Expression of avian pathogenic *Escherichia coli* (APEC) virulence factors, Iss and HlyF, as potential sub-unit vaccine candidates

By

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ABBREVIATIONS USED THROUGHOUT THE DISSERTATION

APEC: Avian pathogenic *Escherichia coli*

CHAPS: 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate

DTT: Dithiothreitol

EK: Enterokinase

FPLC: Fast protein liquid chromatography

GPI: Glycosylphosphatidylinositol

GST: Glutathione S-transferase

IMAC: Immobilized metal ion affinity chromatography

IPTG: Isopropyl-b-D-thiogalactopyranoside

Iss: Increased serum survival protein

IssT: Truncated increased serum survival protein

LB: Luria-Bertani / Lysogeny Broth

LB-Kan: Lysogeny broth /Luria-Bertani broth containing 30 µg/ml Kanamycin

LC-MS/MS: Liquid chromatography-tandem mass spectrometry

PLC: Phospholipase C

PTMs: Post translational modifications

TCA: Trichloroacetate

TMB: 3,3',5,5'-Tetramethylbenzidine

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GENERAL INTRODUCTION

Pathogenic *Escherichia coli* are often classified by their virulence properties such as Enterotoxigenic *E. coli* (ETEC), Enteropathogenic *E. coli* (EPEC) and Enterohemorrhagic *E. coli* (EHEC). *E. coli* found to be virulent in poultry and other avian species are classified as APEC (Avian Pathogenic *E. coli*). Due to the rapidly increasing spread and development of antibiotic resistance, the existence of multi-drug resistance APEC strains, and the potential threat of zoonosis from APEC-contaminated foods, alternative therapeutics and preventative measures are becoming increasingly crucial for the profitability of the poultry industry and ensuring food security.

Various vaccines exist to prevent bacterial diseases in poultry and will start to play a bigger role as antibiotics lose effectivity due to antibiotic resistance and the legislation preventing the use of antibiotics for livestock. There is an increased research interest into the development of novel new generation bacterial vaccines. There are also efforts to explore other potential alternative treatments such as bacteriophage therapy (Borie *et al*., 2009; Oliveira *et al*., 2010; Tsonos *et al*., 2014). Strain and phenotype specificity of bacterial vaccines will also be important in many cases, as some bacterial species are part of the normal commensal microbiota of animals. The challenge will be to target the pathogenic strains but not destroy these commensals. Colibacillosis is such an example, as non-pathogenic strains of *E. coli* exist in the intestines of warm-blooded animals, and their elimination can result in poor gut health and an increased susceptibility to secondary infections (Sullivan *et al*., 2001). This is one of the pitfalls of whole bacterial vaccines, as there are bound to be antigens common to both commensal and APEC. The immune system of the host will thus produce antibodies against these common antigens, therefore the immune system will remove both commensal and pathogenic *E. coli* strains. Therefore, sub-unit vaccines can potentially lead to a much more specific immune response as these vaccines can target specific antigens only present in APEC strains, but a proper understanding of the virulence of APEC is required.

Various virulence genes of APEC have been investigated in literature and their mechanisms elucidated. Although it has been found that no single combination of virulence genes

contributes to the pathogenicity of APEC, some potential minimal predictors have been identified, which allows for the potential development of vaccines targeting these virulence gene products (Johnson *et al*., 2008). Virulence genes of APEC are often involved in iron-acquisition from the host, adhesion to host tissues, invasion of host tissues and protection from host-compounds to improve the survival of the bacterium inside the host and some of these gene products are situated in the outer-membrane of APEC or are secreted from the *E. coli* cell, making them potential immunogens which can be used in vaccine development.

The development of a highly specific sub-unit vaccine would greatly aid the poultry industry, but factors such as cost must also be considered, as no farmer would be willing to use a vaccine which increases the cost of production significantly. A potential sub-unit vaccine must therefore be cost effective, reduce the incidence of disease significantly and be long lasting to make it economically feasible and a competitive product.

In this dissertation, the problem of antibiotic resistance, potential alternatives to antibiotics will be discussed and the initial development of a potential sub-unit vaccine for the control of colibacillosis in the poultry industry will be investigated.

CHAPTER 1

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

Preface

This chapter is presented in the format of a journal review manuscript, as submitted to the journal Avian Pathology upon request to write an *E. coli* review article. This chapter will serve as the literature review to this dissertation and is styled according to the guidelines set by Avian Pathology.

As this article was co-authored by five people their contributions are as follows:

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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, improve 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. Avian Pathogenic *Escherichia coli* (APEC)

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 can be localised or systemic and can include swollen head syndrome, colisepticaemia, air sacculitis, pericarditis, cellulitis, omphalitis, diarrhoea, salpingitis, orchitis, encephalitis, meningitis; which lead to decreased egg yields and an increase in mortalities. Lesions are often found on the organs, but as various bacterial diseases during secondary infections can lead to lesions, *E. coli* must therefore be isolated before colibacillosis can be confirmed (Nolan *et al.,* 2013). 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; Tivendale *et al*., 2010; Mitchell *et al*., 2015).

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)

1.2 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). However, in a study conducted by Kemmett and co-workers, various pathotypes of APEC were identified, indicating that there is high genetic diversity among diseased broilers' APEC isolates, and that the state of the host susceptibility also plays a significant role in which *E. coli* strains with specific pathotypes will lead to disease (Kemmett *et al*., 2013).

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 *neuC* 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.

1.3 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.

1.4 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).

2. Antibiotics: from celebrated discovery to imposed restrictions

2.1 History: The production of the antibiotic penicillin by 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, thanks to collaborative work done between Ernest Chain and Howard Florey with Fleming in the purification of penicillin (Chain *et al*., 2005). 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). These problems of antibiotic resistance and prohibitions of their use in animal industries thus indicate an urgent need for alternative therapeutics to antibiotics.

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 exaggerated 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.

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).

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 continue 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).

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.

3. Vaccines

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.

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 program and in some cases the duration of infection was reduced, showing the importance of improved hygienic practices even when vaccination programmes are in place.

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). It was shown that the deletion only slightly impaired curli fimbriae production and that the strain was avirulent when injected into 1-day old chicks, followed by drinking water vaccination at day 7, leading to protection against a challenge at 6 weeks of age (La Ragione *et al*., 2013).

4. Bacteriophages

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.

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 diphtheria* to virulent 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.

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). As 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).

4.4 Lysogenic phages: The main different lifecycles of bacteriophages are the lytic and lysogenic lifecycles. 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 means 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 lifecycle. Understandably this is an immense amount of work, which is less favourable than identifying alternative sources of lytic phages.

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 phage cocktails 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.

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 formation 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.

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 minutes 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).

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.

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.

4.10 Bacteriophage studies in 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 media 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.

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 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. 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 (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 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.

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. As 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).

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 will ensure that the poultry industry will continue to produce good quality products and ensure food security.

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CHAPTER 2

Immunogenicity evaluation of full-length and truncated transmembrane virulence protein Iss from avian pathogenic *Escherichia coli* (APEC)

Preface

This chapter is presented in the format of a journal manuscript, as submitted to the Journal of Veterinary Immunity and Immunopathology, following the style of this journal for publication. Permission is granted to make use of the submitted article by Elsevier, as an author, as described in the link below: https://service.elsevier.com/app/answers/detail/a_id/565/track/APMM8wolDv8W~bvKGoAa~yKgh4sqMC75Mv_a~zj~PP9T/

This study investigated the expression of the Increased Serum Survival (Iss) protein of avian pathogenic *Escherichia coli* (APEC). As Iss is natively situated in the outer-membrane of *E. coli*, there are hydrophilic (interacting with the aqueous environment) and hydrophobic (situated in the hydrophobic lipid membrane) regions, giving the protein an overall hydrophobic nature. Hydrophobic proteins are generally difficult to express as largely water-insoluble proteins. Therefore, a truncated variant of this protein, with the membrane-bound domain excluded, was also constructed in this chapter, namely IssT.

The main aim of this work was to elucidate whether antibodies raised against either the full Iss protein and truncated variant IssT protein can cross-react with the two proteins. If cross protection is shown, this would indicate that similar epitopes are being presented to the host immune system. Ethics approval (Animal Experiment number 08/2013) was obtained to administer the two versions of the expressed proteins into chickens for the raising of antibodies against both the full length and truncated expressed Iss proteins.

Ninety embryonated specific pathogen-free (SPF) eggs were obtained and hatched with the purpose of raising antibodies against the two *E. coli*-expressed proteins. Unfortunately, due to circumstances beyond our control, only 11 chickens hatched from the SPF eggs and were available for this study. It is clear that this is a very small sample size of chickens, but as the main objective of this part of the experiment was purely the raising of antibodies against the different expressed proteins, it was decided to proceed with the experiment in spite of the small sample number of birds. If this had been an experiment to specifically evaluate the immune response in the birds or any form of protective capabilities of the expressed proteins as a sub-unit vaccine, the sample size of birds would have been totally unacceptable.

Although less significant conclusions could be made due to the small sample size used, the results obtained did provide us with the information required to satisfactory address the objectives described.

Immunogenicity evaluation of full-length and truncated transmembrane virulence protein Iss from avian pathogenic *Escherichia coli* (APEC)

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Abstract

Avian colibacillosis is a disease caused by avian pathogenic *Escherichia coli* (APEC) and is of great economic importance in the poultry industry. The advent of multiple antibiotic resistant strains has resulted in a limitation on the large-scale use of antibiotics for the prevention and treatment of cases of colibacillosis. Furthermore, several bans have been put in place in various countries regarding the use of antibiotics as growth promoters in animal production. Alternative preventative measures and therapeutics are therefore in great demand to control diseases caused by bacterial pathogens. While vaccines are commonplace in the animal production industry, they mostly target viral diseases. There are vaccines against some bacterial pathogens available, however none are second generation (subunit) vaccines.

The aim of the study is to improve on the manufacturing of an APEC Iss antigen as a precursor in the development of a second-generation vaccine, by improving and simplifying the production and costs of preparation while preserving the epitope structure. The recombinant protein and a truncated variant were produced using *E. coli*, and purified before injection into chickens. Serum samples collected from blood at zero, two and four weeks were tested for specificity against the full and truncated forms of the antigen using enzyme-linked immunosorbent assays and western blotting. Antibodies against the full and truncated Iss antigens were detected and the sera showed cross-reactivity between the two expressed antigens, which will allow future research into testing the potential of the soluble truncated variant as a potential immunoprotective agent against Iss-producing APEC infections.

Keywords: Avian pathogenic *Escherichia coli*, APEC, increased serum survival, transmembrane protein, truncated protein

Abbreviations:

APEC: Avian pathogenic *Escherichia coli*

CHAPS: 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate

DTT: Dithiothreitol

FPLC: Fast protein liquid chromatography

GST: Glutathione S-transferase

IMAC: Immobilized metal ion affinity chromatography

IPTG: Isopropyl-b-D-thiogalactopyranoside

Iss: Increased serum survival protein

IssT: Truncated increased serum survival protein

LB: Luria-Bertani

LC-MS/MS: Liquid chromatography-tandem mass spectrometry

TMB: 3,3',5,5'-Tetramethylbenzidine

Introduction

Avian pathogenic *Escherichia coli* (APEC) is the causative agent of avian colibacillosis, a disease which has serious financial implications in global poultry production (Barnes et al., 2003). This has become a more threatening problem over the past decade due to antibiotics becoming either ineffective against resistant strains and therefore banned, or restricted to therapeutic use only (E.U. Commission Press Release, 2005). The world is moving into a post-antibiotic era and alternative therapeutics and preventative measures are thus becoming increasingly important. The current alternative to antibiotic therapeutics primarily involve the use of inactivated bacterial vaccines and modified live vaccines, such as the Poulvac® *E. coli* vaccine for the prevention of colibacillosis (Mombarg et al., 2014). The production of vaccines is moving towards more specific immunogenic targets, such as the use of sub-unit vaccines. It can be speculated that a sub-unit vaccine would serve as a more specific immune stimulus, which is beneficial in the case of *E. coli* since non-pathogenic strains are part of the beneficial microbiota of chickens.

The *iss* gene encodes the "increased serum survival" protein, predicted to be an outer membrane protein (Olsen et al., 2012). It is hypothesised that the gene is derived from the bacteriophage-encoded *bor* gene, as the sequences are highly homologous (Johnson et al., 2008a). The presence of genes coding for *iss* has been correlated to complement resistance and has been recommended as a potential vaccine target to control avian colibacillosis (Binns et al., 1982; Nolan et al., 2003; Lynne et al., 2012). In studies by Lynne and co-workers (Lynne et al., 2006; Lynne et al., 2012) the ability of this antigen to provide protective immunization against APEC was comprehensively demonstrated. The aim of the current study was to further simplify the production of this antigen and reduce the potential costs associated with such production.

The hydrophilic regions of transmembrane proteins are considered the probable epitopic regions, as they are most likely to interact directly with the immune system of the host (Hopp and Woods, 1981). The generally hydrophobic nature of transmembrane proteins can result in insoluble proteins during over-expression in *E. coli* accumulating in inclusion bodies (Singh et al., 2015). Therefore, antigens in candidate vaccines are often truncated versions of the original protein (Flach et al., 2011; He et al., 2014), in which the transmembrane

regions have been truncated (Zhan et al., 2015). A potential drawback to using truncated proteins is potential modifications in secondary and / or tertiary structures, potentially altering the original epitopes of the antigen. This could naturally hinder the use of such a protein in a vaccine, and therefore any serological differences between the native and truncated proteins must be determined. In this study, the full length Iss protein and a truncated form were produced and tested for immune system stimulation as well as cross-reactivity of the serum obtained against the two different protein forms.

Materials and Methods

Amplification of *iss* from *E. coli* strain 1080: The *iss* gene was amplified from the APEC strain 1080, which had previously tested positive for the presence of *iss* and various other virulence genes (Van der Westhuizen and Bragg, 2012). The translated sequence of this strain's *iss* gene is completely homologous to various APEC *iss* genes found on Genbank, including *iss* type 1 as defined by literature (Johnson et al., 2008b). The full length *iss* gene (306 bp, 102 amino acids) was amplified using the primers iss-pet28-FP 5'-CCGCATATGCAGGATAATAAGATGAAAAAAATG-3' and iss-pet28-RP 5'-GTTCTCGAGTTGTGAGCAATATACCCGG-3'; and a truncated form of the *iss* gene (*issT*, 183 bp, amino acid residues 20 – 81 of the full protein) was amplified using the primers truncISS-FP-NdeI 5'-TGTAGACATATGGGATGTGCTCAACAG-3' and truncISS-RP-XhoI 5'-TCATTACTCGAGTCCATTTACGAATGT-3' (Integrated DNA Technologies). The forward and reverse primers were designed with incorporated *Nde*I and *Xho*I restriction recognition sites (underlined) respectively, for cloning into pET28b(+) (Novagen) with both N and C-terminal hexahistidine tags.

Cloning and transformation: Standard molecular biology techniques were carried out according to Sambrook and Russell (2001), and enzymes were applied according to the specifications of the manufacturers. PCR amplicons were sub-cloned into the pGEM®-T Easy Vector System (Promega), using *E. coli* 10-β cells (New England Biolabs). The sequences of the cloned inserts were determined using Sanger sequencing with the SP6-promoter and T7-terminator primers (Integrated DNA Technologies). Consensus sequences were subjected to BLAST searches against the NCBI sequence database (Altschul et al., 1990) for the identity confirmation of the sequences.

The *iss* and *issT* genes were removed from the pGEM®-T Easy vector to the pET28b(+) vector using the *Xho*I and *Nde*I restriction endonucleases (Thermo Scientific). The digested DNA fragments were purified from 2 % LM-sieve agarose gels (Lonza) using the Wizard® SV Gel and PCR Clean-Up System (Promega). The digested gene inserts were ligated into pET28b(+), and the recombinant plasmids were used to transform *E. coli* BL21 (DE3) (New England Biolabs). Henceforth the recombinant strains containing these plasmids will be referred to as BL21-pET28-iss and BL21-pET28-issT respectively.

The plasmids were extracted after a 24-hour incubation at 37 ^{o}C in Luria-Bertani (LB) broth (10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract) containing 50 ug/mL kanamycin. The presence of the inserted *iss* and *issT* regions were confirmed by PCR, and the sequences were reconfirmed using Sanger sequencing with the T7-promoter and T7-terminator primers (Integrated DNA Technologies), which flank the multiple cloning site of pET28b(+).

Protein expression and purification: BL21-pET28-iss and BL21-pET28-issT were inoculated into separate 1 L Erlenmeyer flasks containing 250 mL of ZYP-5052 auto-induction media (Studier, 2005) supplemented with 50 µg/mL kanamycin in duplicate. The flasks were incubated at 20 ^{o}C and 30 ^{o}C for BL21-pET28-iss and BL21-pET28-issT respectively, for 24 hours with shaking at 150 rpm. The cells were centrifuged at 7000 x *g* for 5 minutes and the supernatant removed. Cell pellets were resuspended in wash/binding buffer (50 mM Tris-HCl, 500 mM NaCl, 20 mM Imidazole, pH 7) to a final concentration of 0.1 g wcw/mL. CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate) detergent was added to BL21-pET28-iss to a final concentration of 10 mM to aid in the solubilisation of the hydrophobic Iss for downstream processing. The cells were broken by using a constant cell disruptor at 30 kpsi (2.068 x 10^5 kPa) and the lysate was centrifuged for 90 minutes at 4 ^{o}C at 100 000 x *g*. The cell-free extracts were applied to 1 mL HisTrap^{TM} HP (GE Healthcare) Nickel immobilized metal ion affinity chromatography (IMAC) columns at a flow rate of 1 mL/minute. The columns were washed with 10 mL of wash/binding buffer (supplemented with 10mM CHAPS for the purification of the full Iss followed by elution by a linear gradient from 20 mM to 500 mM imidazole for 25 x 1 mL fractions with wash/binding buffer and elution buffer (50 mM Tris-HCl, 500 mM NaCl, 500 mM Imidazole, pH 7) during fast protein liquid chromatography (FPLC). The elution buffer was supplemented with 10 mM CHAPS for the purification of the full Iss. A final FPLC elution of 5 x 1 mL fractions was performed with elution buffer to remove any remaining proteins bound to the IMAC column.

SDS-PAGE and liquid chromatography-tandem mass spectrometry (LC-MS/MS): Collected FPLC fractions were prepared for SDS-PAGE according to Laemmli (1970). The SDS-PAGE was performed using a 0.75 mm, 12 % 37.5:1 acrylamide/bis-acrylamide (Bio-Rad Laboratories) gel in 1x Tris-HCl-Glycine-SDS buffer (Bio-Rad Laboratories). Precision Plus Protein™ All Blue Prestained Protein Standard (Bio-Rad Laboratories) was used for molecular weight estimation, unless stated otherwise. Electrophoresis was performed at 17 V/cm until the dye-front reached the bottom of the gel.

SDS-PAGE gels were stained and destained with Fairbanks A and Fairbanks D solutions respectively (Fairbanks et al., 1971), according to Wong and co-workers (Wong et al., 2000). The gel images were obtained using a Gel Doc™ EZ Imager (Bio-Rad Laboratories).

Protein bands of interest were excised, reduced with dithiothreitol (DTT), alkylated with 2-iodoacetamide and digested with trypsin (Thermo Scientific) according to the manufacturer's instructions before LC-MS/MS analysis for protein identification.

Protein concentration and quantification: FPLC fractions containing high concentrations of the protein of interest were applied to an Amicon Ultra-4 centrifugal filter unit (Merck-Millipore) with a 10 kDa nominal molecular weight limit and centrifuged at 7000 x *g* for 20 minutes. The protein concentrate was washed on-column with 50 mM Tris-HCl, pH 7.4 (supplemented with 10 mM CHAPS for the full Iss protein) followed by centrifugation at 7000 x *g* for 20 minutes. The protein concentrate was then resuspended to 1 mL final volume with 50 mM Tris-HCl, pH 7.4 (supplemented with 10 mM CHAPS for the full Iss protein).
Concentrated proteins were diluted 10-fold with Tris-HCl buffer pH 7.4 prior to concentration determination using the Pierce™ BCA Protein Assay Kit, using bovine serum albumin (BSA) standards.

Vaccination of chickens: Ethical approval was obtained from the Interfaculty Animal Ethics Committee, University of the Free State, Bloemfontein, Republic of South Africa, to perform immunisation experiments in white leghorn chickens (Animal Experiment number 08/2013).

Ten chickens were hatched, grown under controlled conditions (21 ^{o}C, water and food supplied *ad infinitum*) and divided into three groups. Blood was collected before each subsequent immunisation. The purified proteins, Iss and IssT, were mixed with Freund's incomplete adjuvant (Sigma-Aldrich). Six-week old white leghorn chickens were each injected intramuscularly with approximately 150 µg of the prepared antigen as follows: One group of three chickens received the Iss antigen, a second group of four chickens received the IssT antigen, and a third group of three chickens received Freund's incomplete adjuvant

supplemented with Tris-HCl buffer (pH 7.4) as the control group. Blood samples were collected 14 days post-immunisation prior to the second immunisation, as described above. After a further 14 days, a second batch of blood samples was collected.

All the above collected blood samples were clotted for 30 minutes at room temperature post-bleeding, followed by centrifugation at 2 000 x *g* for 10 minutes at 4 ^{o}C. Serum was collected and stored at -80 ^{o}C until required.

Enzyme-linked immunosorbent assays (ELISA): ELISAs was performed in 96-well Costar® high-binding polystyrene plates (Corning Incorporated). The plates were coated with 100 uL of Iss antigen diluted in phosphate buffered saline (PBS) to a final concentration of 3 µg / mL. Test wells were coated in quadruplicate. Two wells per sample had only PBS added (antigen-free controls) and 2 wells per sample were coated with antigen with no serum added. Coated plates were incubated at 37 ^{o}C for 1 hour, before removal of excess liquid. The plates were then washed six times with 200 uL of PBS-Tween 0.1 % (v/v). The wells were blocked using 200 µL of 3 % (w/v) BSA in PBS-Tween 0.1 % (v/v) for 1 hour at 37 ^{o}C, followed by washing as described before. Serum was diluted 1:1500 in PBS-Tween 0.1 % (v/v) and 100 µL added into all wells (except for appropriate controls) and incubated for 1 hour at room temperature with gentle mixing. After washing as before, 50 uL rabbit-raised anti-chicken peroxidase (Sigma-Aldrich) diluted 1:5000 in PBS-Tween 0.1 % (v/v) was added to all the wells, followed by incubation at room temperature for 1 hour with gentle mixing. The plates were washed as before, before addition of 50 µL 3,3',5,5'-Tetramethylbenzidine (TMB) (Roche) as substrate. After gentle mixing for 15 minutes at room temperature, the reaction was stopped by addition of 50 µL 2 N H_2SO_4 (Merck). Absorbance values of the wells at 450 nm were measured using a BioTek ELx800 plate reader. To determine statistical significance among samples, a standard two-tailed t-test was performed.

Western Blotting: The concentrated IssT and Iss solutions (4 µg and 20 µg of each solution) and a BSA control (10 µg) that had been electrophoresed on SDS-PAGE gels, were transferred to 0.45 µm pore-size nitrocellulose membranes by use of a wet/tank blotting system (Bio-Rad Laboratories) for 1 hour at 100 V in transfer buffer (25 mM Tris-HCl, 190 mM glycine, 20% methanol, pH 8.3). The membrane was rinsed in water before being blocked with BSA 3 % (w/v) in PBS-Tween 0.1 % (v/v) for 1 hour at room temperature. The membrane was washed five times with PBS-Tween 0.1 % (v/v) for 15 minutes per wash. The membrane was then incubated in a 1:1500 dilution of Iss or IssT serum for 1 hour at room temperature. The membrane was again washed five times with PBS-Tween 0.1 % (v/v) for 15 minutes. The membranes were then incubated in the presence of 1:5000 diluted rabbit-raised anti-chicken peroxidase-conjugated secondary antibodies (Sigma-Aldrich) for 1 hour at room temperature. Excess antibodies were removed by washing five times with PBS-Tween 0.1 % (v/v) for 15 minutes. Bound antibodies were visualised by incubation for 15 minutes at room temperature in the presence of the addition of TMB substrate (Roche). The membrane was viewed using a Gel Doc™ EZ Imager (Bio-Rad Laboratories).

Results

Cloning and protein expression: The initial amplification of the *iss* and *issT* gene from strain 1080 resulted in amplicon sizes around 300 bp and 200 bp respectively. The amplicons were cloned into pET28b(+) via sub-cloning in pGEM T-Easy. After sequence analysis, the plasmids were used to transform competent BL21 (DE3) *E. coli* cells.

Protein expression of Iss and IssT in ZYP-5052 auto-induction media was confirmed using SDS-PAGE analyses (Figure 1a, b respectively) and verification using LC-MS/MS. These proteins were then purified using FPLC on Nickel affinity resin, and the obtained fractions were analysed using SDS-PAGE (Figure 2a, b). The fractions containing the highest purity and concentration of the Iss and IssT were concentrated by ultrafiltration, and analysed using SDS-PAGE (Figure 2c). The concentrated protein solutions yielded protein concentrations of 3.4 mg/ml and 3.34 mg/ml for Iss and IssT respectively, as determined using the BCA assay.

Immunogenicity testing: Purified proteins were injected into chickens, followed by collection of serum samples over time. No changes were observed at the site of injection and the birds showed no pathological reaction to the vaccine during the experiment. ELISA assays were performed using the purified proteins and collected serum samples. Statistically different values were obtained at days 14 and 28 for the two antigens against the control values was observed (Figure 3). The standard deviations of values obtained between chickens injected with either protein was unfortunately high, which is probably attributable to the small sample size. From the values obtained from the day 14 samples it was clear that Iss and IssT showed an immune response statistically more significant than the control. At day 28, chickens from both Iss and IssT showed the presence of both anti-Iss and anti-IssT antibodies with levels similar to that obtained on day 14 for the IssT protein

SDS-PAGE was duplicated (Figure 4c, d), with one Coomassie stained as described and the other further analysed by Western blotting, using serum samples obtained from administration of both Iss (Figure 4a) and IssT (Figure 4b). Although purified, some impurities in the Iss and IssT protein samples resulted in the co-production of some non-specific antibodies. Cross-reactivity of IssT to anti-Iss antibodies and Iss to anti-IssT antibodies was demonstrated, indicating similar epitopes being present on the Iss and IssT proteins (Figure 4a, b).

Discussion

A post-antibiotic era is looming that will change the way humans treat and prevent infections in the future (World Health Organization, 2014). Various vaccines exist to prevent diseases in humans, pets and livestock, and these vaccines will start to play a bigger role as antibiotics lose effectivity due to antibiotic resistance. Bacterial vaccines are however currently receiving more attention, as well as other potential alternative treatments such as bacteriophage therapy (Borie et al., 2009; Oliveira et al., 2010; Tsonos et al., 2014). Safe, cost-effective and highly specific vaccines against bacterial diseases are becoming increasingly necessary for prevention of infections before disease. Strain and phenotype specificity of bacterial vaccines will be important in many cases, as some bacterial species are part of the normal commensal microbiota of animals. Colibacillosis is such an example, as non-pathogenic strains of *E. coli* exist in the intestines of warm-blooded animals, and their elimination can result in poor gut health and an increased susceptibility to secondary infections (Sullivan et al., 2001). Researchers have discovered various gene-products specific to APEC that could be tested as vaccine targets, which would discriminately target pathogenic strains without affecting commensal *E. coli* strains (Johnson et al., 2008a). Sub-unit vaccines hold an advantage over conventional vaccines which use whole (and sometimes living) cells to stimulate the host's immune response, as they can lead to more specific responses and carry no risk of reversion to pathogenicity sometimes encountered with live vaccines (Hooke et al., 1985, Hanley, 2011).

The Iss protein was previously expressed for use as a sub-unit vaccine, and showed a humoral response and a decrease in lesion scores in challenged birds (Lynne et al., 2006; Lynne et al., 2012). The production process involved both time consuming methods (refolding of the protein) and various, often specialised or expensive reagents (IPTG, glutathione S-transferase-affinity resin (GST), inclusion body solubilisation kits to obtain the purified protein.

In this study, both the full length and truncated versions of Iss elicited immune stimulation leading to antibody production in the vaccinated chickens. During the production of IssT in this study, the non-requirement for isopropyl-β-D-thiogalactopyranoside (IPTG - typically

~5,50 USD* / L of growth medium at 0,1 mM final concentration in comparison to α-Lactose at ~1 USD* / L of growth medium at 0,3 M), as well as inclusion body solubilisation kits and detergents (typically ~27 USD* / g inclusion body pellet), allowed for simplified and cost-effective production of this antigen. Furthermore, the use of a poly-histidine tag on the protein and the relevant affinity resins that are used for purification of such tagged-proteins, further reduce the costs (typically ~0,875 USD* / mg protein binding capacity) involved when compared to the use of other affinity resins, such as GST (typically ~6.9 USD* / mg protein binding capacity). GST-facilitated purification does however carry the advantage of being highly specific and thus leads to more pure protein elutes. The truncated form of the protein, IssT, is expressed in a soluble form which simplifies purification and reduces the associated cost and time, as no detergents, solubilisation kits or protein refolding kits are required. The cross-reaction between serum samples obtained from vaccination with Iss and IssT proteins was encouraging, as it indicated that the epitopes found on the two proteins are similar enough for IssT to be used as a potential vaccine candidate in future research instead of the full protein, which should bring down production costs. In our experiments, we also achieved a higher antibody titre with the IssT protein after 14 days (Figure 3), leading to a similar titre at day 28 even though reimmunization was performed on day 14. The significantly higher reaction of the IssT protein in comparison to the Iss protein on day 14 could be attributed to the more purified IssT in the solution, as similar total protein concentrations were used so a higher molar concentration of the IssT protein will be present in comparison to the less pure Iss preparation used.

This potentially lead to the full Iss protein requiring 28 days and the booster at day 14 to reach similar levels. Although the truncated form is expected to provide similar protection against an APEC infection as the full-length protein, this remains to be tested in future research that includes post-vaccination challenging with APEC.

*as of January 2017

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Figures and legends:

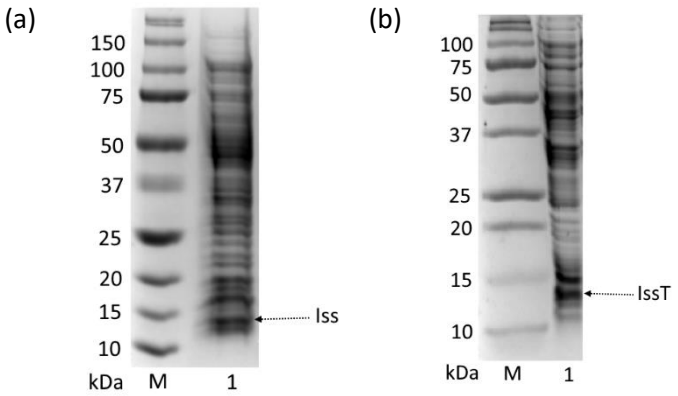

Figure 1: SDS-PAGE showing cell-free lysates of (a) BL21-pET28-iss and (b) BL21-pET28-issT in lane 1 of each gel respectively. Arrows indicate the protein of interest on each gel at around 14 kDa and 13 kDa respectively. Lane M represents the prestained Precision Plus Protein™ molecular weight standard.

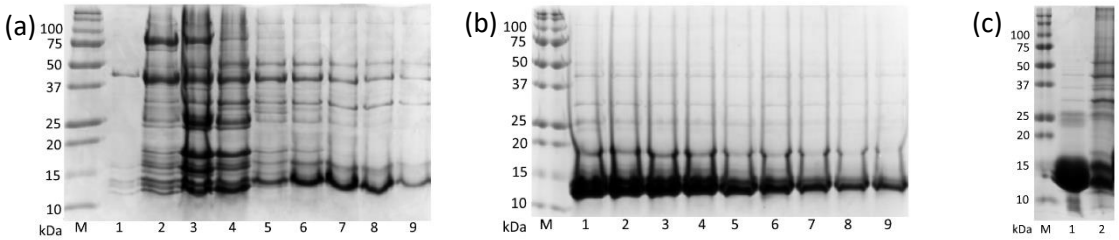

Figure 2: SDS-PAGE indicating FPLC fractions using IMAC from (a) BL21-pET28-iss, (b) BL21-pET28-issT, and (c) the concentrated proteins of IssT (lane 1) and Iss (lane 2) that were obtained after ultra-filtration. Lane M represents the prestained Precision Plus Protein™ molecular weight standard.

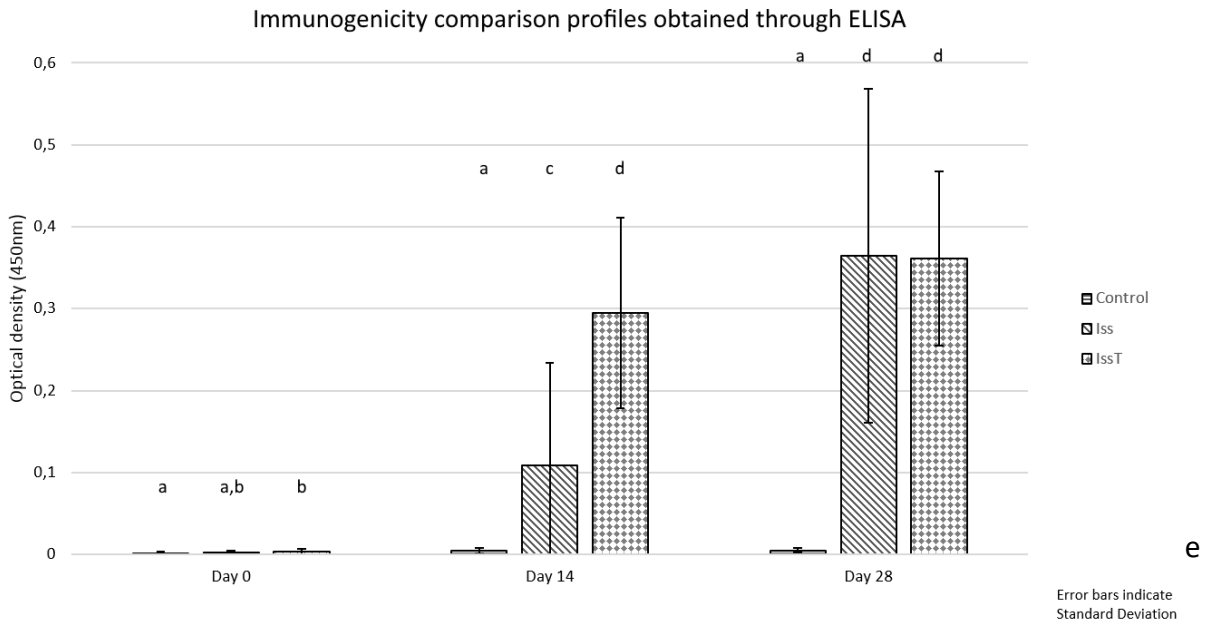

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Figure 3: Summarised ELISA results indicating the average optical density among the chickens of each group at different time intervals (indicated on the horizontal axis). Standard deviations are indicated as error bars. Statistically significantly similar results are indicated by alphabetical letters (a – d) above each column for $p < 0,05$.

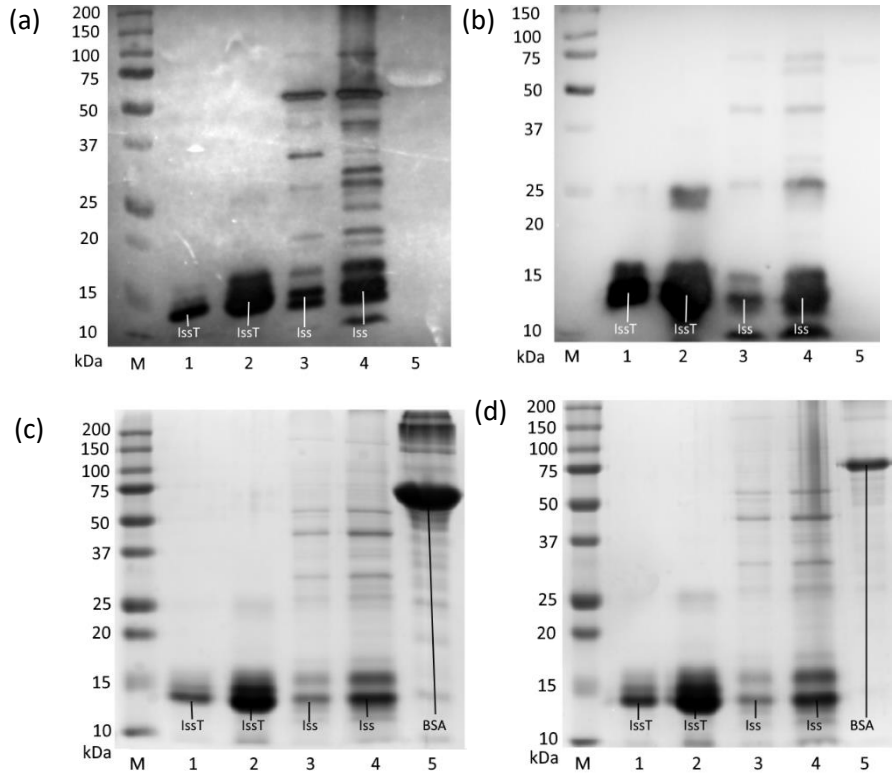

Figure 4: Western blots obtained with serum from chickens vaccinated with (a) Iss and (b) IssT proteins, and their corresponding SDS-PAGE gels (c) and (d) respectively. Lines with annotations indicate the appropriate protein bands. Lanes 1 and 2 on each gel contains the concentrated IssT at lower (4 ug) and higher (20 ug) concentrations respectively, followed by lanes 3 and 4 which contains lower (4 ug) and higher (20 ug) concentrations of the full Iss protein respectively. Lane 5 contains BSA as a negative control for the western blots. Lane M represents the prestained Precision Plus Protein^{TM} molecular weight standard.

Co-purified proteins can be seen on the SDS-PAGE gels (c) and (d) above the indicated bands which lead to non-Iss and non-IssT antibodies being produced as seen in (a) and (b) as bands above the indicated bands are also visible during western blotting.

CHAPTER 3

Expression of increased serum survival virulence protein of avian pathogenic *Escherichia coli* in the yeast strain *Yarrowia lipolytica* Po1h using surface display and secretion expression vectors

Preface

This chapter is presented in the format of a full thesis chapter.

This study investigated the expression of the Increased Serum Survival (Iss) protein of avian pathogenic *Escherichia coli* (APEC) in a *Y. lipolytica* yeast expression system patented by our research group. *Y. lipolytica* has been classified as a GRAS organism, making it an ideal system for expressing sub-unit vaccines, especially as it can then be used as a whole or lysed yeast in vaccine preparations, adding potential nutritional benefits to the poultry.

Unfortunately, expressing outer-membrane proteins can be a daunting task, as can be witnessed in this chapter.

Abstract

In this study, heterologous protein expression of *iss* in a *Yarrowia lipolytica* expression system, patented by this research group, was attempted for the development of a potential sub-unit vaccine against avian colibacillosis. One of the main advantages of using *Y. lipolytica* for protein expression is that this yeast is Generally Regarded As Safe (GRAS), therefore this system is an ideal candidate for use in downstream applications, such as vaccine development. Two similar vectors in *Y. lipolytica* were used for expression in this study; one which expresses the inserted protein on a GPI-anchor on the surface of the yeast and another which secretes the protein into the extracellular environment. An enterokinase proteolytic cleavage site was additionally incorporated into the surface-display system and a hexahistidine affinity tag into the secretion system. Protein expression was attempted, with proteins expected to be over-expressed during the late exponential phase under the hp4d promotor. No protein expression was observed for the surface display system, nor could the protein of interest be visualised after removal of the Iss protein using enterokinase and phospholipase C enzymes for cleavage from the cell surface. Secretion of the protein was also not observed, even after protein precipitation was performed on the extracellular environment to concentrate the protein. The hexahistidine tagged secreted protein could also not be purified and concentrated by use of immobilised metal-ion affinity chromatography. Lastly, the transformants were diagnosed using western blotting, using anti-Iss antibodies raised in Chapter 2, to confirm the presence of the Iss protein. No protein was detected for the surface-display and secretion transformants, despite the presence and sequence of the *iss* gene having been confirmed in these transformants. Either the Iss protein was expressed in sub-detectable concentrations, or protein expression failed completely due to unknown reasons. Investigating the use of a truncated form of the Iss protein, as done during Chapter 2, and using a *Y. lipolytica* strain which has Ylt1 retrotransposon sites for directed integration could lead to future successful *iss* expression.

Abbreviations and additional information

Enterokinase enzyme, light chain from New England Biolabs: 1 unit is defined as the amount of enzyme required to cleave 25 µg of a MBP-EK-paramyosin-ΔSal substrate to 95 % completion in 16 hours at 25 °C in a total reaction volume of 25 µl.

Phospholipase C from Sigma Aldrich: One unit will liberate 1.0 µmole of water soluble organic phosphorus from egg yolk L-α-phosphatidylcholine per min at pH 7.3 at 37 °C.

EK – Enterokinase

GPI – Glycosylphosphatidylinositol

PLC – Phospholipase C

IMAC – Immobilized metal ion affinity chromatography

TCA - Trichloroacetate

LB-Kan – Lysogeny broth /Luria-Bertani broth containing 30 µg/ml Kanamycin

PTMs – Post translational modifications

Introduction

Avian Pathogenic *Escherichia coli* (APEC) causes the disease colibacillosis in many avian species, which causes economic losses in the poultry industry (Barnes *et al*., 2003). The common clinical symptoms include swollen head syndrome, septicaemia, air sacculitis, pericarditis and cellulitis, which lead to decreased egg yields and possibly bird death.

Virulence in *E. coli* is caused by virulence genes in the genome as well as on extra-chromosomal structures such as plasmids (Dozois *et al*., 2003). Many varieties of virulence encoding genes exist, and some have been associated with colibacillosis. The genes act individually or polygenically, with varying frequencies observed in clinical isolates (Delicato *et al*., 2003; Vandekerchove *et al*., 2005). Certain combinations of virulence genes, specifically *iroN, iutA, iss, ompT* and *hlyF*, have been found to occur more frequently in pathogenic strains, due to some conservation on various virulence-encoding plasmids (Johnson *et al*., 2008). The virulence genes *iroN, iutA, iss, ompT, traT* and *etsC* encode for

proteins that have been predicted to be situated on the outer-membrane, and can thus be potential vaccine targets (Olsen *et al*., 2012). The genes *iroN, iutA* and *iss* fulfil both the criteria of Johnson *et al.* (2008) and Olsen *et al*. (2012) in that they are conserved among APEC isolates and their proteins are situated on the APEC cell surface This subset of virulence genes therefore represent ideal targets for the development of a vaccine against APEC in the poultry industry.

Y. lipolytica has been extensively investigated by researchers for heterologous expression of proteins from bacteria (e.g*. E. coli* β-glucoronidase) (Bauer *et al*., 1993), fungi (e.g. *Saccaharomyces cerevisiae* invertase) (Nicaud *et al*., 1989), plants (e.g. *Zea Mays* cytokinin oxidase I) (Madzak *et al*., 2001), mammals (e.g. Bovine cytochrome P450 17α) (Juretzek *et al*., 1995-1998), humans (e.g. Epidermal growth factor) (Hamsa *et al*., 1998) and viruses (e.g. Bacteriophage P1 Cre recombinase and Hepatitis B virus pre-HBs antigen) (Richard *et al*., 2001; Hamsa & Chattoo, 1994); making it a very versatile yeast for foreign protein production.

The *Y. lipolytica* strain Po1h used in this study, is a derivative of strain Po1d. Strain Po1d has high protein secretion characteristics, can utilise sucrose due to the presence of a recombinant invertase, and lacks the native *Y. lipolytica* alkaline extracellular protease (AXP). Po1h further lacks the native acid extracellular protease (AEP) and has only a single auxotrophy, for uracil. Furthermore, integration of foreign DNA can take place non-homologously using zeta-based vectors, which occurs as random integration in Po1h that lacks Ylt1 retrotransposon sites (Mauersberger *et al*., 2001; Thevenieau, 2007).

The promotor hp4d used for expression in the zeta-based pINA1317 vector in *Y. lipolytica*, is derived from the alkaline extracellular protease (*XPR2*) gene promotor, p*XPR2,* which natively secretes very large amounts of the proteases. An upstream activating sequence of p*XPR2* (UAS1) was used to create a hybrid promotor called hp4d, which contains four tandem copies of UAS1 followed by the TATA box of the *LEU2* promoter. This hybrid promotor allows for strong expression regardless of the nitrogen and carbon sources present in the growth medium, is pH independent, and growth-phase dependent (expression occurs primarily in the late exponential to early stationary phases) (Madzak *et al*., 2000).

In this study, the production of a heterologous expressed Iss protein in *Y. lipolytica* strain Po1h for potential vaccine use, will be investigated.

Materials and Methods

Gene *iss* Design and Synthesis

The reference sequence of the *iss* gene was obtained from NCBI (GenBank Accession number: NC_011964.1). The sequence was codon-optimized for expression in *Yarrowia lipolytica* and synthesised by GeneArt (Life Technologies). ATG and TAG codons were removed from the beginning and end of the sequence respectively to allow translation from the upstream ATG included in the secretion signal peptide, as well as to incorporate the downstream anchoring protein in pINA1317-YlCWP110 plasmid (Figure 1). Restriction sites for *Sfi*I and *Hind*III were added upstream and downstream from the trimmed sequence respectively for cloning into this plasmid.

Restriction digest of the synthesized plasmid 1206849_iss_pMA-T

The synthesized gene was supplied in a plasmid named 1206849_iss_pMA-T. Restriction digests were performed on the plasmid: *Sfi*I digestion was performed with 10 µl (1 µg) of plasmid DNA, 2 µl 5x Tango Buffer (Thermo Fischer Scientific), 1 µl *Sfi*I (Thermo Fischer Scientific) and 7 µl H_2O. The reaction mixture was incubated for one hour at 50 ^oC and 0.5 µl of this reaction mixture was removed and stored at -20 ^oC. Thereafter, *Hind*III digestion was performed with the remaining reaction mixture with the addition of 0.6 µl Tango buffer, 1 µL *Hind*III and 3.9 µl H_2O, followed by one hour incubation at 37 ^oC. Afterwards, 0.5 µl was removed and stored at -20 ^oC.

The two 0.5 µl fractions were analysed using electrophoresis (4.5 V/cm) in a 1 % (w/v) agarose gel to confirm complete digestion by both restriction enzymes.

The described restriction digest procedure was repeated for pINA1317-YlCWP110 (Patent number P4573ZACO), a plasmid which allows fusion of expressed proteins of interest to an external Glycosylphosphatidylinositol (GPI) anchor on the cell wall.

The reaction mixtures were separated on a 2 % (w/v) Low Melt Agarose gel by electrophoresis (3.5 V/cm). The bands representing the *iss* gene insert and the restriction digested pINA1317-YlCWP110 were excised from the agarose gels. The pINA1317-YlCWP110 plasmid was purified using a GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare), which uses a chaotropic buffer and a glass fiber matrix, following the manufacturer's protocol, while the *iss* gene gel fragment was stored at -20 °C.

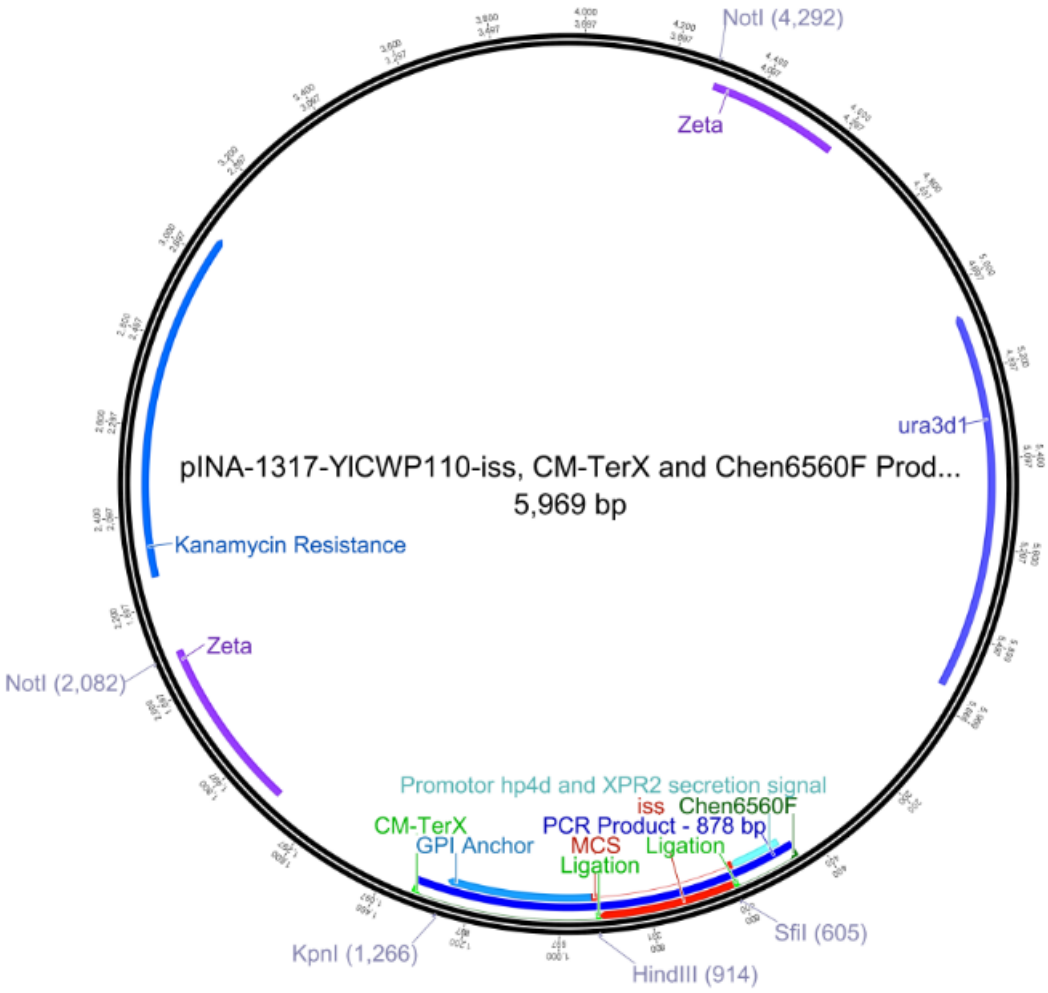

Figure 1: *In silico* plasmid map of pINA1317-YlCWP110 containing the *iss* gene ligated into the MCS using enzymes *Sfi*I (605 bp) and *Hind*III (914 bp) as indicated. The GPI-anchor sequence is indicated downstream of *iss* and the promotor hp4d containing the *XPR2* secretion signal is indicated upstream of *iss*. The "PCR Product" annotation indicates the PCR amplicon obtained when the primer pair Chen6560F and CM-TerX is used, producing an amplicon around 878 bp in size. *Not*I restriction sites are indicated at 2082 bp and 4292 bp for the removal of the bacterial backbone including the kanamycin resistance gene for selection in *E. coli*. The Zeta-sites for random integration of the yeast expression cassette into Po1h and the *ura3d1* marker for selection in Po1h cells are also indicated.

Ligation into pINA1317-YlCWP110 and transformation into RbCl-competent *E. coli* TOP10 cells

The ligation reaction contained 6 µl (60 ng) of pINA1317-YlCWP110, 100 µl of the melted agarose containing the *iss* gene, 15 µl Ligation buffer, 10 µl T4 ligase (New England Biolabs) and 19 µl H_2O. The ligation reaction was carried out at 37 ^oC for one hour.

Competent *E. coli* TOP10 (Invitrogen) cells were transformed with the ligation reaction mixture according to Sambrook and Russell (2001) and 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 for 16 hours.

Plasmid Extraction and Sequencing Confirmation

A single kanamycin-resistant *E. coli* colony was picked from the selective agar plate, inoculated into 5 ml LB containing 30 µg/ml kanamycin and incubated for 16 hours at 37 ^oC. Plasmids were extracted from the cells by use of a Qiagen Plasmid miniprep kit (Whitehead Scientific). A restriction digest was performed to confirm that the plasmids contained the insert, using *Sfi*I and *Hind*III as described before with 200 ng (10 µl) of extracted plasmid DNA. The digest was analysed using gel electrophoresis (4.5 V/cm) on a 1 % (w/v) agarose gel to confirm the insert's presence. PCR was performed with primer pairs Chen6560F (5'-GATCCGGCATGCACTGATC-3') and CM-terX (5'-GAACCTCGTCATTGATGGAC-3') (Patent number P4573ZACO) on the undigested pINA1317-YlCWP110 plasmid using the following cycling conditions: Initial denaturation at 94 ^oC for 5 minutes, 35 cycles of 94 ^oC for 30 seconds, 51 ^oC for 45 seconds and 72 ^oC for 75 seconds, followed by a final elongation stage at 72 ^oC for 7 minutes. The sequencing reaction was prepared using the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fischer Scientific) according to the manufacturer's specifications, followed by an EDTA/Ethanol clean-up and precipitation. The prepared samples were submitted for Sanger sequencing at the University of the Free State, Bloemfontein, Republic of South Africa. The sequences were aligned to the expected sequence of the codon-optimised *iss* gene and the GPI-anchor sequence present in pINA1317-YlCWP110.

Restriction Digest for the removal of the bacterial expression cassette

A *Not*I (Thermo Fischer Scientific) digest was performed on the plasmid for the removal of the bacterial expression cassette. The reaction mixture, containing 18 µl (3.6 µg) of extracted plasmid, 3 µl 6x Orange Buffer (Thermo Fischer Scientific), 1.5 µl *Not*I and 7.5 µl H_2O; was incubated for one hour at 37 ^oC. Electrophoresis (3.5 V/cm) was performed using a 2 % (w/v) low melt agarose. The gel band containing the yeast expression cassette was excised and purified using an illustra GFX Gel extraction kit (GE Healthcare) according to the manufacturer's protocol.

Transformation of the yeast expression cassette into *Y. lipolytica* Po1h and confirmation

The *Not*I digested DNA was used to transform *Y. lipolytica* Po1h cells using the one-step lithium acetate transformation according to the method described by Chen *et al*. (1997). The transformed yeast cells were then plated onto YNB agar plates (15 g/L bacteriological agar, 1.5 g/L yeast nitrogen base without amino acids and ammonium sulphate, 10 g/L glucose and 5 g/L ammonium sulphate) for the selection of Ura-positive transformants.

Whole-cell PCR was performed on 12 transformants to confirm the presence of the insert. Electrophoresis (4.5 V/cm) was performed with 1 % (w/v) agarose gel. The approximate 900 bp bands, corresponding to the expected amplicon length (Figure 1), were excised, cleaned with the illustra GFX Gel purification DNA kit (GE Healthcare) and used as template for the sequencing reaction using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The samples were submitted for Sanger sequencing at the University of the Free State, Bloemfontein, Republic of South Africa. The sequences were analysed and aligned using Geneious Pro R9 (http://www.geneious.com, Kearse *et al*., 2012).

Growth, protein expression and scanning electron microscopy (SEM) analysis of *iss* in *Y. lipolytica* Po1h

A 10 ml YPD pre-inoculum of one of the stored isolates was prepared in a 50 mL culture flask and incubated at 22 ^oC on a rotary shaker at 105 rpm for 17 hours. A main culture of 50 ml YPD in a 250 mL flask was inoculated with the pre-culture to an OD_{600nm} value of 0.1, followed by incubation as before. OD_{600nm} measurements were taken every 24 hours and 10 mL of YPD was added every day from the second day onwards. A growth curve was constructed to determine the onset of expression, which is expected in the late exponential

phase. Thereafter, untransformed Po1h and selected transformants named I5, I6, I7 and I8 were grown up in 5 mL YPD for 48 hours with shaking at 20 ^{o}C.

A sample from the untransformed strain Po1h and transformant I5 were prepared for SEM through fixation with 3 % (v/v) glutardialdehyde and 1 % (w/v) osmium tetroxide, ethanol dehydration, critical point drying, mounting on sample holder stubs and gold sputter-coating. Micrographs were taken with a Shimadzu SSX-550 Superscan SEM (University of the Free State, Bloemfontein, Republic of South Africa) to look for any cell-surface differences between the untransformed Po1h and the pINA1317-YlCWP110 transformant cells.

SDS-PAGE of protein products from *iss* in pINA1317-YlCWP110

The cultures of transformants I5, I6, I7 and I8, obtained from the expression procedure above, were disrupted by using a constant cell disruptor at 30 000 psi (2.068 x 10^5 kPa) and centrifuged at 7 000 x *g* for 10 minutes. The resultant pellets were resuspended in 1 x Laemmli sample buffer, the supernatants used to dilute 4 x Laemmli sample buffer to 1 x, and a YPD broth sample was included as a negative control. After boiling the samples at 95 ^{o}C for 5 minutes, they were loaded onto a 10 % polyacrylamide SDS gel and electrophoresed at 120 V in Tris-HCl Tricine SDS (TTS) buffer. The gel was stained and destained with Fairbanks A and Fairbanks D solutions (Fairbanks *et al*., 1971) respectively according to a protocol described by Wong *et al*. (2000). Destained gels were visualised using a Bio-Rad Gel Doc EZ system.

Addition of an enterokinase protease cutting site between Iss and GPI-Anchor

An enterokinase site was added between the expressed protein and the cell wall anchoring protein by incorporatoin by PCR. The constructed plasmid (pINA1317-YlCWP110-iss) containing the *iss* gene was used as a template and amplification was performed with forward primer iss-FP 5'-AGCTCGGCCGTTCTGGCCCAGGACAAC-3' (including *Sfi*I site, underlined) and reverse primer issEK-RP 5'-AATTAAAAGCTT(**CTTGTCGTCGTCGTC**)CTGAGAGCAG-3' (including *Hind*III site, underlined and the enterokinase (EK) recognition site, in bold). PCR cycling conditions were as follows: Initial denaturation at 94 ^{o}C for 5 minutes, 32 cycles of 94 ^{o}C for 30 seconds, 58 ^{o}C for 45 seconds and 72 ^{o}C for 30 seconds, followed by a final elongation stage at 72 ^{o}C for 7 minutes. Restriction digestions, ligations, sub-cloning and sequencing were all performed as

described previously. Thereafter, *Y. lipolytica* Po1h was transformed using the lithium acetate transformation procedure described previously. Eight colonies were picked, named S1 – S4 and T1 – T4 and whole cell PCR and sequencing confirmation was performed to confirm the incorporated enterokinase site by analysis using Geneious R9 (http://www.geneious.com, Kearse *et al*., 2012).

Removal of Iss from *Y. lipolytica* cell wall using enterokinase (EK) and phospholipase-C (PLC)

Po1h control cells and transformants I5 (containing only *iss*) and T1 (containing *iss* with an EK-site) were cultivated as described previously. After 7 days of cultivation, 1 mL of cells was harvested through centrifugation and washed twice with EK buffer (20 mM Tris-HCl, 50 mM NaCl, 2 mM $CaCl_2$, pH 8.0). Thereafter, the washed cells were resuspended in 1 mL of EK buffer containing 2 µL (32 units) of EK light chain (New England Biolabs). The reaction was incubated at 25 ^{o}C for 16 hours and a supernatant sample was prepared for SDS-PAGE.

In addition, 3.32 units of PLC was added to 2 mL of the above culture before washing incubated for 20 hours at 37 ^{o}C with mild agitation, and a supernatant sample was prepared for SDS-PAGE.

Electrophoresis of the samples was performed using SDS-PAGE and thereafter the gel was stained and destained with Fairbanks A and Fairbanks D solutions as described previously, and visualised using a Bio-Rad Gel Doc EZ system.

Cloning and transformation of the secretion vector pINA1317

The *iss* gene was incorporated into the secretion vector, pINA1317, after PCR amplification from the 1206849_iss_pMA-T plasmid with the forward primer set iss-FP 5'-AGCTCGGCCGTTCTGGCCCAGGACAAC-3' (additional *Sfi*I restriction site underlined) and iss-GA-Sec-RP 5'-GAAGGCCCATGAGGCCAGTCTTGTGCTCCATTAATTAAGGTACCTTATCACTGAGAGCA-3'
(additional *Kpn*I restriction site underlined) for incorporation into the pINA1317 yeast secretion vector. The cycling conditions used were: Initial denaturation at 94 ^{o}C for 5 minutes, 35 cycles of 94 ^{o}C for 30 seconds, 60 ^{o}C for 45 seconds and 72 ^{o}C for 30 seconds, followed by a final elongation step at 72 ^{o}C for 7 minutes. The PCR product and vector were digested with *Kpn*I and *Sfi*I restriction enzymes in 1x Tango buffer (Thermo Fischer Scientific) successively.

Reaction clean-ups, ligations and sub-cloning into *E. coli* TOP10 (Invitrogen) was performed as described previously. Sequences were confirmed using the Chen6560F and CM-terX primer pair for Sanger sequencing. *Not*I digests were performed on the resultant plasmids and the yeast backbone was purified using a GFX Gel extraction kit according to the manufacturer's protocol, before performing lithium acetate yeast transformation on *Y. lipolytica* strain Po1h as described previously. The transformed yeast cells were plated onto YNB agar plates and incubated at 25 ^{o}C for 3 – 4 days. Six transformants (Named 16_1, 16_2, 16_3, 9_1, 9_2 and 9_3) were randomly selected for whole cell PCR and sequencing, using the Chen6560F and CM-terX primer pair as described previously, to confirm the presence of the *iss* gene.

Screening secretion vector transformants for Iss secretion

The iss secretion transformant 16_1 and the non-transformed Po1h strain were each inoculated into 5 mL YPD media and grown overnight as pre-inoculums. Two 250 mL flasks containing 50 mL YPD media and one tablet of cOmplete™ EDTA-free Protease Inhibitor Cocktail (Roche) were inoculated from the pre-inoculums and incubated with shaking for 11 days. The cultures were centrifuged and the supernatants treated using four different chemicals: trichloroacetate (TCA), sodium hydroxide, ammonium sulphate and acetic acid, to precipitate extracellular proteins.

TCA precipitation: TCA was added to a final concentration of 10 % (w/v) to the sample, incubated on ice for 30 minutes and centrifuged for 10 minutes at 20 000 x *g* at 4 ^{o}C. The supernatant was discarded and the pellet washed twice with -20 ^{o}C acetone and left to dry.

Sodium hydroxide precipitation: A final concentration of 0.5 M of sodium hydroxide was prepared with the supernatant, centrifuged for 10 minutes at 20 000 x *g* at 4 ^{o}C and the pellet washed twice with -20 ^{o}C acetone and left to dry.

Ammonium sulphate precipitation: Ammonium sulphate crystals were added to the supernatant with constant stirring until saturation was reached. The solution was then centrifuged for 10 minutes at 20 000 x *g* at 4 ^{o}C and the pellet washed twice with -20 ^{o}C acetone and left to dry.

Acetic Acid preparation: A final concentration of 50 % of glacial acetic acid was prepared with the supernatant, centrifuged for 10 minutes at 20 000 x *g* at 4 ^{o}C and the pellet washed twice with -20 ^{o}C acetone and left to dry.

The resultant pellets were resuspended in 1 x Laemmli sample buffer, boiled and the proteins separated through SDS-PAGE. The gel was stained and destained with Fairbanks A and Fairbanks D solutions respectively as described previously, and visualised using a Bio-Rad Gel Doc EZ system.

Cloning, transformation, expression and purification of a modified secretion vector pINA1317 containing a C-terminal hexahistidine tag

The variant plasmid pINA1317-6xHIS was constructed by Dr C.W. Theron, University of the Free State, Bloemfontein, Republic of South Africa, containing a gene insert unrelated to this project.

The 1206849_iss_pMA-T and pINA1317-6xHIS plasmids were digested with *Sfi*I and *Hind*III, followed by purification and ligation of the relevant DNA fragments. *E. coli* TOP10 was transformed with the ligation mix and cultivated on LB-Kan agar. Colonies were picked and screened using whole-cell PCR with the Chen6560F and CM-terX primers as previously described. The plasmid containing the insert was linearized using *Xba*I as an additional *Not*I site is present just before the *Kpn*I cutting site (Figure 2), which is problematic as *Not*I, as discussed previously, is used to remove the bacterial expression cassette of the pINA1317 vector. The *Xba*I-linearized vector containing the *iss* gene with a C-terminal hexahistidine tag was transformed into *Y. lipolytica* Po1h and cultivated for 3 – 5 days on YNB agar. Four colonies designated Xba1, Xba2, XbaD and Xba9 were isolated and screened for the presence of the insert using the Chen6560F and CM-terX primers. Positive amplicons from samples Xba2 and XbaD were sequenced and aligned to *in silico* determined sequences using Geneious R9 (http://www.geneious.com, Kearse *et al*., 2012).

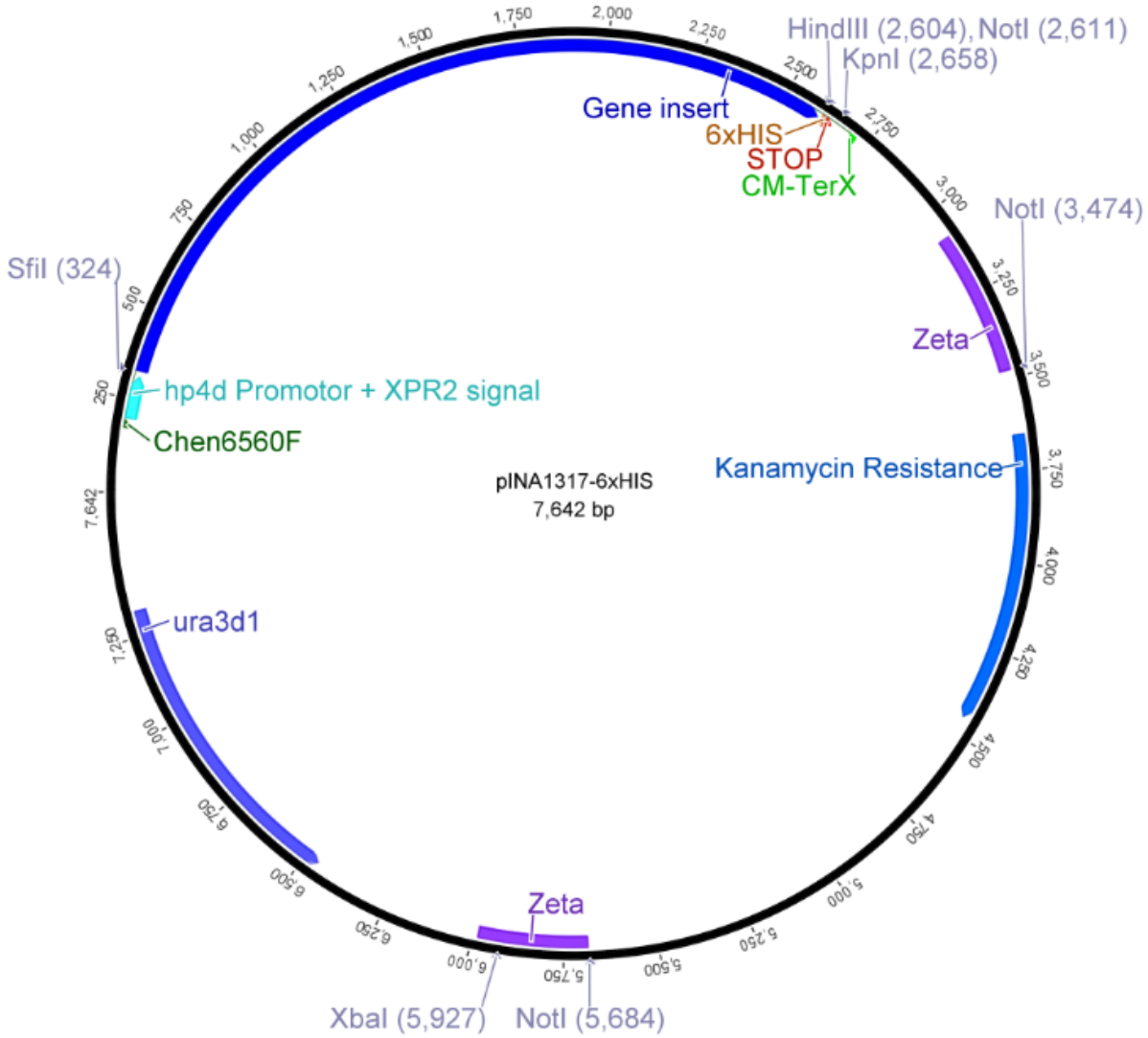

Figure 2: *In silico* plasmid map of the secretion pINA1317-6xHIS constructed by Dr C.W. Theron from the University of the Free State, Bloemfontein, Republic of South Africa. The gene insert indicated is unrelated to this study and is replaceable with *iss* by using *Sfi*I (324 bp) and *Hind*III (2658 bp) digestion, with retention of the hexahistidine sequence downstream of the gene.

Protein expression was performed by incubating clone Xba2 for 48 hours in 250 ml YPD with shaking at 20 ^{o}C. The cells were pelleted by centrifugation and the supernatant dialysed against IMAC binding buffer (0.5 M NaCl, 20 mM Imidazole and 50 mM Tris-HCl, pH 7.4) overnight through a 10 kDa molecular weight-cutoff SnakeSkin™ Dialysis Tubing (Thermo Fisher Scientific). The dialysed sample was applied to a 5 mL Cobalt-immobilized metal ion affinity chromatography (IMAC) column and washed with wash buffer (0.5 M NaCl, 20 mM Imidazole and 50 mM Tris-HCl, pH 7.4) for 10 column volumes (50 mL) using an ÄKTA FPLC

(GE Healthcare). Elution of any bound protein was performed using a linear gradient of imidazole from 20 mM to 500 mM in 5 mL fractions. The fractions were separated on SDS-PAGE and the gel was stained and destained with Fairbanks A and Fairbanks D solutions (Fairbanks *et al*., 1971) respectively according to Wong *et al*. (2000).

Western blotting analysis of *Y. lipolytica* clones expressing *iss*

Western blotting analysis of *Y. lipolytica* clones was performed using anti-Iss chicken-raised antibodies against *E. coli*-expressed Iss protein (Chapter 2). *Yarrowia lipolytica* strain Po1h transformants containing the *iss* gene in plasmid pINA1317-YlCWP110 (I5, I6, I7), the iss gene with an EK cutting site in pINA1317-YlCWP110 (T1, T2, S1), the iss gene in the secretion vector pINA1317 (9_1) and the *iss* gene followed by a hexahistidine tag in the secretion plasmid pINA1317 (Xba9, XbaD) were incubated in 5 mL YPD at 30 ^{o}C for 48 hours. Cells containing the pINA1317-YlCWP110 cell surface display plasmid were disrupted by using a constant cell disruptor at 30 000 psi (2.068 x 10^5 kPa) and centrifuged at 10 000 x *g* for 10 minutes. The supernatants were prepared for SDS-PAGE. Cells containing the pINA1317 protein secretion plasmids were centrifuged at 10 000 x *g* for 10 minutes and the supernatants prepared for SDS-PAGE. Duplicate 10 % polyacrylamide gels were prepared and each of the samples loaded onto both gels and SDS-PAGE performed. One gel was stained and destained with Fairbanks A and Fairbanks D solutions (Fairbanks *et al*., 1971) respectively according to Wong *et al*. (2000).

The western blotting protocol described by Bio-Rad laboratories was used as a guideline to perform western blotting (Bio-Rad Laboratories, General Protocol for Western Blotting, http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6376.pdf). The remaining unstained gel was transferred onto a 0.45 µm nitrocellulose membrane, sandwiched in filter paper and the proteins transferred onto the membrane by applying a voltage of 300 V for 1 hour in cooled transfer buffer (25 mM Tris-HCl, 190 mM glycine and 20 % methanol, pH 8.3) with the gel on the anode and the membrane on the cathode using a wet/tank blotting system (Bio-Rad Laboratories). The membrane was briefly stained with Ponceau S solution (0.2 % w/v Ponceau S and 5 % v/v glacial acetic acid) to confirm adequate transferral of proteins onto the membrane. The stain was rinsed off with multiple washes of Tris-buffered saline with Tween 20 (TBST) (20 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.1 % Tween 20). The membrane was blocked with TBST containing 3 % BSA (w/v) for 1

hour at room temperature to avoid any background fluorescence. Chicken-raised polyclonal antibodies containing anti-Iss antibodies from Chapter 2 were diluted 1:1500 in TBST and incubated overnight at 4 ^{o}C on the membrane. The membrane was rinsed three times with TBST containing 3 % BSA (w/v) to remove excess antibodies and incubated for 1 hour with 1:5000 TBST containing 3 % BSA diluted HRP-conjugated rabbit-raised anti-chicken antibodies at room temperature. Thereafter the membrane was briefly rinsed with TBST and incubated with 3,3',5,5'-Tetramethylbenzidine (TMB) (Roche) for 15 minutes to allow the horseradish peroxidase (HRP) to oxidise the TMB substrate to 3,3',5,5'-tetramethylbenzidine diamine which is blue and can be viewed with a Bio-Rad Gel Doc EZ imager system.

Results

pINA1317-YlCWP110 with a codon-optimised increased serum survival gene

The synthesized gene supplied in the 1206849_iss_pMA-T vector by GeneArt was successfully removed through the *Sfi*I and *Hind*III double digest (Figure 3) and the pINA-1317-YlCWP110 plasmid linearized with compatible sticky-ends (Figure 4). The products from the double digest were purified (Figure 5), ligated and successfully transformed into *E. coli* TOP10 cells as colonies were present on LB-Kan plates after 16 hours of incubation.

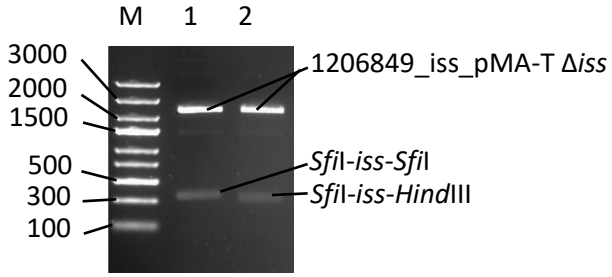

Figure 3: 1 % Agarose gel, indicating the GeneRuler™ Express DNA Ladder molecular weight marker (in bp) in lane M. Lane 1 shows the product (*Sfi*I-*iss*-*Sfi*I) obtained after the *Sfi*I restriction digestion of plasmid 1206849_iss_pMA-T and lane 2 the subsequent *Hind*III restriction digestion thereof (*Sfi*I-*iss*-*Hind*III). Lane 1 and 2 also show the linearized plasmid (1206849_iss_pMA-T Δ*iss*) with the *iss* gene removed.

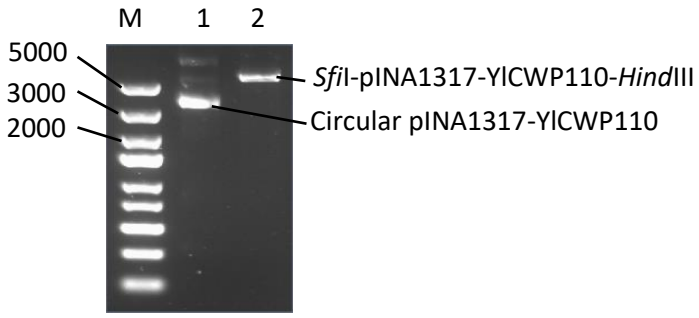

Figure 4: 1 % Agarose gel, indicating the GeneRuler™ Express DNA Ladder molecular weight marker (in bp) in lane M. Lane 1 shows the circular undigested pINA1317-YlCWP110 plasmid and lane 2 the linearized form (*Sfi*I-pINA1317-YlCWP110-*Hind*III) of the plasmid after *Sfi*I and *Hind*III restriction digestion.

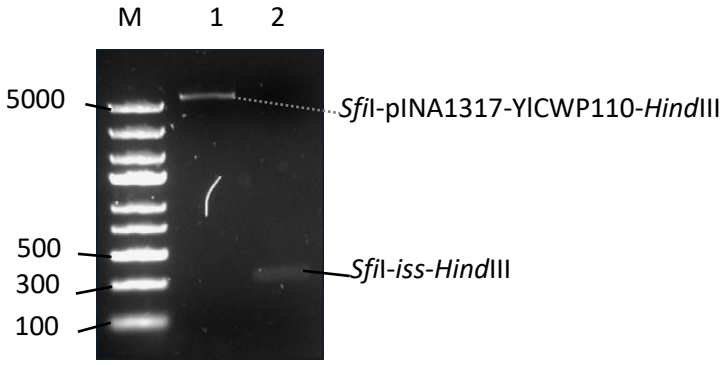

Figure 5: 1 % Agarose gel, indicating the GeneRuler™ Express DNA Ladder molecular weight marker (in bp) in lane M. Lane 1 shows the purified *Sfi*I and *Hind*III restriction digested product of pINA1317-YlCWP110 with lane 2 indicating the same for the restriction digestion product of *iss* (~310 bp) removed from plasmid 1206849_iss_pMA-T.

Plasmids were extracted from liquid cultures of nine randomly picked colonies and the inserts confirmed through a double digest with *Sfi*I and *Hind*III (Figure 6). The PCR performed on the extracted plasmids with the Chen6560F and CM-TerX primers yielded the expected band size of approximately 878 bp (Figure 7). The sequence obtained from the PCR product yielded high quality sequence data reads (forward and reverse) that were assembled, trimmed and aligned to the *in silico* designed plasmid (Figure 8) in Geneious R9. The extracted plasmids were digested with *Not*I (Figure 9) which yielded the expected band sizes of approximately 2214 bp and 3763 bp and PCR-gel clean-up performed on the yeast

backbone fragment sized 3763 bp. Clone 1's purified band (Lane 2 in Figure 10) showed the highest yield on the gel and was used during lithium acetate transformation of *Y. lipolytica* Po1h.

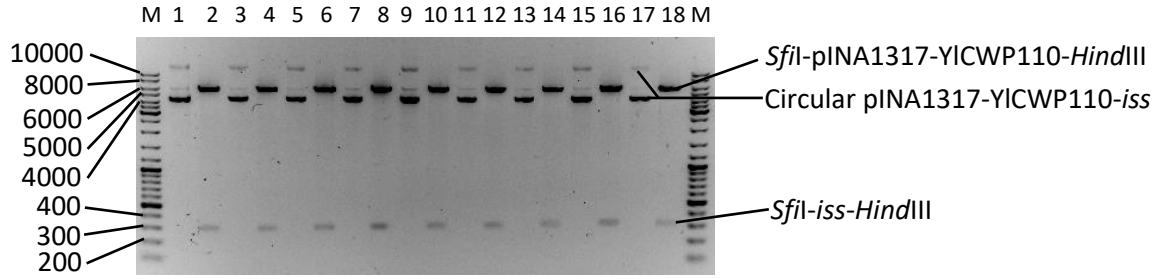

Figure 6: 1 % Agarose gel, indicating the GeneRuler™ DNA Ladder Mix molecular weight marker (in bp) in lanes M. Lanes 1, 3, 5, 7, 9, 11, 13, 15 and 17 indicate the nine extracted plasmids (Clones 1 – 9) from transformed *E. coli* TOP10 isolates with the suspected pINA1317-YlCWP110 plasmid containing the *iss* gene insert. Lanes 2, 4, 6, 8, 10, 12, 14, 16 and 18 were loaded with the plasmids of the previous lanes respectively which were restriction digested with *Sfi*I and *Hind*III to linearize the plasmid (~5600 bp) and remove the *iss* insert (~310 bp) in the MCS of pINA1317-YlCWP.

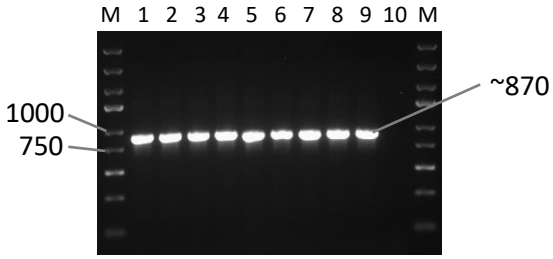

Figure 7: 1 % Agarose gel, indicating the GeneRuler™ Express DNA Ladder molecular weight marker (in bp) in lanes M. Lanes 1 – 9 contained the PCR product (~870 bp) obtained with the primer pair Chen6560F and CM-TerX amplified from nine plasmid extractions (Clones 1 – 9) from *E. coli* TOP10 pINA1317-YlCWP110-*iss* transformants. Lane 10 contained the no-template PCR control.

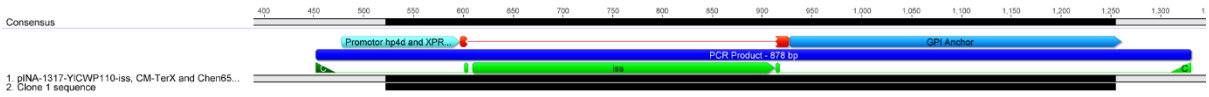

Figure 8: Nucleotide alignment performed with Geneious R9 of the *in silico* derived sequence of pINA1317-YlCWP110-*iss* and the sequence obtained for Clone 1 from *E. coli* TOP10 transformed with pINA1317-YlCWP110-*iss*. The black bars indicate homology between the sequences and white bars indicate dissimilar sequences. Complete homology between the two sequences is indicated by the black bar in the "Consensus" row for the overlapping region which includes the entire "iss" and partial "Promotor hp4d and XPR…" and "GPI-anchor" regions as indicated by the colour annotations.

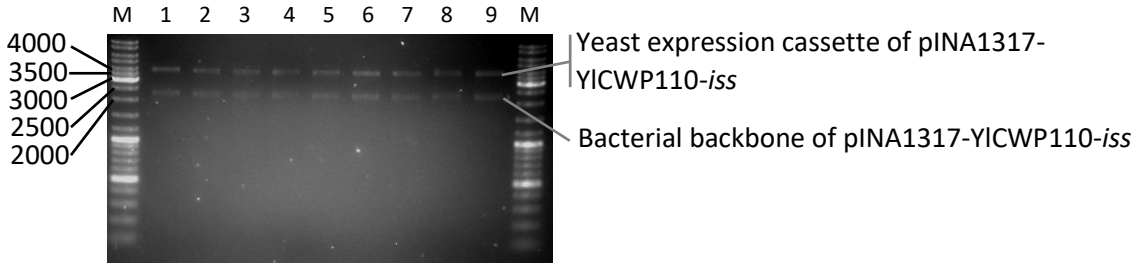

Figure 9: 1 % Agarose gel, indicating the GeneRuler™ DNA Ladder Mix molecular weight marker (in bp) in lanes M. Lanes 1 – 9 contains the nine plasmid extractions (Clones 1 – 9) from *E. coli* TOP10 pINA1317-YlCWP110-*iss* transformants digested with *Not*I. The bacterial backbone of the plasmid (~2200 bp) and the yeast expression cassette containing *iss* (~3700 bp) are indicated.

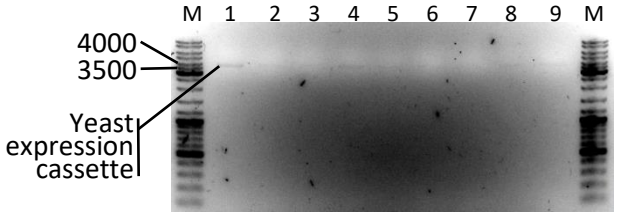

Figure 10: 1 % Agarose gel, indicating the GeneRuler™ DNA Ladder Mix molecular weight marker (in bp) in lanes M and the purified yeast expression cassette (~3700 bp) of pINA1317-YlCWP110-*iss* of Clones 1 – 9 in lanes 1 – 9 respectively. Concentrations of the bands in lanes 1 – 9 are very low indicated by very faint bands.

Twelve yeast colonies were selected from YNB agar plates, which were expected to be positive transformants since untransformed Po1h is Orotidine 5'-phophate decarboxylase (URA) deficient. The flanking-region whole-cell PCR however only yielded only four positive amplifications (Figure 11) on transformants named I5, I6, I7 and I8, from which high-quality

sequences were obtained for the amplicons from lane 5 and 6 (transformants I5 and I6) (Figure 11). The alignment of the sequences from I5 – I8 with the *in silico* determined sequence (Figure 12) yielded 83.7 % consensus sequence coverage for I6 and 77.1 % for I5 with 100 % homology in both cases. For both I5 and I6 the entire *iss* gene sequence was included. With isolates I7 and I8 only a single direction of useable short sequence reads was obtained. 100 % homology for these small regions were obtained during alignment, however neither aligned with the entire *iss* gene. Isolate I5 was used for the subsequent experiments.

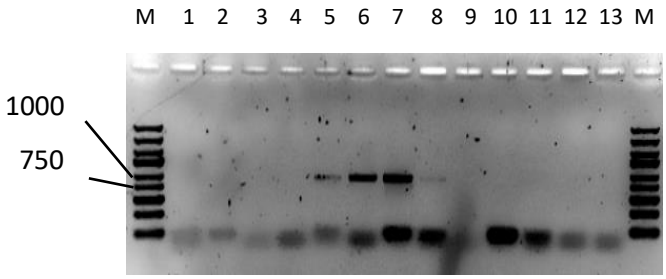

Figure 11: 1 % Agarose gel, indicating the GeneRuler™ Express DNA Ladder molecular weight marker (in bp) in lanes M and PCR products (~870 bp) are visible for yeast transformants (Isolates I5 to I8) using the primer pair Chen6560F and CM-TerX in lanes 5 – 8. Lanes 1 – 4 and 9 – 12 were PCR-negative (Isolates 1 – 4, 9 – 12). Lane 13 contained the no-template PCR control.

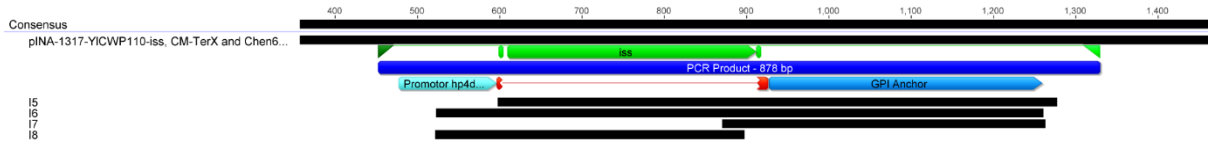

Figure 12: Nucleotide alignment performed with Geneious R9 of the *in silico* derived sequence of pINA1317-YlCWP110-*iss* and the sequence data obtained for *Y. lipolytica* Po1h transformants I5, I6, I7 and I8 containing the yeast expression cassette of pINA1317-YlCWP110-*iss*. The black bars indicate homology between the sequences and white bars dissimilar sequences. Complete homology was obtained for all the sequences compared to the pINA1317-YlCWP110-*iss* sequence. Sequence coverage for I5 is 77.1% of the 878 bp PCR product, I6 at 83.7 % and both covered the entire *iss* gene region as annotated in light green. I7 and I8 sequences were of lower quality and only a single useable read was obtained for each and are thus not representative of consensus sequences. Neither I7 nor I8 gave complete coverage of the entire *iss* gene region.

Optimal expression levels under the hp4d promotor are expected before the onset of stationary phase growth according to Madzak *et al*. (2000), therefore a simple growth curve

experiment was performed on the transformant I5 and the untransformed *Yarrowia lipolytica* strain Po1h (Figure 13) to determine subsequent minimum incubation times required before expecting product formation.

The pINA1317-YlCWP110 plasmid is expected to lead to the production of GPI-anchored proteins externally on the exterior of the yeast cell wall. Therefore, to observe any possible changes in the cell wall surface, SEM was performed on the untransformed Po1h *Y. lipolytica* strain (Figure 14(a)) and the pINA1317-YlCWP110-*iss* transformed I5 isolate (Figure 14(b)). A rough surface of tiny protrusions was observed on the transformed yeast cells, while the untransformed yeast cells displayed a typical smooth surface.

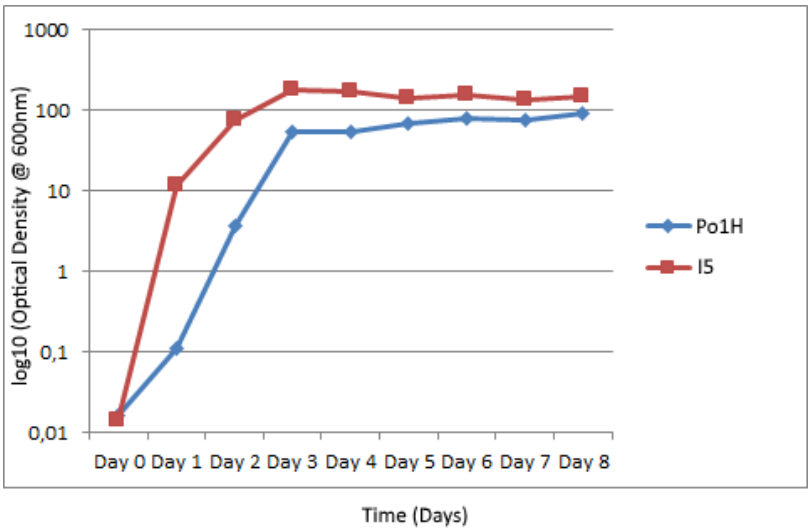

Figure 13: Semi-log growth curved based on optical density readings at 600 nm of a single experimental run over the course of 8 days with yeast transformant I5 and the untransformed *Y. lipolytica* strain Po1h in YPD media. Stationary phase is reached between 2 and 3 days with both cell-lines with I5 reaching higher OD readings earlier on likely due to the presence of the URA gene from the yeast expression cassette used for selection purposes which will give I5 a growth advantage over Po1h.

The SDS-PAGE gel (Figure 15) performed on the expressing yeast did not yield a protein band of the expected size (11 kDa). It was then decided to incorporate an enterokinase proteolytic cutting site between the *iss* gene and the GPI-anchor gene in the vector, to allow for the removal of the protein from the cell surface.

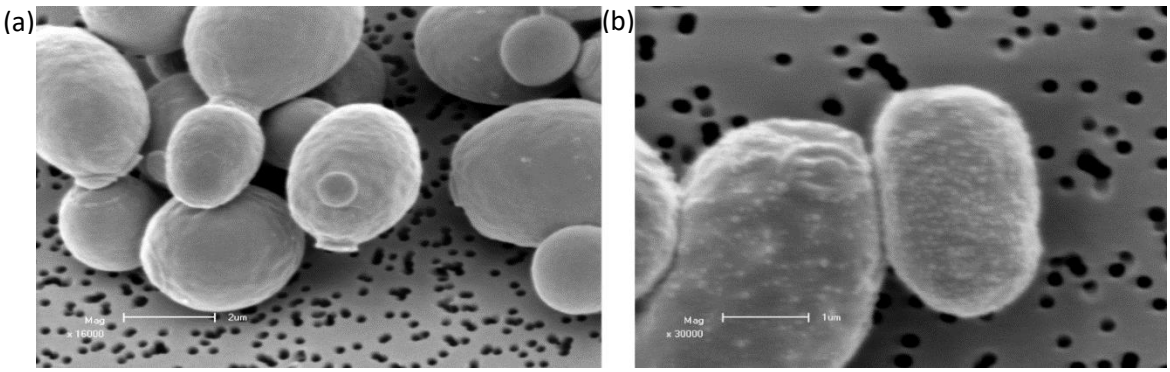

Figure 14: Scanning electron microscope micrograph of *Y. lipolytica* Po1h (a) and the pINA1317-YlCWP110-*iss* transformant I5 (b) after 48 hours of growth. A smooth cell surface was obtained with the untransformed cells. In contrast, the transformed cells showed the cells covered in tiny protrusions, speculated to be due to the presence of the GPI-anchor being expressed and transported to the cell surface.

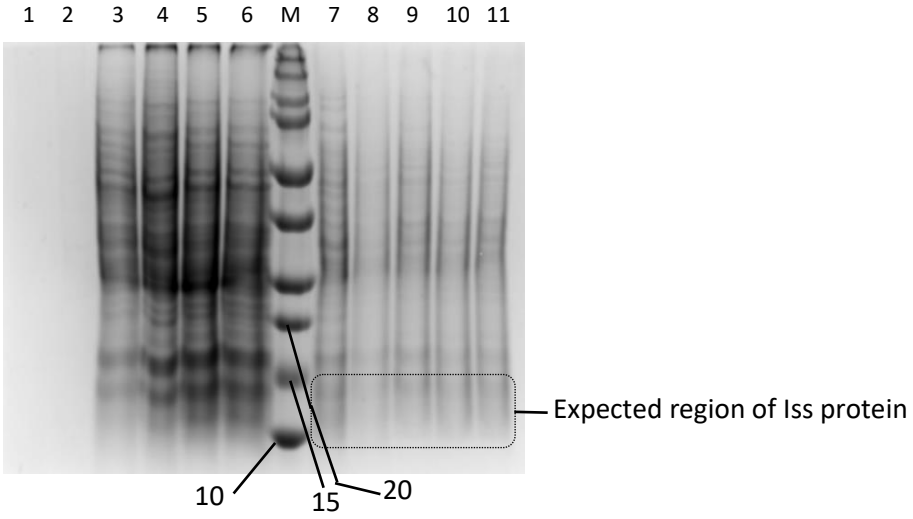

Figure 15: A Coomassie stained 10 % SDS-PAGE gel of cell disrupted lysates of pINA1317-YlCWP110-*iss Y. lipolytica* Po1h transformants I5, I6, I7, I8 and the untransformed strain as a negative control for *iss* expression and YPD as a no-cell media control. Lane M indicates the Bio-Rad Precision Plus Protein™ Standard as a molecular weight marker (in kDa). The indicated lanes contained the following samples: Lane 1 contained YPD broth as the media control, lane 2 the supernatant of the disrupted Po1h control, lanes 3 – 6 the supernatant from the disrupted transformants I5 – I8 respectively, lane 7 the pellet from the disrupted Po1h control and lanes 8 – 11 the pellets obtained from the disrupted transformants I5 – I8 respectively. No protein bands were present in the YPD media control and unexpectedly neither in the Po1h control supernatant. The transformant supernatants (lanes 3 – 6) contained water-soluble proteins in high concentrations and of different sizes. The water-insoluble cell pellets (lanes 7 – 11) gave faint nearly identical protein profiles for both the transformants (lanes 8 – 11) and the Po1h control (lane 7). The Iss protein has a theoretical mass of 11 kDa, is expected to be water-insoluble due to natively being a membrane-associated protein and attached to water-insoluble yeast cell walls, although no distinct bands are present in the vicinity (as indicated) that differed from the Po1h control in the pellets obtained after cell disruption and centrifugation (lanes 7 – 11).

Incorporation of an enterokinase proteolytic cutting site into pINA1317-YlCWP110 with a codon-optimised increased serum survival gene

Iss was successfully re-amplified by PCR using the primers to incorporate a EK cutting-site amplified successfully (Figure 16). The pINA1317-YlCWP110 plasmid and the purified PCR product digested with *Sfi*I and *Hind*III were successfully ligated and transformed into *E. coli* TOP10 cells However, only two colonies named “S” and “T”, were present on the LB-Kan plate. The presence of the insert in the plasmids extracted from the two colonies was confirmed using PCR (Figure 17) and sequencing of these products from the extracted

plasmid from two colonies showed 100 % homology with the expected sequence (Figure 18).

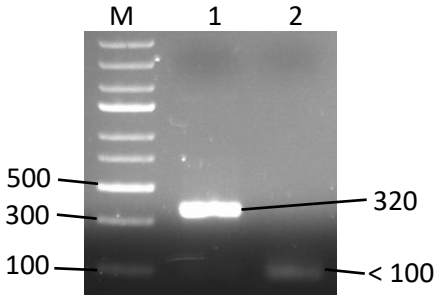

Figure 16: 1 % Agarose gel, indicating the GeneRuler™ Express DNA Ladder molecular weight marker (in bp) in lane M, the PCR product (~320 bp) obtained with primer pair iss-FP and issEK-RP using pINA1317-YlCWP110-*iss* as template in lane 1 with a no-template PCR control in lane 2. Some primer dimers (< 100 bp) are present in lane 2 likely due to a lack of DNA template to bind to.

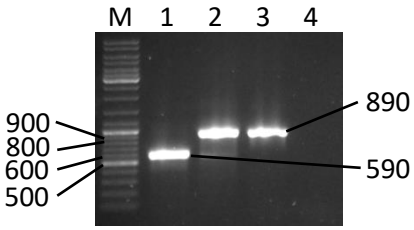

Figure 17: 1 % Agarose gel, indicating the GeneRuler™ DNA Ladder Mix molecular weight marker (in bp) in lane M, the PCR products in lanes 1 (~590 bp), 2 and 3 (~890 bp) obtained with primer pair Chen6560F and CM-TerX. The template used in lane 1 is pINA1317-YlCWP110 without any insert, lanes 2 and 3 used extracted plasmids pINA1317-YlCWP110-*iss*EK from clones "S" and "T" respectively. lane 4 contained the no-template control of the PCR.

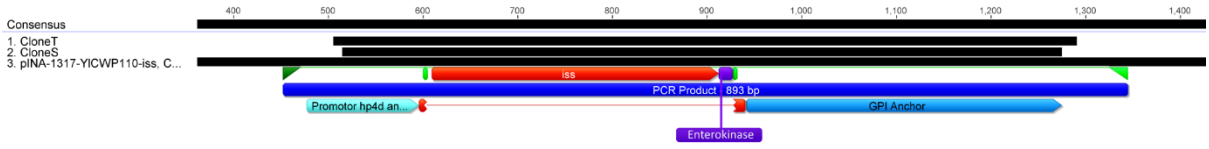

Figure 18: Nucleotide alignment performed with Geneious R9 of the *in silico* derived sequence of pINA1317-YlCWP110-*iss*EK and the sequence data obtained for *E. coli* TOP10 transformants "T" and "S" containing the plasmid pINA1317-YlCWP110-*iss*EK. The black bars indicate homology between the sequences and white bars dissimilar sequences. Complete homology was obtained for both the sequences when compared to the pINA1317-YlCWP110-*iss*EK sequence. Sequence coverage for "T" is 87.8 % of the 893 bp PCR product, "S" at 84.9 % and both covered the entire *iss* gene region as annotated in red with the EK cutting site as annotated in purple.

The yeast backbone (Figure 19) was successfully transformed into chemically competent *Y. lipolytica* Po1h cells after *Not*I digestion and eight transformed colonies were observed after 48 hours of incubation on YNB agar, named S1, S2, S3 and S4 from the "S" colony's plasmid and similarly T1 to T4 for the "T" colony's plasmid. The whole-cell PCR performed on the yeast isolates yielded the correct amplicon size on four of the isolates, namely S1, S4, T1 and T2, (Figure 20). The sequences obtained for the four amplicons shared 100 % sequence homology with the *in silico* determined sequence across the entire *iss* gene and the EK cutting site from the plasmid, although sequence coverage for the sequence of S4 was limited due to low sequence quality (Figure 21).

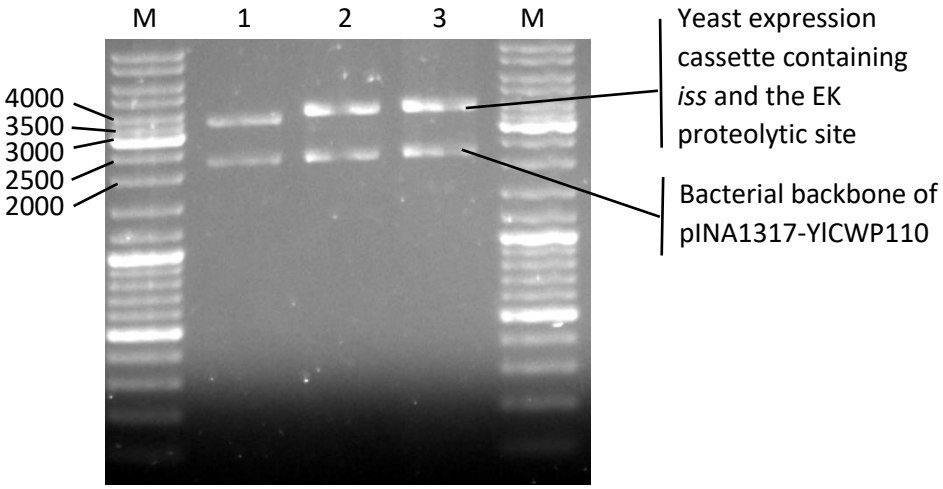

Figure 19: 1 % Agarose gel, indicating the GeneRuler™ DNA Ladder Mix molecular weight marker (in bp) in lanes M. Lane 1 contains the empty pINA1317-YlCWP110 plasmid and lanes 2 and 3 contains the pINA1317-YlCWP110-*iss*EK plasmids extracted from transformants "S" and "T" from *E. coli* TOP10 cells respectively after being digested with *Not*I to separate the bacterial backbone from the yeast expression cassette. The bacterial backbone of the plasmid (~2200 bp) and the yeast expression cassette containing *iss* (~3800 bp) are indicated. The yeast expression cassette from the control empty plasmid (lane 1) is observably smaller (~3600 bp) than that of the plasmids with *iss* and the EK recognition site inserted as can be seen in lanes 2 and 3.

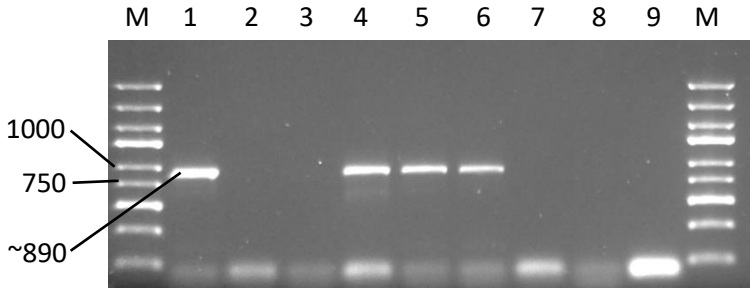

Figure 20: 1 % Agarose gel, indicating the GeneRuler™ DNA Express Ladder molecular weight marker (in bp) in lane M and the PCR products of an estimated size of ~890 bp in lanes 1, 4, 5 and 6 obtained with primer pair Chen6560F and CM-TerX on *Y. lipolytica* transformed with pINA1317-YlCWP110-*iss*EK yeast expression cassettes from *E. coli* transformants "S" (lanes 1 – 4; transformants S1, S2, S3 and S4 respectively) and "T" (lanes 5 – 8; transformants T1, T2, T3 and T4 respectively). Lane 9 contains the PCR no-template control.

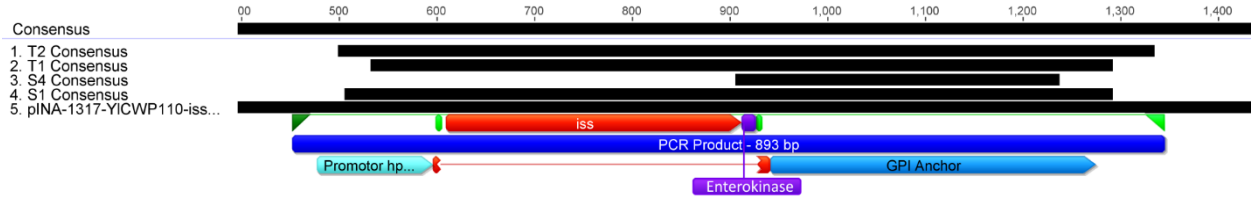

Figure 21: Nucleotide alignment performed with Geneious R9 of the *in silico* derived sequence of pINA1317-YlCWP110-*iss*EK and the sequence data obtained for *Y. lipolytica* Po1h transformants T2, T1, S4 and S1 containing the yeast expression cassette of pINA1317-YlCWP110-*iss*EK. The black bars indicate homology between the sequences and white bars dissimilar sequences. Complete homology was obtained for all the sequences when compared to the pINA1317-YlCWP110-*iss*EK sequence. The presence of the EK proteolytic site (purple annotation) was confirmed for all four sequences. T2, T1 and S1 had high quality sequences which covered the entire "iss" region as annotated in red and "GPI-anchor" region as annotated in blue. T2, T1, S4 and S1 had 93.4 %, 84.4 %, 36.8 % and 87.8 % query coverages respectively of the expected 893 bp PCR product (dark blue annotation).

The prepared supernatants from the EK and PLC reactions performed on transformant T1 were electrophoresed on SDS-PAGE, but no protein bands were observed (results not shown).

Incorporation of iss into the secretion vector with pINA1317.

The PCR using the primer pair to incorporate the *Sfi*I and *Kpn*I sites for the cloning of the *iss* gene into the secretion vector pINA1317 amplified successfully (Figure 22). The PCR product was successfully ligated into the pINA1317 vector as colonies were obtained when propagated in *E. coli* TOP10 cells on LB-Kan agar plates. The inserts were confirmed with the extracted plasmids through PCR (Figure 23) and two amplicons' sequences aligned to the *in silico* determined sequences (Figure 24).

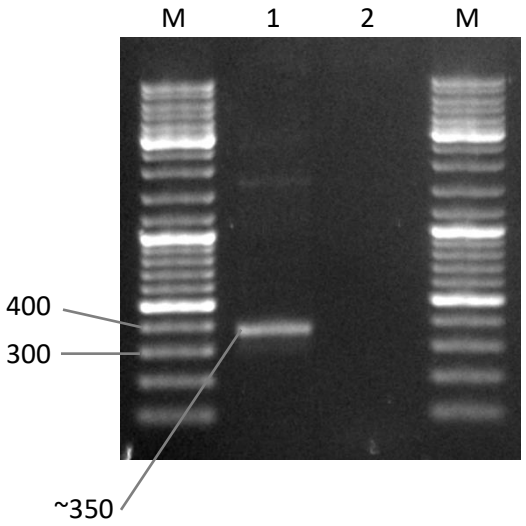

Figure 22: 1 % Agarose gel, indicating the GeneRuler™ DNA Ladder Mix molecular weight marker (in bp) in lanes M, the PCR product (~350 bp) obtained with primer pair iss-FP and iss-GA-Sec-RP using plasmid 1206849_iss_pMA-T as template DNA to incorporate a *Kpn*I restriction site on the downstream end of *iss* in lane 1. The no-template PCR control was loaded in lane 2.

The *Not*I-digested yeast backbone (Figure 25) from the two isolates (9 and 16) were purified and successfully transformed into *Y. lipolytica* Po1h cells, as indicated by the confirmation PCR amplicons (Figure 26) except for two of the three reactions of the isolate 9 derived plasmid transformants. The sequences obtained for colonies 9_2, 16_1, 16_2 and 16_3 were aligned with the *in silico* derived sequences to confirm homology (Figure 27). Complete homology was obtained for the aligned sequences which covered the entire *iss* gene except in the case of 16_3 (98 % coverage) due to low sequence quality, and this data was obtained only from the forward sequencing reaction.

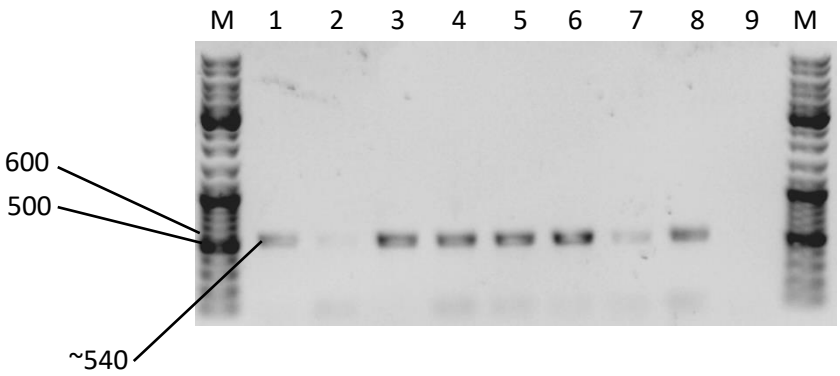

Figure 23: 1 % Agarose gel, indicating the GeneRuler™ DNA Ladder Mix molecular weight marker (in bp) in lanes M, the PCR products (~540 bp) obtained in lanes 1 - 8 with primer pair Chen6560F and CM-TerX on the pINA1317-*iss* plasmid extracted from *E. coli* TOP10 transformant clones named from "9" – "16" respectively. Lane 9 contained the no-template control of the PCR.

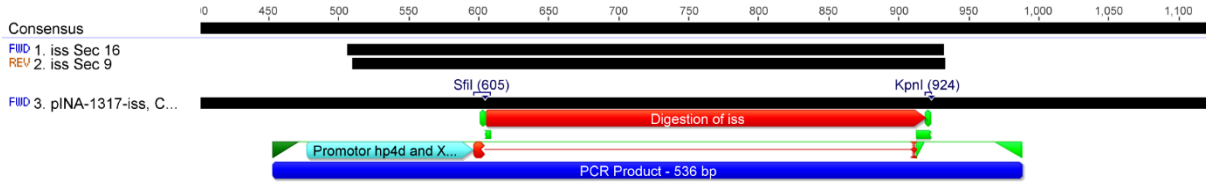

Figure 24: Nucleotide alignment performed with Geneious R9 of the *in silico* derived sequence of pINA1317-*iss* and the sequence data obtained for clones 16 ("iss Sec 16") and 9 ("iss Sec 9") *E. coli* transformants. The black bars indicate homology between the sequences and white bars dissimilar sequences. Complete homology was obtained for both sequences when compared to the pINA1317-*iss* sequence. Coverages of 79.5 % and 78.9 % of the expected 536 bp PCR product was obtained for clones 16 and 9 respectively and in both cases the entire *iss* gene (red annotation named "digestion of iss") was covered by the sequences obtained.

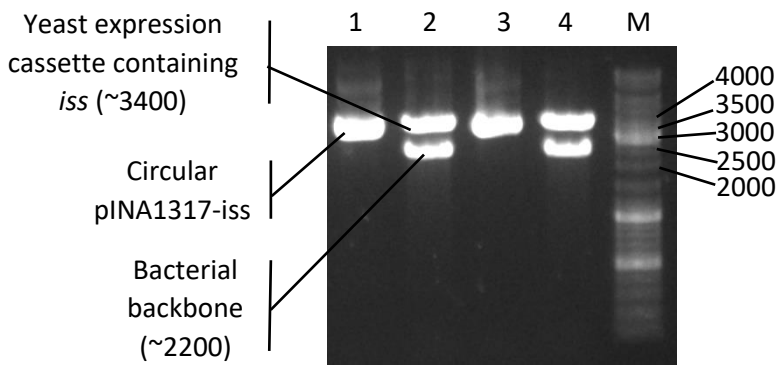

Figure 25: 1 % Agarose gel, indicating the GeneRuler™ DNA Ladder Mix molecular weight marker (in bp) in lane M. Lanes 1 and 3 contains the undigested circular plasmids pINA1317-*iss* from clones "9" and "16" respectively. Lanes 2 and 4 contains the *Not*I digested pINA1317-*iss* plasmids extracted from transformants "9" and "16" from *E. coli* TOP10 cells respectively to allow the removal of the bacterial backbone. The bacterial backbone of the plasmid (~2200 bp) and the yeast expression cassette containing *iss* (~3400 bp) are indicated for "9" (lane 2).

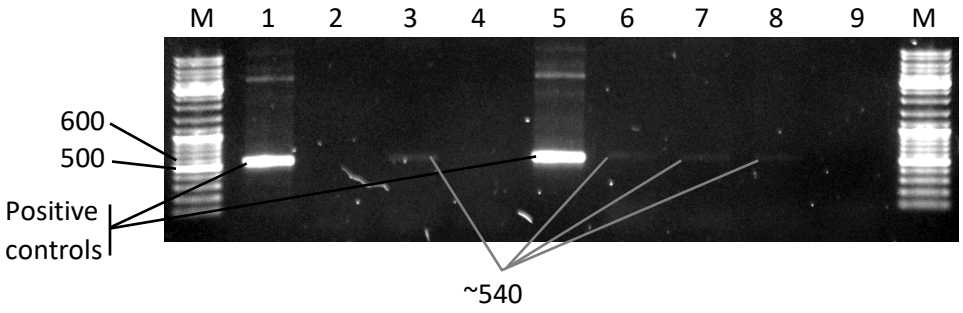

Figure 26: 1 % Agarose gel, indicating the GeneRuler™ DNA Express Ladder molecular weight marker (in bp) in lanes M and the PCR products of an estimated size of ~540 bp in lanes 1 and 5 obtained with primer pair Chen6560F and CM-TerX using pINA1317-*iss* plasmids from clones "9" and "16" as templates as positive PCR controls. Lanes 2 – 4 and 6 – 8 display *Y. lipolytica* Po1h transformants 9_1 – 9_3 and 16_1 – 16_3 respectively as templates for the PCR. Lane 9 contains the PCR no-template control. Transformants 9_2, 16_1, 16_2 and 16_3 amplified successfully as indicated in lanes 3, 6, 7 and 8 respectively.

Expression and secretion of the Iss protein was determined using SDS-PAGE, although no protein bands in the region of 11 kDa were observed in comparison between the negative control (untransformed strain Po1h) and the 16_1 transformant during TCA, NaOH, ammonium sulphate and acetic acid precipitation of the supernatant growth media of the yeast cells (Figure 28).

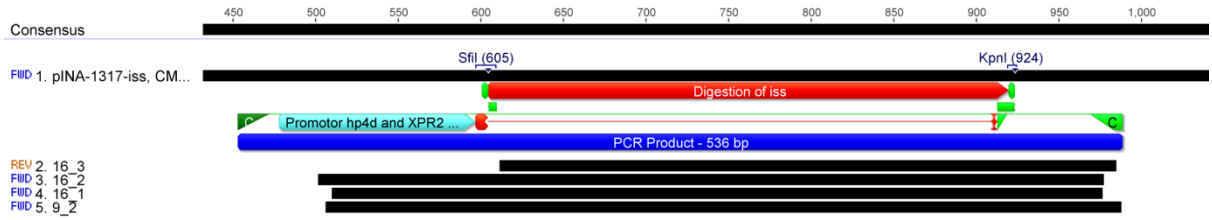

Figure 27: Nucleotide alignment performed with Geneious R9 of the *in silico* derived sequence of pINA1317-*iss* and the sequence data obtained for *Y. lipolytica* Po1h transformants 16_1, 16_2, 16_3 and 9_2. The black bars indicate homology between the sequences and white bars dissimilar sequences. Complete homology was obtained for all sequences when compared to the pINA1317-*iss* sequence. Coverages of 86.9 %, 88.8 %, 69.6 % and 89.7 % were obtained for sequences obtained from transformants 16_1, 16_2, 16_3 and 9_2 respectively. Low sequence coverage was obtained for 16_3 as a consensus region could not be reached between the forward and reverse sequencing reactions due to a low-quality reverse sequence and is therefore not represented by a consensus sequence obtained. The rest of the sequences covered the entire *iss* gene indicated in red as "Digestion of iss" confirming the gene's presence in these transformants.

Incorporation of *iss* into a C-terminal hexahistidine tagged secretion vector, pINA1317-6xHIS

The insert present in the supplied secretion vector pINA1317-6xHis was successfully replaced with the iss gene using *Sfi*I and *Hind*III. Transformation of *E. coli* TOP10 was performed and colonies were obtained on LB-Kan agar plates of which the sequences of the PCR products (Figure 29) confirmed the presence of the insert with a downstream hexahistidine-tag (Figure 30).

The plasmid was linearized using the *Xba*I restriction enzyme and was transformed into Po1h, giving rise to various yeast colonies on YNB agar plates. The screening for the insert through PCR and sequencing on four randomly picked yeast colonies are shown in Figure 31. Alignment of the sequenced products from two colonies (XbaD and Xba2) shown in Figure 32 indicates 100 % homology to the *in silico* predicted sequence. Protein purification of the supernatant after expression and secretion using IMAC, however yielded no proteins in the eluate (results not shown).

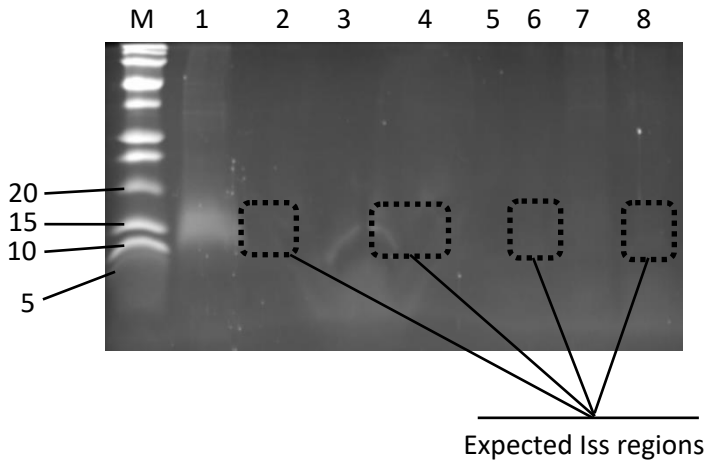

Figure 28: A Coomassie stained 10 % SDS-PAGE gel of precipitated supernatants of negative-control untransformed *Y. lipolytica* Po1h growth media and pINA1317-*iss* transformed *Y. lipolytica* Po1h transformant 16_1 growth media after 11 days of incubation and expected secretion of Iss. Lane M indicates the Bio-Rad Precision Plus Protein™ Dual Xtra Prestained Protein Standards as a molecular weight marker (in kDa). Lanes 1, 3, 5 and 7 contains the TCA, sodium hydroxide, ammonium sulphate and acetic acid precipitates of the untransformed *Y. lipolytica* Po1h control strain's growth media, while lanes 2, 4, 6 and 8 contains the respective precipitates for transformant 16_1. The regions where the Iss protein (~11 kDa) is expected be present in 16_1 are indicated. Protein bands present in these regions and absent in the control strain's lanes indicate potential Iss production, but such bands were absent, with all the precipitation methods performed.

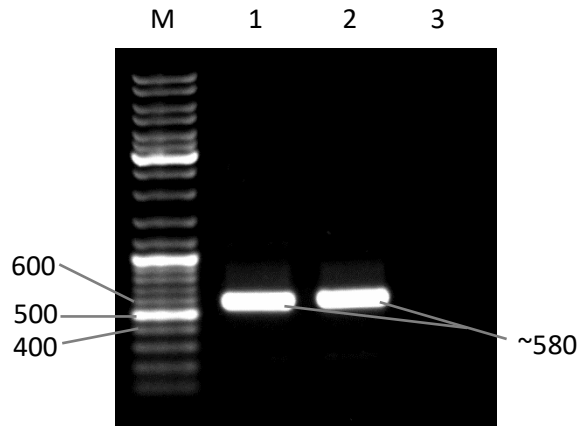

Figure 29: 1 % Agarose gel, indicating the GeneRuler™ DNA Ladder Mix molecular weight marker (in bp) in lane M, the PCR products (~580 bp) obtained in lanes 1 - 2 with primer pair Chen6560F and CM-TerX on the pINA1317-6xHIS-*iss* plasmid extracted from *E. coli* TOP10 transformant clones. Lane 3 contains the no-template control.

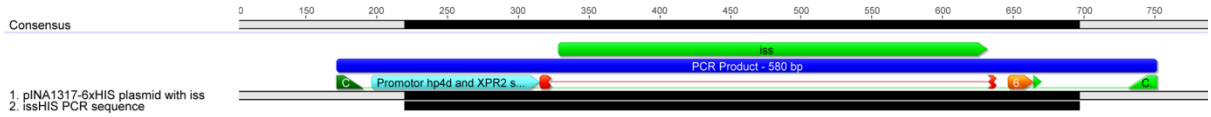

Figure 30: Nucleotide alignment performed with Geneious R9 of the *in silico* derived sequence of pINA1317-6xHIS-*iss* and the sequence data obtained from the *iss* gene sub-cloned into the pINA1317-6xHIS plasmid. The black bars indicate homology between the sequences and white bars dissimilar sequences. Complete homology was obtained for the sequence when compared to the pINA1317-6xHIS-*iss* sequence. A coverage of 82.2 % was obtained when compared to the expected PCR product (indicated in blue as "PCR Product – 580 bp") region. Complete coverage was obtained for the *iss* gene (indicated in light green as "iss") and the hexahistidine region (indicated in orange as "6." downstream from "iss") when compared to the *in silico* determined sequence.

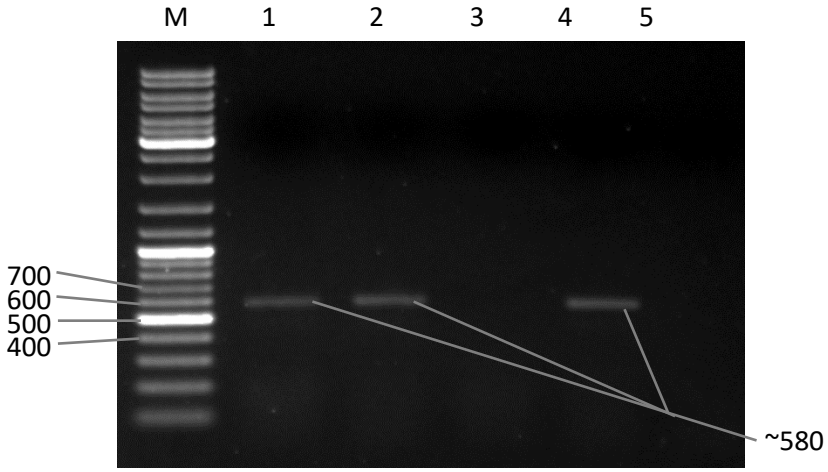

Figure 31: 1 % Agarose gel, indicating the GeneRuler™ DNA Ladder Mix molecular weight marker (in bp) in lane M and the PCR amplicons of an estimated size of 580 bp in lanes 1, 2 and 4 obtained with primer pair Chen6560F and CM-TerX on *Y. lipolytica* Po1h transformants transformed with the pINA1317-6xHIS-*iss* plasmid linearized with *Xba*I. Lanes 1 to 4 contains the PCR reaction mixture from transformants XbaD, Xba2, Xba3 and Xba9 respectively followed by the no-template PCR control in lane 5.

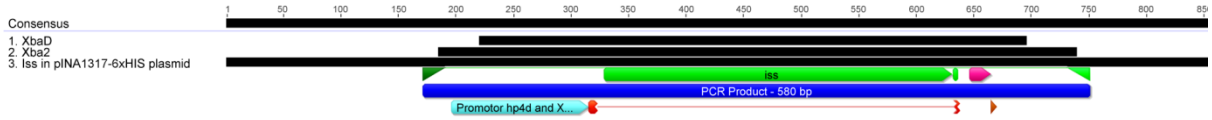

Figure 32: Nucleotide alignment performed with Geneious R9 of the *in silico* derived sequence of pINA1317-6xHIS-*iss* and the sequence data obtained from *Y. lipolytica* Po1h transformants XbaD and Xba2 containing the linearized pINA1317-6xHIS-*iss* plasmid. The black bars indicate homology between the sequences and white bars dissimilar sequences. Complete homology was obtained for the two sequences when compared to the pINA1317-6xHIS-*iss* sequence. Coverages of 82.0 % and 95.5 % were obtained for XbaD and Xba2 respectively in comparison to the expected 580 bp amplicon size (indicated in blue as "PCR Product – 580 bp") and covered the entire *iss* gene (green annotation "iss") region and hexahistidine tag (pink annotation downstream of "iss") region.

Western blotting analysis of Iss-producing yeast transformants

The stained SDS-PAGE is shown in Figure 33(a). Proteins are clearly visible for the pINA1317-YlCWP110 based transformants (I5, I6, I7, T1, T2 and S1) while the pINA1317 secretion-based transformants (9_1, Xba9 and XbaD) did not show any visible protein bands. The Ponceau S stained membrane after transfer showed intense red bands for the pINA1317-YlCWP110 transformants, confirming adequate transfer of the proteins to the membrane. The western blot membrane showed very faint bands (Figure 33b), most likely from non-specific binding of the polyclonal chicken primary antibodies, in the cell surface display transformants. No bands were observed for the secretion-based transformants. No over-expressed proteins are visible at the 11 kDa (non-His-tagged Iss estimated size) nor in the 13 kDa region (His-tagged Iss estimated size) nor are any significantly HRP-reacting bands visible. This indicated a negative result for the presence of proteins similar in nature to the Iss protein produced during *E. coli* over-expression to which the antibodies could bind.

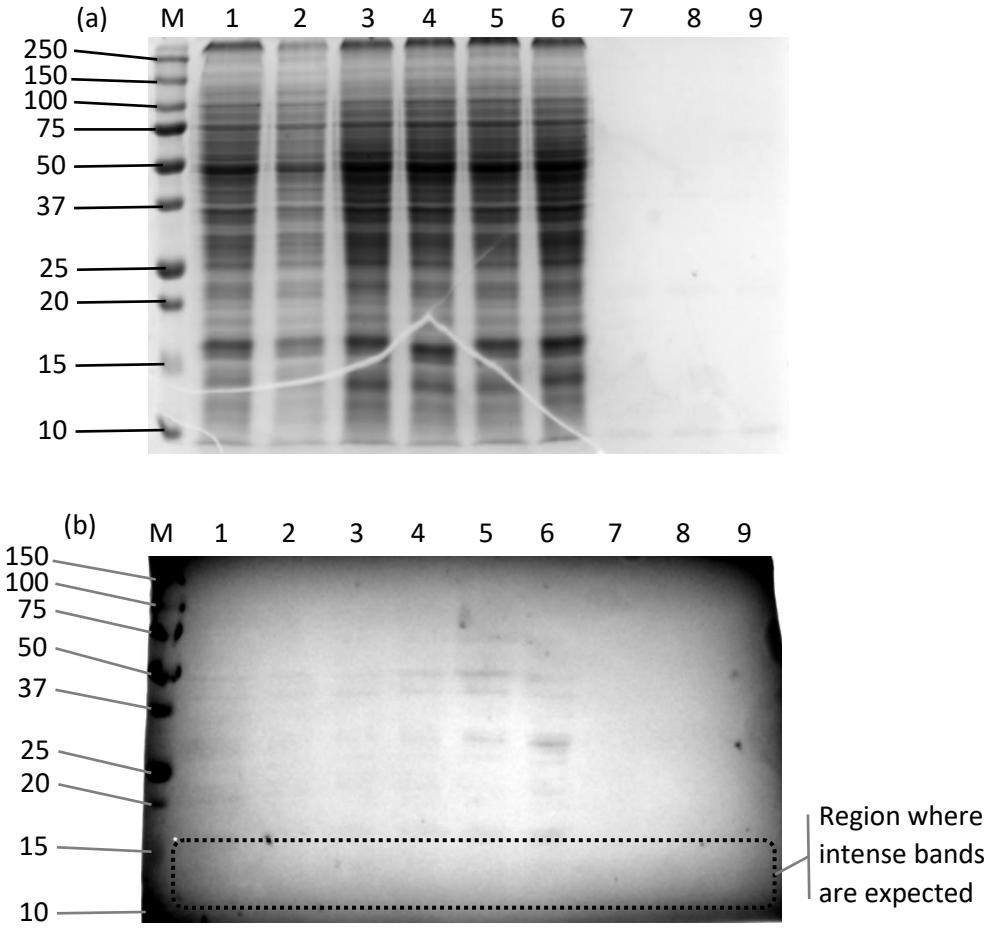

Figure 33: (a) A Coomassie stained 10 % SDS-PAGE gel of cell-disrupted and growth-media supernatants of *Y. lipolytica* Po1h pINA1317-based *iss* transformants and (b) nitrocellulose membrane used in western blotting a duplicate gel to (a). Bio-Rad Precision Plus Protein™ Prestained Protein Standards was loaded in lane M (values in kDa), lanes 1 – 6 contains cell-disrupted transformants I5, I6, I7, T1, T2 and S1 respectively while lanes 7 – 9 contains the supernatant of YPD growth media from transformants 9_1, Xba9 and XbaD respectively after 48 hours of incubation. No intense bands were observed in (b) in the region where the Iss protein should be present as indicated. Some faint bands were observed at molecular weights over 15 kDa, possibly due to non-specific binding as polyclonal chicken primary antibodies were used.

Discussion

Y. lipolytica has been successfully used to express proteins from various organisms from different phyla of life (Bauer *et al*., 1993; Nicaud *et al*., 1989; Kopecný *et al*., 2005; Juretzek *et al*., 1995-1998; Hamsa *et al*., 1998; Richard *et al*., 2001; Hamsa & Chattoo, 1994). However, limitless varieties of protein structures, characteristics and functions exist, meaning that there could potentially be countless proteins that cannot be expressed successfully without either modifications to the gene sequence or to the expression system used. Even if gene translation is successful, it does not guarantee whether the structure of the protein is correctly folded or whether potential post-translational modifications (PTMs) will hamper downstream applications (Madzak *et al.,* 2004).

In this study, various yeast transformants were obtained in which the yeast expression cassette was confirmed to be present without any signs of mutation. The *iss* (increased serum survival) gene was codon-optimised before synthesis by GeneArt, potentially ruling incompatible codon usage out as a factor. *Iss* expression was tested with a surface display system that allows the fusion to a GPI-anchor peptide. The gene is fused to a secretion signal from p*XPR2* to facilitate the transport of the protein out of the cell. Colonies are selected on YNB agar, as strain Po1h is an uracil auxotroph and will only be able to grow if a complementary *URA3* gene is inserted. No clear signs of expression were observed through SDS-PAGE of the cell-disrupted expressing cells after 48 hours of growth, nor during the attempted removal of Iss by enterokinase cleavage from the GPI-anchor sequence after insertion of an EK cleavage site. Phospholipase C (a phosphatidylcholine cholinephosphohydrolase) cleaves the phosphate bond on phosphatidylcholine and other glycerophospholipids through hydrolysis, and is thus expected to cleave GPI-anchors, which should release any bound proteins into the surrounding environment (Müller *et al*., 2012). This was however not observed with the surface displayed Iss protein, which could be due to very low concentrations of Iss expression, which cannot be visualised through SDS-PAGE with Coomassie staining. If this was the case, such low yielding transformants would in any case not be very useful in the production of subunit vaccines. Another reason could be the inaccessibility of the GPI-anchor when it is fused with the Iss protein, preventing the enterokinase or phospholipase C from cleaving the Iss protein. Due to the potential

complications surrounding the GPI-anchored expression, the use of a system allowing secretion into the growth medium Iss was then attempted.

Culture supernatants were chemically treated to precipitate extracellular proteins, but unfortunately no proteins in the expected size range were detected using SDS-PAGE. Immobilized metal-affinity chromotography (IMAC) was attempted to concentrate the protein when fused to a hexahistidine tag. After IMAC however, Iss was still not detected in the now 13 kDa region of the SDS-PAGE gel. Western blotting was attempted as a more sensitive protein detection method.

Anti-Iss antibodies from Chapter 2, which were raised in chickens against an *E. coli*-expressed Iss protein, were used for western blot analysis against an entire range of transformants, including: surface displayed Iss with and without an enterokinase cleavage site, and supernatants from Iss secretion transformants with and without poly-histidine-tags. Iss was not detected in any of the transformants samples tested during western blotting. A potential limitation to the western blot analysis is that it is not known whether the yeast-produced Iss would have the correct surface epitopes to bind anti-Iss antibodies raised from an *E. coli*-expressed system, as protein folding conditions could differ. In addition, there is the possibility of interference by post-translational modifications (PTMs) such as glycosylation (Madzak *et al*., 2004). Conclusively one cannot state that Iss was not produced using this yeast expression system, but the expression is either too low for detection, the protein is highly unstable, or the proteins are folded differently to the native *E. coli* host expression and are thus unable to bind to the antibodies, as observed through western blotting.

There are a variety of potential reasons for low or failed protein expression, such as: unrecognised or rare codons in the expression host, RNA secondary structure preventing translation-machinery from functioning properly, protein misfolding, insolubility of the expressed protein, inclusion-body formation, secretion issues, post-translational processing problems, the presence of proteases, unstable proteins and specifically in Eukaryotes – problems during the decondensation of the gene to be expressed from the tightly packaged chromosomal structure, especially when random integration of the gene of interest is used (Smith & Robinson, 2002; Alves & Dobrowsky, 2017; Duong-Ly & Gabelli, 2014; Felsenfeld *et*

al., 2002). Problems during heterologous protein expression is thus a highly complex problem and extremely difficult to troubleshoot and will vary from the protein to be expressed and the expression system used. In the case of the Iss protein, it is an outer-membrane protein of *E. coli* which therefore includes hydrophobic regions which could make the protein insoluble, which could lead to the formation of inclusion bodies. It could potentially also be binding to lipid structures inside the yeast cell due to the hydrophobic regions present in the protein. One potential work-around to this could be to express a truncated variant of the protein, as was done in Chapter 2, to increase the solubility of the protein to see if this rectifies the problems with Iss over-expression in *Y. lipolytica*. If this does not solve the issue, using a strain of *Y. lipolytica* which carry Ylt1 retrotransposons, such as strain E129 (Madzak *et al*., 2004), for controlled integration into the genome when using zeta-based pINA-vectors, could also be attempted to see if random integration was the cause of the protein expression related issues observed in this study.

Stepwise troubleshooting is therefore required when heterologous protein expression fails and one must meticulously decide whether the extra costs and time involved in solving potentially complex issues in such projects are worth the potential benefits/outcomes in the end.

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CHAPTER 4

Regulation of outer-membrane proteins (OMPs) A and F, during *hlyF*-induced outer-membrane vesicle (OMV) biosynthesis.

Preface

This chapter is presented in the format of a journal manuscript, as submitted to the Journal BMC Microbiology, following the style required by this journal for publication.

Haemolysin F was originally thought to be a putative avian haemolysin, which could have been a target antigen for sub-unit vaccine development, which was the initial plan. During this Ph.D. study, new literature, which was published in 2016, which claimed that this classification was incorrect, and that the HlyF protein is located in the cytoplasm and involved during outer-membrane vesicle biogenesis. HlyF is, however, highly prevalent in APEC strains and it has been shown in literature that outer-membrane vesicles play a role in virulence. Biogenesis of OMVs is still a topic of interest as various mechanisms have been hypothesised. In this study, we tested one of the proposed mechanisms, to gain new insight into HlyF-induced OMV biogenesis as its involvement in biogenesis is newly described.

Regulation of outer-membrane proteins (OMPs) A and F, during *hlyF*-induced outer-membrane vesicle (OMV) biosynthesis.

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Abstract

Background:

Gram-negative bacteria actively secrete outer membrane vesicles into the surrounding environment and these vesicles have been shown to play various physiological and protective roles such as carrying antibiotic-degrading enzymes and acting as decoys against host defences, therefore promoting the pathogenesis of the bacterium. It has been shown that avian pathogenic *Escherichia coli* strains can increase vesicle biosynthesis through the acquisition of the *hlyF* gene but the effect this has on the cell by scavenging outer-membrane associated proteins (OmpA, OmpF) into the vesicles during vesicle release have not yet been investigated.

Results:

Relative quantitative real-time PCR data obtained from *hlyF* expressing and non-expressing cells showed that during *hlyF* induction, *ompF* showed a nearly 2-fold down regulation relative to the non-expressing cells during the entire 24 hours, while *ompA* was expressed at the same level as the non-expressing cells during the first 8 hours of expression. At 24 hours post-*hlyF* expression, *ompA* was up-regulated 4-fold.

Conclusions:

The regulatory effects of the newly described outer-membrane vesicle biosynthesis-related gene, *hlyF*, on *E. coli* has not previously been investigated. As *hlyF*-induced vesicles contain OmpA and OmpF scavenged from the bacterial outer-membrane, potential regulatory effects on the host was investigated. An increase in *ompA* expression and an insignificant decrease in *ompF* expression was observed during *hlyF* induction demonstrating that *hlyF*-related biosynthesis is not related to decreased *ompA* expression, which is one of the potential mechanisms discussed in literature for biosynthesis. Outer-membrane vesicle biosynthesis during *hlyF* over-expression could potentially be accomplished through a different mechanism(s).

Keywords

hlyF, OMV, biogenesis, gene regulation, ompA, ompF, APEC, haemolysin F

Background

Gram-negative bacteria actively secrete outer membrane vesicles (OMVs), which are nanosized lipid vesicles with periplasmic proteins and surface associated membrane proteins [1][2]. The production of OMVs have been shown to play various physiological and protective roles and are secreted by bacteria throughout their lifecycles [3][4]. Secreted OMVs accomplish these roles through interacting with the environment of the bacterium during survival situations such as the enzymatic neutralization of human serum [5], acting as decoys to be targeted by antibiotics and carrying antibiotic-degrading enzymes [6] and through the formation of biofilms [7], therefore promoting the pathogenesis of the bacterium.

It has been shown that some avian pathogenic *Escherichia coli* (APEC) species can increase OMV biosynthesis through the acquisition of the *hlyF* gene [8]. Haemolysin F (*hlyF*) as the name suggests, was alleged to be an avian haemolysin often associated with avian pathogenic *Escherichia coli* [9]. The erroneous naming of this haemolysin was due to the haemolytic activity gained by *E. coli* MG1655 cells after the introduction and subsequent over-expression of *hlyF*. This was later shown by Murase et al. [8] to have been due to the transport of a native haemolysin, cytolysin A which is natively produced by this strain, within *E. coli* MG1655 to the extracellular environment by the *hlyF*-induced OMVs. Literature indicates that *hlyF* is an important virulence factor and of pathological importance in screening of potential APEC isolates [10][11].

Subsets of genes in various Gram-negative bacteria have been found to be associated with OMV biosynthesis and their functions on the cells have been investigated [12][13][14]. However, the association of *hlyF* over-expression with OMV biogenesis has newly been described and therefore the effects on the expressing cell is partial [8]. During OMV biosynthesis, outer-membrane proteins are incorporated into the OMVs, where proteins are scavenged from the cell-membrane, potentially leading to the loss of functions attributed by these cell wall proteins, unless there are regulatory changes involved in the expression levels of the genes to counteract the scavenging of these cell wall / membrane proteins. This study thus investigates the gene-regulation of two abundant outer-membrane proteins, namely outer-membrane proteins A and F, encoded by *ompA* and *ompF* associated with

hlyF-induced OMVs. Outer-membrane protein A has been shown to be involved in stabilizing the structure of the peptidoglycan and the outer-membrane of *E. coli* [15], which can potentially be influenced by cell-membrane destabilization during OMV formation and release. The gene of Outer-membrane protein F has been shown to be regulated by the *ompR* gene product during osmotic pressures applied to *E. coli* cells [16], so during OMV production, speculatively, it is unlikely to be influenced by *hlyF* expression unless the formation of OMV influences the osmolarity of the cell, by some other unknown mechanism.

Methods

Production of Haemolysin F pET22b(+) constructs in *E. coli* BL21 (DE3) cells

The *hlyF* gene was amplified from the APEC strain 1080, which had previously tested positive for the presence of *hlyF* [17]. The translated sequence of the *hlyF* gene of this strain shares 99,7 % amino acid homology to the reference sequence in the NCBI database (Accession number NC_011964.1). The *hlyF* gene was amplified using the primers hlyF-pet22-FP 5'-GGGAATTCATGGATCCTCGTCTTGATG-3' and hlyF-pet22-RP 5'-GGCTCGAGTTATTTAAAATCAACTTCCATTTGTTG-3' (Integrated DNA Technologies). The forward and reverse primers were designed with incorporated *Eco*RI and *Xho*I restriction recognition sites (underlined) respectively, for cloning into pET22b(+) (Novagen).

Standard molecular biology techniques were carried out as described by literature [18], and enzymes were applied according to the manufacturers' specifications. Amplicons from PCR were sub-cloned into the pGEM®-T Easy Vector System (Promega), using *E. coli* 10-β cells (New England Biolabs). The sequences of the cloned inserts were determined using Sanger sequencing with the SP6-promotor and T7-terminator primers (Integrated DNA Technologies). Consensus sequences were subjected to BLAST searches against the NCBI sequence database [19] for the confirmation of the sequences.

The *hlyF* gene was transferred from the pGEM®-T Easy vector into the pET22b(+) vector using the *Eco*RI and *Xho*I restriction endonucleases (Thermo Fisher Scientific). The digested DNA fragments were purified from 2 % LM-sieve agarose gels (Lonza) using the Wizard® SV Gel and PCR Clean-Up System (Promega). The digested gene inserts were ligated into

pET22b(+), and the recombinant plasmids were used to transform *E. coli* BL21 (DE3) (New England Biolabs).

The plasmids were extracted using the Qiagen QIAprep Spin Miniprep kit (Whitehead Scientific) after a 24-hour incubation period in Luria-Bertani (LB) broth (10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract) at 37 ^{o}C supplemented with 100 ug/mL ampicillin. The presence of the inserted *hlyF* region was confirmed by PCR, and the sequences were obtained using Sanger sequencing with the T7-promotor and T7-terminator primers (Integrated DNA Technologies), which amplifies the cloning site of pET22b(+). As a control, an empty pET22b(+) was cloned into *E. coli* BL21 (DE3) as described above.

Transmission electron microscopy (TEM) analysis of OMV production

The transformed cells described above were inoculated into two LB media tubes containing 100 ug/mL ampicillin. One of the tubes received IPTG to a final concentration of 1 mM to induce *hlyF* expression while another tube served as control. Both tubes were incubated at 37 ^{o}C for 16 hours. Cell pellets were harvested through centrifugation and mixed with bacteriological agar. Small cubes were cut (1 mm^3), fixed with osmium tetroxide and glutaraldehyde and dehydrated sequentially with increasing acetone concentrations. The dehydrated cubes were impregnated into epoxy and subjected to an ultramicrotome for sectioning to roughly 50 nm slices. The sections were placed on a copper grid and stained with uranyl acetate and lead citrate followed by transmission electron microscopy (TEM) analysis on a Philips CM100 TEM (Philips Electron Optics).

RNA extraction and quality analysis:

Total RNA was extracted and purified using the Qiagen RNeasy Mini Kit (Whitehead Scientific) following the manufacturer's specifications and an on-column DNase (RNase-free DNase set by Qiagen) was performed to remove any contaminating DNA from the extracted RNA. Concentrations of the extracted total RNA was measured using a NanoDrop ND-1000 spectrophotometer. RNA quality was determined by a 2100 Agilent Bioanalyzer using the Agilent RNA 6000 Pico Kit according to the manufacturer's specifications.

cDNA synthesis using random oligos:

cDNA was synthesized from the extracted RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) as per the manufacturer's instructions.

Primer design for qPCR assays:

Primer pairs as listed in Table 1 were designed for regions within the genes of interest and analysed with the Oligo Analyzer® v3.1 tool (Integrated DNA Technologies) to yield primer pairs with melting temperatures close to 58 ^{o}C to allow for optimal annealing of the primer pairs during the same cycling conditions and with thermodynamic attributes hindering the formation of hairpins and dimers.

Relative Quantitative Real-Time PCR (qPCR):

The assays were performed in biological duplicates and technical triplicates. The qPCR was performed as per the manufacturer's instructions using the LightCycler® FastStart DNA Master^{PLUS} SYBR Green I kit (Roche) on a LightCycler® 2.0 (Roche) capillary instrument. Each experimental run was performed with the primer pairs for the selected reference gene, *ompA* and *ompF* target genes, and no-template controls (NTC) for each primer pair. The following temperature cycling conditions were used for all reactions: An initial denaturation step at 95 ^{o}C for 10 minutes, followed by amplification, which included 95 ^{o}C for 10 seconds, 55 ^{o}C for 10 seconds and 72 ^{o}C for 7 seconds. Melting curve analysis was performed at 65 ^{o}C for 1 minute followed by 0.1 ^{o}C/s increases up to 95 ^{o}C. All the data was analysed on the LightCycler® 2.0 software version 4.1 (Roche).

Standard curves:

Standard curves for all the qPCR primer pairs were performed in triplicate by creating two-fold dilution series of cDNA with five data points. The efficiency values for each primer pair were calculated on the LightCycler® 2.0 software version 4.1.

Reference gene selection:

Zhou et al. [20] conducted a study during which microarray data was obtained for 63 *E. coli* strain BL21 (DE3) transformants during the over-expression of 63 distinct recombinant proteins [20]. This data was used to select for constitutively expressed genes during various conditions to select for candidate reference genes for qPCR. Three genes were identified as reliable and novel reference genes, namely *cysG*, *idnT* and *hcaT*, as ideal genes to monitor during transcription analysis during recombinant protein expression studies in *E. coli*. These three genes respectively encode for a sirohaem synthase, L-idonate and D-gluconate transporter and a putative 3-phenylpropionic transporter and were tested in this study.

The triplicate Cq values obtained for the three reference genes' (*idnT*, *cysG* and *hcaT*) standard curves were analysed on the RefFinder tool to select a stable reference gene for this study [21].

Experimental setup:

Gene expression profiles for the three genes (*cysG, ompA, ompF*) were carried out on the cDNA synthesised from the total RNA extracted from lactose-induced transformed *E. coli* cells either with or without (as control) *hlyF* inserted into in the pET22b(+) plasmid at three different time points post-induction – 4, 8 and 24 hours. This process was repeated to serve as a biological replicate. The data obtained were used to calculate the relative expression ratios during this 24 hours of gene expression.

Calculation of relative expression ratios:

The relative expression ratios of the *ompA* and *ompF* genes were calculated relative to the reference gene during both *hlyF* expressing and non-expressing cells by using the Pfaffl equation (Equation 1) [22]. The Pfaffl equation incorporates qPCR Cq values from two different conditions, in this case *hlyF* expressing and non-expressing cells, of the target two genes and reference gene to indicate any potential effects of the different conditions on the target genes' expression.

Results

Cloning and transformation into *E. coli* 10-β and BL21 (DE3) was successful with both the empty pET22b(+) vector and the pET22b(+) containing the PCR-amplified *hlyF* insert. Sequence data obtained from the pET22b(+) containing the insert confirmed the correct orientation of the gene and no mutations which could result in the protein not being transcribed and translated correctly and 99.5 % homology was obtained with the *hlyF* reference sequence (Accession number NC_011964.1).

The effect of HlyF protein production was tested by preparing the IPTG-induced and uninduced transformant for TEM to observe any OMV-like structures. Transmission electron microscope micrographs indicated various electron-transparent extracellular membranous vesicles in the sample treated with 1 mM IPTG while the untreated cells show minimal extracellular material near the bacterial cells (Figure 1).

The standard curves generated during qPCR for each gene produced efficiency values between 1.83 and 2.0 for the primer pairs. The Cq values from the standard curves for the potential reference genes, which were subjected to the RefFinder tool indicated that *idnT* and *cysG* were the most stable of the three reference genes and *cysG* was ultimately selected as the reference gene of choice for this study due to the lower expression levels of *idnT*.

The Cq values obtained from the qPCR of the target genes *ompA* and *ompF* with *cysG* as the reference gene were analysed using the Pfaffl equation and the data plotted in Figure 2. Outer-membrane protein F showed a nearly 2-fold down regulation (0.616-fold change as shown in Table 2) during the 24 hours under *hlyF* expressing conditions while *ompA* was expressed at the same level as the non-expressing cells during the first 8 hours of expression. At 24 hours post-*hlyF* expression however, *ompA* was up-regulated 4-fold (4.099-fold change in Table 2).

Discussion

Outer membrane vesicles are extracellular lipid structures produced by Gram negative bacteria and have been linked to virulence [8], antimicrobial resistance [3][23] and resistance to bacteriophages [3]. The recently observed effect of outer-membrane vesicle formation during *hlyF* overexpression and the potential benefits a cell gains from the biogenesis of OMVs, as discussed, gives a potential explanation as to why this gene has been highly associated with APEC [10].

The OMVs produced during *hlyF* overexpression, similarly reported as with other *E. coli* OMV biosynthesis processes, contain various proteins from the host cell found in the periplasmic space and the outer membrane of the cell wall [1][2]. Outer-membrane protein A is required by the cell to maintain membrane-peptidoglycan stability [24][25]. The OmpA protein was observed in OMVs formed during *hlyF* overexpression, even though the HlyF protein itself could not be detected, and is expected to have been scavenged from the membrane of expressing cells. Therefore, the cell membrane stability would be compromised unless the cell compensates for the loss by increased expression of *ompA* or by some other unknown mechanism. Outer-membrane protein F, which is also present in *hlyF*-induced OMVs, is involved during changing osmotic pressure conditions of cells [16]. As they are also scavenged from the host cell during OMV formation, this could lead to the cell being susceptible to changes in osmotic pressure unless there is a mechanism in place to counter this. Therefore, scavenging of proteins such as OmpA and OmpF from the cell wall can compromise the cell integrity and ability to survive osmotic stresses. However, gene expression regulation could potentially be playing a role in replenishing the cell wall with these proteins. In contrast, it has been shown that low levels of expression of *ompA* can also lead to increased OMV production [26] – which is expected as a lack of OmpA will lead to a destabilized cell wall due to decreased peptidoglycan-outer-membrane interactions, however this was not the case as supported by the qPCR data obtained in this study nor was any cell wall destabilisation observed during TEM. There is the additional possibility that *hlyF* over-expression leads to decreased *ompA* expression, which leads to OMV formation due to this effect. As OmpF is only situated in the outer membrane and does not directly

interact with the peptidoglycan, it is doubtful that a change in *ompF* expression will have a similar role.

In this study, qPCR results show that OMV biogenesis during *hlyF* overexpression is not related to a decrease in *ompA* expression and is therefore speculated to be accomplished through a different mechanism. We hypothesise that the eventual increase in *ompA* expression observed between 8 and 24 hours was possibly due to some as yet uncharacterised mechanism in the cells to stabilize the cell wall to prevent extensive cellular leakage and cell lysis.

The expected decrease in OmpF protein abundance on the outer cell membrane, due to the scavenging process of OMV biosynthesis, would likely leave the cells more susceptible to osmotic stresses. An insignificant down regulation of a less than 1 log fold-change as shown in Figure 2 in *ompF* expression levels was observed during the three intervals tested during *hlyF* overexpression in comparison to the control. Therefore, the data does not support the possibility of a compensatory mechanism to counteract the scavenging of this protein.

Conclusions

The regulatory effects of the newly described OMV biosynthesis-related gene *hlyF* on *E. coli* has not been fully investigated. As *hlyF*-induced OMVs contain OmpA and OmpF scavenged from the host cell, potential regulatory effects on the host was investigated. An increase in *ompA* expression and an insignificant decrease in *ompF* expression was observed. It can be speculated that the increase in *ompA* expression is to counteract the scavenging of OmpA from the cell, which is necessary to maintain the outer-membrane-peptidoglycan interaction to stabilise the cell wall. From the results, it can also be deduced that OMV biosynthesis is not related to decreased *ompA* expression during *hlyF* overexpression, which is one of the potential mechanisms as shown in literature for OMV biosynthesis [27]. Outer-membrane vesicle biosynthesis during *hlyF* overexpression is thus potentially accomplished through a different mechanism(s).

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

All data generated or analysed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

WA conducted all experiments, analysed the data obtained and was the main author of the manuscript. CW, CB and RR gave scientific input on experimental data and on the analysed data. CW, CB and RR made editorial changes and revised the manuscript. CB contributed to the analysis of the data obtained. All authors read and approved the manuscript.

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Not Applicable

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Figures:

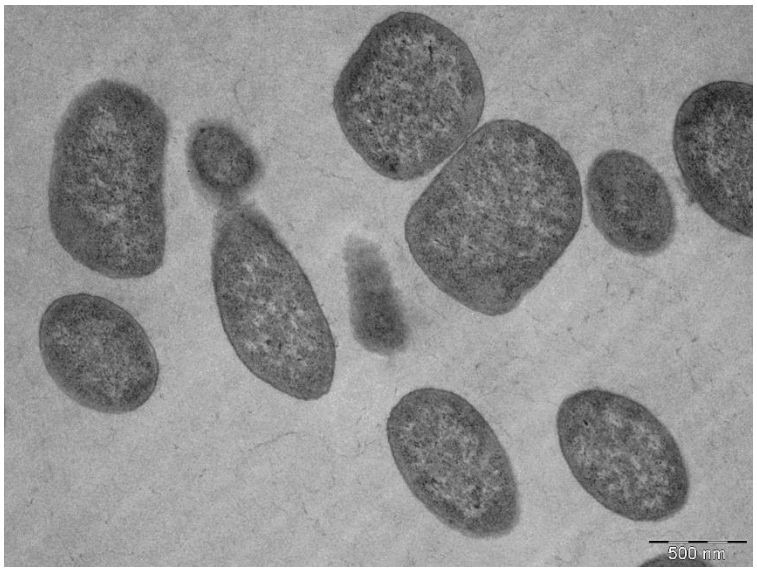

Figure 1: TEM view of a section of control cells (A) showing no visible OMVs in the vicinity of the cells. Cells over-expressing *hlyF* under IPTG induction (B) with a visible OMV being released from the cell as indicated by the arrow.

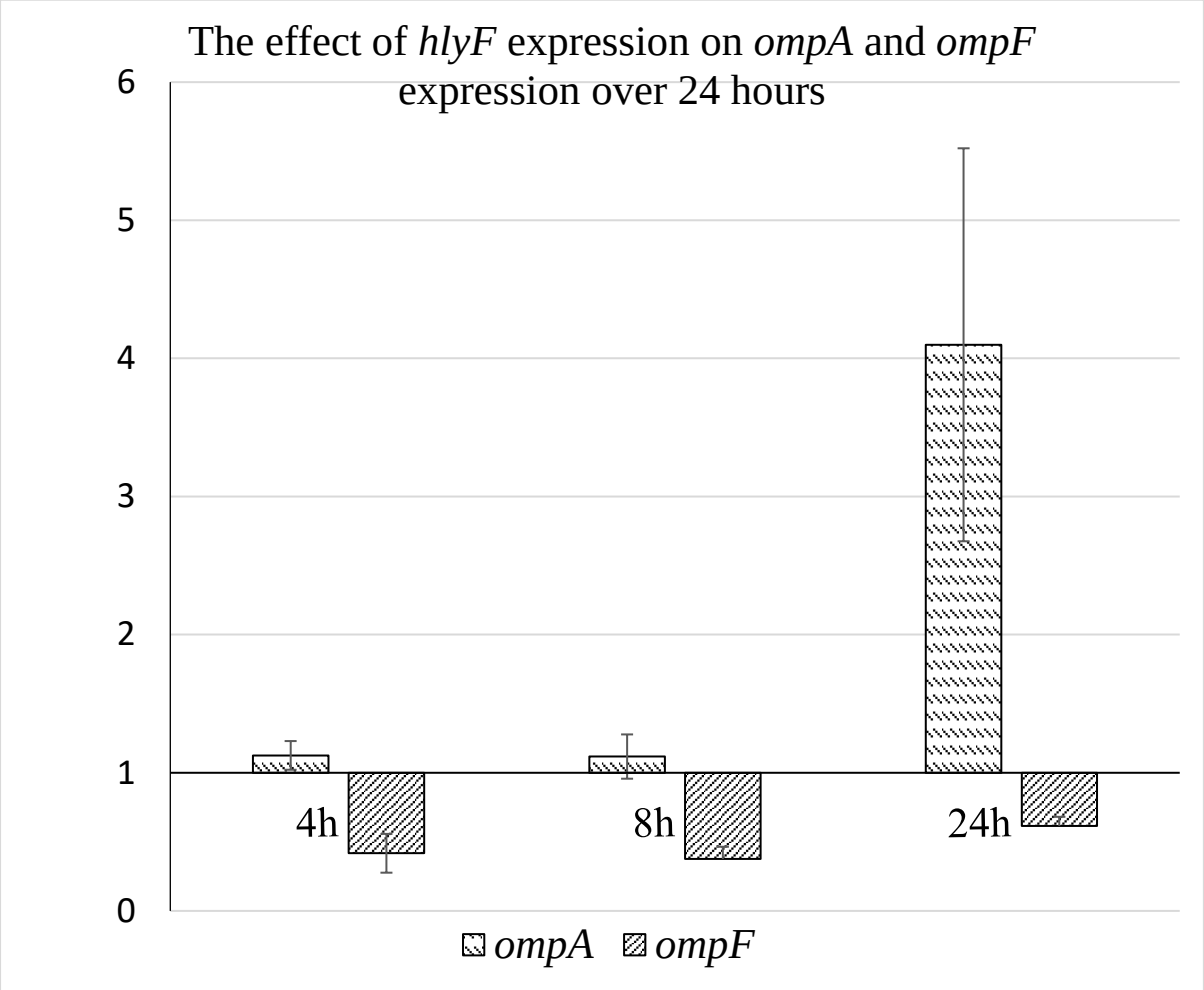

Figure 2: Bar graph with standard deviations indicated by error bars, showing the fold change in gene expression levels relative to the reference gene during *hlyF* expressing and non-expressing conditions, according to the Pfaffl equation. An overall down regulation for *ompF* can be observed for the three time intervals, while *ompA* stays constant until a period between 8 and 24 hours is reached, leading to strong upregulation of the gene.

Equations:

$$ratio = \frac{(\mathrm{E_{target}})^{\Delta Cq_{target}(control-sample)}}{(\mathrm{E_{reference}})^{\Delta Cq_{reference}(control-sample)}}$$

Equation 1: The Pfaffl equation adapted from Pfaffl [22]. Ratio is the relative expression ratio of the target gene of interest (*ompA* or *ompF*) to that of the reference gene (*cysG*). E_{target} is the PCR efficiency value of the target gene (*ompA* or *ompF*) obtained from its standard curve. ΔCq_{target} is the difference between the Cq values of the target gene (*ompA* or *ompF*) obtained in the control sample and the experimental sample. $E_{reference}$ is the PCR efficiency value of the reference gene (*cysG*) PCR obtained from its standard curve. $\Delta Cq_{reference}$ is the difference between the Cq values of the reference gene (*cysG*) obtained in the control sample and the experimental sample.

Tables:

Target gene	Primer name	Primer sequence (5' – 3')	Category	Melting Temperature (°C)	Reference
hlyF	hlyF-pet22-FP	GGGAATTCATGGATCCTCGTCTTGATG	Cloning	59,1	This study
	hlyF-pet22-RP	GGCTCGAGTTATTTAAAATCAACTTCCATTTGTTG		58,6	
cysG	cysG-qPCR-FP	TTGTCGGCGGTGGTGATGTC	Reference	60,3	Zhou *et al*., 2011
	cysG-qPCR-RP	ATGCGGTGAACTGTGGAATAAACG		57,9	
hcaT	hcaT-qPCR-FP	GCTGCTCGGCTTTCTCATCC	Reference	58,7	Zhou *et al*., 2011
	hcaT-qPCR-RP	CCAACCACGCTGACCAACC		59,3	
idnT	idnT-qPCR-FP	CTGTTTAGCGAAGAGGAGATGC	Reference	55,5	Zhou *et al*., 2011
	idnT-qPCR-RP	ACAAACGGCGGCGATAGC		58,9	
ompA	ompA-qPCR-FP	TGGACCAACAACATCGGTGAC	Target	57,6	This study
	ompA-qPCR-RP	CAACTACTGGAGCTGCTTCGC		58,3	
ompF	ompF-qPCR-FP	TGCTTATGGTGCCGCTGAC	Target	58,2	This study
	ompF-qPCR-RP	CGTAGTTCGACTGCCAGGTAG		57,7	

Table 1: Primer sequences and target regions are tabulated for the six primer pairs used in this study. The source of each primer pair is indicated in the last column.

	Biological Replicate 1			Biological Replicate 2			Mean Relative Expression Fold[1] Change		
Gene	Relative Expression at 4h	Relative Expression at 8h	Relative Expression at 24h	Relative Expression at 4h	Relative Expression at 8h	Relative Expression at 24h	4h	8h	24h
ompA	1.022	0.967	2.676	1.230	1.278	5.521	1.126	1.123	4.099
ompF	0.277	0.465	0.550	0.558	0.287	0.681	0.418	0.376	0.616

[1]Fold change values < 1 indicate a decrease in expression, = 1 no change in expression and > 1 indicates increase in expression levels relative to the control cells lacking *hlyF*

Table 2: Data of the relative expression fold changes for each gene at the different time intervals are indicated for each biological replicate. Each value was calculated from the mean of triplicate Cq values used in the Pfaffl equation. The mean fold change is indicated in the last three columns.

GENERAL DISCUSSION AND CONCLUSIONS

Avian pathogenic *Escherichia coli* virulence has been extensively studied, to the point that potential minimal predictor virulence genes have been identified which can be used as a tool to easily identify most APEC strains. Of these minimal predictors, two genes were chosen as potential sub-unit vaccine candidates against avian colibacillosis. These were increased serum survival (*iss*) and hemolysin F (*hlyF*), as they were expected to be exposed to the immune system of the chicken host.

The increased serum survival protein (Iss) has been shown to be situated in the outer-membrane of APEC strains and would therefore expose a potential epitope for recognition by the immune system (Olsen *et al*., 2012). As the protein is membrane-bound, hydrophobic regions will be present in the section(s) of the protein which interact with the lipid-rich membrane, while any part exposed to the extracellular environment, which are predicted epitopic regions, should be hydrophilic. Expression of hydrophobic-rich proteins can lead to water-insoluble proteins which can prevent efficient over-expression and the formation of inclusion bodies. During a study by Lynne and co-workers (2006) Iss over-expression experiments performed in *E. coli* BL21 DE3 lead the formation of inclusion bodies. Urea is often used for solubilisation of inclusion bodies, but this has the drawback of denaturing the protein of interest. Therefore, refolding is required which can lead to incorrectly folded proteins if the conditions are not optimal. Even if proper refolding is achieved, this leads to added costs and time to obtain protein suitable for vaccine developmental and production purposes.

Two alternative methods for obtaining water-soluble Iss protein were attempted: the inclusion of a zwitterionic detergent to attempt to solubilise the inclusion bodies and hydrophobic proteins, and secondly to create a truncated Iss protein with the suspected hydrophobic membrane-interacting regions removed, leaving only the expected epitopic water-soluble region intact. Both methods would simplify the production process as no refolding of the protein is required.

The full protein, solubilised in detergent, and the truncated protein were successfully expressed and purified using IMAC, and immunisation performed in chickens to obtain antibodies against the respective proteins. As the Iss protein was modified to create the truncated Iss, the protein could fold differently in such a way that different epitopes would be created. To see whether this was the case, cross-reaction experiments were performed with both western blotting and ELISA. In both cases, it was clear that the antibodies raised against the full Iss protein could bind to the truncated protein and *vice versa*. This meant that for vaccine development, the simplified and cheaper process of producing water-soluble truncated Iss proteins could potentially be used, simplifying and reducing downstream costs during production. It was also found that the truncated protein purified better during IMAC, giving it an even further promising quality for downstream processing, especially since a highly pure *E. coli*-expressed protein will be essential for product development and registration.

Yarrowia lipolytica has been extensively studied and used for the successful production of heterologous proteins for various applications. One of the main benefits of using *Y. lipolytica* for the heterologous expression of an *E. coli* protein, is that it is Generally Regarded as Safe (GRAS). This allows the entire yeast, for instance, to be used as added protein while expressing the protein of interest. Also, GPI-anchors can be used to display the protein of interest on the surface of the yeast, allowing it to be accessed by the immune system of the host. The *iss* gene from the NCBI reference database was used as a reference to have a *Y. lipolytica* codon-optimised gene synthesized with restriction sites compatible with the pINA1317-YlCWP110 vector. Although successful transformants were obtained, with the gene sequence intact, no protein expression was observed in comparison to the control cells. An enterokinase proteolytic cleavage site was then integrated between the *iss* and GPI-anchor sequence, which would allow for the removal of the surface displayed Iss protein. Unfortunately using enterokinase, and even phospholipase C which is expected to cleave the GPI anchor, no Iss protein was observed during SDS-PAGE. It was then decided to then move over to a secretion-based pINA1317 vector, hoping that attaching the gene to the GPI-Anchor was maybe causing unknown problems during translation or somewhere downstream from that. The secretion vector was expected to lead to a build-up of the Iss

protein outside of the cells, in the growth media, although this was not observed. Protein concentrating methods such as precipitation using acids, salting out and acetone were attempted to no avail. The iss gene was then hexahistidine tagged, allowing for the use of IMAC to isolate the Iss protein from the growth media. Again, no Iss was obtained using this method. Finally, to determine if any Iss was present in the cells, supernatant or cell surface, transformants from the various transformations and vectors were used in a western blot, using anti-Iss antibodies raised in chickens from Chapter 2. Besides from some slight non-specific binding, no bands in the expected region of 11 - 13 kDa was observed. It was concluded that the Iss protein was produced at either an undetectable level during *Y. lipolytica* protein expression, or that the antibodies obtained from *E. coli* expressed Iss antigen used during the chicken immunisation experiment recognised a different epitope to that on the yeast-expressed Iss due to protein folding differences or potential glycosylation of the protein. However, since no Iss protein was detected through SDS-PAGE, it is more likely that the protein is just produced in too low quantities for any detection or not at all.

Hemolysin F (*hlyF*) was originally described as an avian haemolysin, secreted from the expressing cell to break down red blood cells to make iron-rich heme available to the bacterium for cellular processes (Torres & Payne, 1997). It has been shown, in March 2016, to be involved in the biogenesis of outer-membrane vesicles, in the cytosol of the bacterium, and therefore unlikely to be directly involved as an immunostimulant and not useful as a vaccine immunogen (Murase *et al*., 2016). Outer-membrane vesicles have, however, been shown in literature to play important roles in the pathogenesis of Gram-negative bacteria (Manning & Kuehn, 2001; Grenier & Bélanger, 1999; Yonezawa *et al.*, 2009). As this was a newly described OMV-biosynthesis-involved protein and the effects of *hlyF*-expression on the cell have not yet been extensively studied, it was still included in this study as a topic of interest even though it could not be directly used as a target for sub-unit vaccine development. The outer-membrane vesicles produced during *hlyF* overexpression mostly consisted of outer-membrane proteins A and F. These proteins were most likely scavenged during the biogenesis of the OMV from the cell, and could therefore influence the cell wall properties should regulation of the *ompA* and *ompF* genes not counter this effect. Relative quantitative PCR (qPCR) was performed on *hlyF*-induced and non-induced

cells to compare gene regulation of the *ompA* and *ompF* genes, with the aim of potentially uncovering the mechanism of *hlyF*-induced OMV biogenesis and the effect this has on the cell. A four-fold increase in *ompA* expression and an insignificant decrease in *ompF* expression was observed over a 24-hour period during *hlyF* induction, demonstrating that *hlyF*-related biosynthesis is not related to a decrease in *ompA* expression, which is one of the potential mechanisms of OMV biogenesis as discussed in literature (Deatherage *et al.*, 2009). It was therefore concluded that OMV biosynthesis during *hlyF* over-expression could potentially be accomplished through a different mechanism. As no increase in *ompF* expression was observed as a compensatory mechanism to the scavenging of the OmpF protein from the cell membrane, it could lead to the expressing cell to become more sensitive to osmotic stresses as this is the role of OmpF according to literature (Kawaji *et al*., 1979).

In conclusion, alternative therapies and prevention methods are required in the fast-approaching post-antibiotic world. Bacteriophage therapy has potential but also some downfalls regarding the non-linear pharmacokinetics required for therapeutic products. Bacteriophage-based products such as the use of endolysins show promise as broad-range therapeutics, although the efficacy against Gram-negative bacteria could pose a problem due to the protective lipopolysaccharide layer around the cell, but research into overcoming this is showing promise. Whole-bacteria-based vaccines have been shown to be effective, but more specific vaccines, such as sub-unit vaccines, are required to prevent the removal of commensal bacteria from the host by the host immune system. This requires adequate knowledge of the virulence factors contributing to bacterial pathogenicity. Secreted and outer-membrane virulence-associated antigens can then be used as targets for sub-unit vaccine development. The Iss protein of APEC has been shown to be immunogenic in chickens and successful attempts at improving protein expression, through simplified and cheaper processes, have been made using a truncated protein to increase solubility, while maintaining cross-reactivity to full-Iss antiserum. Whether the full and truncated Iss proteins give protective immunity against APEC remains to be seen and will require further research which will include an APEC challenge post-vaccination with these proteins. In addition, having the Iss protein expressed in the *Y. lipolytica* expression patented by this research

group would be advantageous as any products developed in this system are covered by the patent and the use of a whole-yeast expressing this protein can be easily administered as a feed additive and protein supplement. Although expression of Iss failed in *Y. lipolytica* during this study, future research can investigate the use of the truncated *iss* gene for expression or using a different *Y. lipolytica* strain. Although the aim was to also use the HlyF protein as a potential sub-unit vaccine, it was discovered during the course of this doctoral degree, that this will not be possible due to new literature being published. This created the opportunity to potentially learn more about the mechanisms involved during the newly described hlyF-induced OMV biosynthesis as this could lead to novel research. The exact mechanism of biosynthesis could not be elucidated; however, it was concluded that it is not through decreased *ompA* expression and that *hlyF*-induced OMV biosynthesising cells could be vulnerable to osmotic stresses due to the scavenging of OmpF from the outer-membrane of the cell. Future research is required to test whether this holds true and could lead to novel therapeutics being developed.

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SUMMARY

Avian pathogenic *Escherichia coli* (APEC) is the causative agent of colibacillosis in poultry and leads to economic losses in the poultry industry. Due to rising concerns of antibiotic resistance and antibiotic carry-over into food, bans have been implemented on antibiotic use in animal production. The process of discovering new antibiotics and having them registered and approved can take up to eight years, and their application will most likely be limited to human-use. Alternative therapies for the control of bacterial diseases in animals, including poultry, are therefore becoming increasingly important. Alternative treatment options could include the use of bacteriophages, bacteriophage enzymes, essential oils and vaccines. Bacterial vaccines are generally based on whole bacterial cells, and in the case of commensal bacteria such as *E. coli*, this can lead to poor gut health. Therefore, the need for highly specific bacterial vaccines are required, such as sub-unit vaccines containing only antigens found in pathogenic strains of *E. coli*.

Various virulence genes have been found in APEC that contribute to the pathogenicity of the strains. Five of these genes have been found to be highly prevalent in most clinical cases of avian colibacillosis. In this study, two of these genes were selected as potential candidates for sub-unit vaccine development, namely increased serum survival (*iss*) and haemolysin F (*hlyF*).

The increased serum survival gene, an outer-membrane protein of APEC, was successfully expressed in *E. coli* BL21 (DE3) using the pET28b(+) vector system, although the protein was mostly water-insoluble due to hydrophobic N -and C-terminals. The sequence of *iss* was then modified to create a truncated version of the gene encoding the hydrophilic region of the gene, also the potential epitope of the Iss antigen, to improve solubility during over-expression. Water-soluble truncated Iss was produced in conjunction with the full Iss protein, solubilised using a zwitterionic detergent, purified using the hexahistidine regions flanking the inserted gene in the pET28b(+) vector system. The purified proteins were mixed with adjuvant and injected into chickens raise antibodies against the two expressed proteins. The antibodies obtained from the chickens were used to perform western blotting and ELISA and both proteins were confirmed to be immunogenic. Furthermore, the obtained serum was cross-reacted with the full and truncated forms of the protein,

indicating potentially similar epitopes, showing promise of using the highly water-soluble truncated Iss protein for potential future vaccine development.

Yarrowia lipolytica heterologous protein expression was also attempted with the full *iss* gene. However, no expressed protein was detected using SDS-PAGE, so alternative methods of expression, purification and isolation were attempted. An enterokinase proteolytic cleavage site was introduced between the *iss* gene and the GPI-anchor sequence, the secretion vector pINA1317 and the C-terminus hexahistidine region bearing pINA1317 secretion vector were used as alternatives methods to express the full *iss* gene sequence. These methods failed to produce the desired results. Western blotting using antiserum raised against *E. coli* expressed protein could also not detect any expressed Iss protein. It was then concluded that a yet unknown issue is preventing adequate, if not any, expression of the *iss* gene in *Y. lipolytica* and further research is required.

During the study, published literature indicated that the putative avian haemolysin F, was not a haemolysin but an enzyme involved in outer-membrane vesicle (OMV) biogenesis. This was validated by our failure to detect haemolytic activity after *hlyF*-overexpression and the presence of OMVs observed by TEM. As this was very recent research into the involvement of this gene in OMV biogenesis, the impact of the expression of the *hlyF* gene was investigated regarding the regulation of outer-membrane proteins which are scavenged during OMV release. Relative quantitative PCR was used to compare *hlyF*-expressing and non-expressing cells, and it was shown that *ompA* expression is increased during *hlyF* expression, while *ompF* expression remained nearly the same, which could lead to osmotic-stress susceptible cells during *hlyF* induction. It was concluded that a decrease in *ompA* expression is not involved in the mechanism of *hlyF*-induced OMV biogenesis, contrary to one of the biogenesis mechanisms described in literature.

Sub-unit bacterial vaccines could be the future method of preventing bacterial diseases in the poultry industry. Alternative methods such as bacteriophage therapy might not be possible due to the non-linear pharmacokinetics observed, which will hinder registration of these products. Sub-unit vaccines should elicit highly specific immune responses to virulence-related antigens and not target commensal bacteria. Various antigens accessible to the immune system of the host are outer-membrane or cell wall-associated proteins,

protein expression of these antigens could pose problems such as high costs for production and problems during the development of expression systems for these proteins, even complete failure of expression. Knowledge and correct characterisation of virulence-related proteins is also essential, as seen with the HlyF protein expressed in this study which is not directly suitable for vaccine production as previously thought. Future work is required in the optimisation of protein expression parameters, and chicken challenge studies with APEC will need to be conducted to determine if protective immunity is gained by vaccination with the *E. coli*-expressed Iss proteins. It will also be highly advantageous if the problems encountered during protein expression in *Y. lipolytica* could solved and the yeast-expressed Iss protein tested during challenge studies with APEC.

KEYWORDS

APEC, vaccine, sub-unit, truncated, hlyF, OMV, antibiotic resistance, antibiotic alternatives, colibacillosis, *Yarrowia lipolytica*, heterologous protein expression

RESEARCH OUTPUTS

Publications:

Van der Westhuizen, W.A. & Bragg, R.R. (2012). Multiplex Polymerase Chain Reaction for Screening Avian Pathogenic *Escherichia coli* for Virulence Genes. *Avian Pathology* **41**(1), pp33-40.

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Van der Westhuizen W.A., Theron C.W., Boucher C.E. & Bragg R.R. Regulation of outer-membrane proteins (OMPs) A and F, during *hlyF*-induced outer-membrane vesicle (OMV) biosynthesis. **Submitted manuscript**

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"There is no end to education. It is not that you read a book, pass an examination, and finish with education. The whole of life, from the moment you are born to the moment you die, is a process of learning."

-Jiddu Krishnamurti