomycin C induction. The use of these three methods should have induced any inducible prophages within the bacterial host. However, a lack of induced plaques was observed in all three methods; even with various modifications. Furthermore, of the plaques that were observed, only a few were passaged successfully with subsequent induction. Of these, a maximum of three sequential passages were successful before further plaques could no longer be observed. Whether this is related to the mosaic and modular makeup of bacteriophage genomes (Juhala *et al*., 2000) and the difficulty that comes with remnants of prophages remaining in bacterial genomes, or whether the necessary pressure was not achieved to move the genetic switch of inducible prophages, is uncertain. An attempt at overcoming the lack of phages was to extract genetic material from the bacteriophage particles that were within the observed plaques and then to amplify the whole genome using Phi29 polymerase. This polymerase uses rolling circle amplification and with random hexamer primers can amplify seemingly endless amounts of whole genome at 30 °C. However, this did not result in amplified bacteriophage genome from the induced plaques.

An alternative direction of the study involved the pursuit of isolated bacteriophage endolysins as an alternative aspect of bacteriophage therapy. Endolysin proteins have the advantage of bacteria having shown no resistance against endolysins, compared to whole bacteriophages (Schmelcher & Loessner, 2016).

Bacteriophage endolysins, in conjunction with holins, represent a system that is widely available to dsDNA bacteriophages, although their precise mechanisms may differ. This system allows mature and packaged bacteriophages to leave their bacterial host cells. This is achieved by the holins forming hydrophobic holes within the cell membrane of the bacterial cell through which the endolysin can cross to reach the cell wall and begin digesting the cell wall.

Recombinant protein production was considered as a favourable means of producing useful quantities of these proteins. Two expression systems were used in this study, namely *E. coli* and *Y. lipolytica* expression systems. The bacterial expression system yielded good expression of endolysin protein, which was observed on SDS-PAGE and confirmed via LC-MS/MS. The bacterially expressed endolysin, containing a 6x Histidine-tag, was successfully purified. The His-tag purified endolysin was tested against bacterial cells that had been treated with permeabilising acids, initially yielding a difference between treated *L. garvieae* cells compared to untreated cells. However, after removal of imidazole (that was used to remove the His-tagged protein from the Nickel column) using dialysis, the endolysin did not have an effect on the bacterial cells. Thus, the inhibiting effect of the imidazole was observed. Bacteriophage endolysin was also tested in conjunction with two commercial poultry antibacterial products: Biotronic® Top Line and Virukill®. The idea involved using the commercial products as lipopolysaccharide permeabilising agents and then treat the cells with unpurified endolysin. This could allow for lower concentrations of the products to be used, while attaining antibacterial effects without the need for concentrating endolysin. Initial results were promising, with log count differences between treated and untreated cells being observed. However, after all the biological and technical repeats the difference between the control and the tests were negligible, except for the combination of Virukill® and endolysin treated *E. coli*, for which statistical significant differences were observed. However, even this was a very small difference and cannot conclusively prove that the combination had an effect.

Although the holin protein was not observed on SDS-PAGE, its effects could be seen in the freeze-thaw as well as autolysis experiments. It is unclear why the holin was not observed on SDS-PAGE after extraction using ionic buffers, which should solvate membrane proteins (Carlone *et al*., 1986; Bläsi *et al*., 1990; Chang *et al*., 1995).

The yeast expression system of *Yarrowia lipolytica* was used as it is an efficient expression host of various heterologous proteins. This industrial workhorse has another benefit over bacterial expression in that it is Generally Regarded as Safe (GRAS). Two applications of this yeast were investigated: one for the cell-surface anchored display of the expressed proteins and the other was secretion of the expressed protein. With cell-surface display, the expressed protein is presented on the surface of the cell on a GPI-anchor. This has advantages as the protein is then externally exposed and may come into contact with possible substrates in the yeast environment. The secretory system allows for easy access to the expressed protein without breaking the cells open. Though

both systems showed cells successfully transformed with the codon-optimised endolysin gene, the attempts to purify and concentrate the proteins were unsuccessful.

To concentrate the proteins, precipitation methods using acids, salting out and acetone were used. However, none of these methods were successful. Prior to concentration of proteins that were expressed with the cell-surface display system, attempts were made to cleave the GPI anchor off and the proteins concentrated using precipitation methods and ultrafiltration columns, but this was unsuccessful.

The novelty of the first part of the research was the induction of the prophages from lysogeny to lysis with the intention of modifying the genomes to make the bacteriophage remain lytic. This is different as most research involve the use of virulent bacteriophage in phage therapy. This approach initiated the potential broadening of bacteriophages previously unused in phage therapy. The novel approach of the heterologous expression of bacteriophage lambda endolysin and holin proteins was their use in conjunction with commercial bacterial permeabilising products (Biotronic® Top Line and Virukill®) with the intention to improve upon the product by adding bacteriophage-derived endolysin to aid in the disruption of the bacterial cells.

To complete this work, there may still be opportunity to use the concentrated and purified recombinant endolysin in conjunction with the commercial products. Future research could divert to enzymes from other bacteriophages and investigate other bacterial strains. The use of different aspects of bacteriophage endolysins should also be looked into as the combination of bacteriophage endolysin cell wall-binding proteins and their enzymatically active areas can lead to improved host range as well as diverse substrate specificity. As with the work of Kauffmann *et al*. (2018), it would also be beneficial to search under dsDNA non-tailed bacteriophage which have - in their study - showed to have a broader host range when compared to tailed bacteriophage. One specific area that should be investigated is MRSA (Methicillin resistant *S. aureus*) as the importance of this bacteria grows with each infection that cannot be cured.

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Appendix B - Research outputs

Publications:

Bragg, R.R., Van der Westhuizen, W., Lee, J-Y., Coetsee, E. & Boucher, C.E. (2014). Bacteriophages as potential treatment option for antibiotic resistant bacteria. *Advances in Experimental Medicine and Biology* **807**, 97-110.

Van der Westhuizen, W.A., Lee, J-Y., Theron, C.W., Boucher, C.E. & Bragg, R.R. Avian Pathogenic *Escherichia coli* (APEC): Review on the control and prevention of colibacillosis. **Submitted to Avian Pathology.**

Chapters in books:

Bragg, R.R., Boucher, C.E., van der Westhuizen, W.A., Lee, J-Y., Coetsee, E., Theron, C.W. & Meyburgh, C.M. (2016). The potential use of bacteriophage therapy as a treatment option in a post antibiotic era. Chapter 15 in *Antibiotic Resistance*, 1st Edition, Elsevier, *In Press*.

Conferences:

International Union for Microbiological Societies (IUMS) 2017, Singapore, Singapore
"*In vitro* test for bacterial treatment using heterologously expressed bacteriophage lambda endolysin and permeabilising agents."
J-Y.Lee, C.W. Theron, C.E. Boucher and R.R. Bragg.

South African Society for Microbiology (SASM) 2016, Umhlanga, South Africa
"Bacteriophage lambda endolysin expression for Avian Pathogenic *Escherichia coli* treatment."
J-Y. Lee, C.W. Theron, C.E. Boucher and R.R. Bragg.

Virology Africa 2015, Cape Town, South Africa
"The expression of endolysin from bacteriophage lambda for the treatment of Avian colibacillosis" -
J-Y. Lee, C.W. Theron, C.E. Boucher and R.R. Bragg.

World Veterinary Poultry Association (WVPA) 2015, Cape Town, South Africa
"Expressed bacteriophage lambda endolysin to assay against Avian Pathogenic *Escherichia coli* as a potential antibacterial treatment"
J-Y. Lee, C.W. Theron, C.E. Boucher and R.R. Bragg.

International Union for Microbiological Societies (IUMS) 2014, Montréal, Canada
"The investigation and application of phage endolysin as antibacterial treatment for Gram-negative bacteria; focusing on Avian Pathogenic *Escherichia coli*"
J-Y. Lee, C.E. Boucher and R.R. Bragg.

South African Society for Microbiology (SASM) 2013, Bela-Bela, Limpopo, South Africa
"The investigation and application of phage endolysin as antibacterial treatment for Gram-negative bacteria; focusing on Avian Pathogenic *Escherichia coli*"
J-Y. Lee, C.E. Boucher and R.R. Bragg.

World Veterinary Poultry Association (WVPA) 2013, Nantes, France
"The investigation and application of phage endolysin as antibacterial treatment for Gram-negative bacteria; focusing on Avian Pathogenic *Escherichia coli*"
J-Y. Lee, C.E. Boucher and R.R. Bragg.

South African Society for Microbiology (SASM) 2011, Cape Town, South Africa
"Investigation into Temperate Bacteriophage as Possible Agents for the Treatment of Bacterial Infections"
J-Y. Lee, J. Albertyn and R.R. Bragg.