Virulence factors of *Lactococcus garvieae* isolated from South African rainbow trout

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Declaration

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Table of Contents

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List of Tables

List of Figures

List of Equations

Non-International System of Units Abbreviations

Abbreviation	Definition
aa	Amino acid
ACN	Acetonitrile
AIX	Ampicillin, IPTG, X-gal
Amp	Ampicillin
APS	Ammonium persulfate
ATCC®	American Type Culture Collection®
BCA	Bicinchoninic acid
BHI	Brain-heart infusion
BLAST	Basic Local Alignment Search Tool
BLASTP	Protein-protein BLAST
bp	Base pair
BSA	Bovine serum albumin
C-terminus	Carboxyl terminus
$CaCl_2$	Calcium chloride
CDS	Coding sequence
CPS	Capsular polysaccharide
Da	Daltons
DMF	N,N-dimethyl formamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate

DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EPS	Extracellular polysaccharide
EtBr	Ethidium bromide
g	Gravitational force
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
His-tag	Polyhistidine-tag
IMAC	Immobilised metal-affinity chromatography
IPTG	Isopropyl β-D-1-thiogalactopyranoside
Kana	Kanamycin
KG-	Capsulated (non-agglutinating) *Lactococcus garvieae*
KG+	Non-capsulated (agglutinating) *Lactococcus garvieae*
LA PCR	Long accurate polymerase chain reaction
LAB	Lactic acid bacteria
LB	Luria-Bertani medium
LPS	Lipopolysaccharide
$MgCl_2$	Magnesium chloride
MS	Mass spectrometry
MSCRAMM	Microbial surface components recognising adhesive matrix molecules
m/v	Mass per volume
MW	Molecular weight
N-terminus	Amino terminus
NCBI	National Centre for Biotechnology Information

OD	Optical density
ORF	Open reading frame
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pfu	Plaque-forming units
$RbCl_2$	Rubidium chloride
RPL	Random peptide library
rDNA	Ribosomal deoxyribonucleic acid
RT	Room temperature
SAROTUP	Scanner and reporter of target unrelated peptides
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
SOC	Super optimal broth with catabolite repression
SSC	Saline sodium citrate
TAE	Tris-acetate-ethylenediaminetetraacetic acid
Taq	Thermus aquaticus DNA polymerase
TB	Transformation buffer
TBS	Tris-buffered saline
TEM	Transmission electron microscopy
TEMED	Tetramethylethylenediamine
*T*m	Melting temperature
Tris	2-amino-2-hydroxymethyl-propane-1,3-diol
TUP	Target unrelated peptide

U	Units
UV	Ultraviolet
v/v	Volume per volume
w/v	Weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

1. Literature review

Aspects of this literature review have been published:

Meyburgh, C. M., Bragg, R. R. and Boucher, C. E. (2017) *Lactococcus garvieae*: An emerging bacterial pathogen of fish. *Diseases of Aquatic Organisms*, 123(1), pp. 67–79.

1.1. INTRODUCTION

Increasing pressure is being placed on aquaculture since the increasing demand for aquaculture products cannot be satisfied solely by wild fisheries. In the past century, an acceleration in the expansion of aquaculture as an industry has been observed and, as the fastest growing agricultural sector worldwide, the aquaculture industry is currently responsible for the production of 50% of consumable fish worldwide. Despite these demands, quality standards need to be maintained. Infectious disease caused by viruses, bacteria, protozoa and trematodes, cause severe fiscal loss in aquaculture (Austin and Austin, 2012). Since the initial description of a Gram-positive coccus implicated in septicaemia in the rainbow trout *Oncorhynchus mykiss* (Walbaum) in Japan (Hoshino *et al.*, 1958), the number of reports on streptococcal isolates associated with fish disease has increased worldwide (Boomker *et al.*, 1979; Wallbanks *et al.*, 1990; Toranzo *et al.*, 1994; Michel *et al.*, 1997). Based on phenotypic similarities, etiological agents of these diseases were initially assigned to the genus *Streptococcus.* Advancements in genotyping methods allowed its reclassification into the separate genera *Enterococcus* (Kusuda *et al.*, 1991), *Vagococcus* (Wallbanks *et al.*, 1990; Michel *et al.*, 1997) *Carnobacterium* (Wallbanks *et al.*, 1990) and *Lactococcus* (Doménech *et al.*, 1993; Eldar *et al.*, 1996). Presently, it is believed that certain species (*Vagococcus salmoninarum* and *Lactococcus piscium*) cause streptococcosis solely in salmonid fish when water temperatures are below 15°C, while other species are responsible for streptococcal outbreaks in cultured freshwater and marine fish when water temperatures rise above 15°C (Eldar and Ghittino, 1999). This review concerns a bacterium, *Lactococcus garvieae*, grouped in the latter category.

1.2 LACTOCOCCOSIS IN AQUACULTURE

1.2.1. Symptoms and clinical signs

Lactococcosis is defined as a systemic hyperacute infection with the occurrence of widespread haemorrhaging (Austin and Austin, 2012). The earliest symptoms of infection include anorexia, melanosis and erratic swimming. Other external signs include uni- or bilateral exophthalmia, swollen abdomens and anal prolapsus (Eldar and Ghittino, 1999; Bekker *et al.*, 2011). At necropsy, accumulation of ascitic fluid in the peritoneal cavity, congestion of internal organs, enlargement of spleen and liver and exudate covering the brain are observed (Bragg and Broere, 1986; Eldar and Ghittino, 1999). During macroscopic examination, extensive haemorrhaging is commonly observed, caused by injury to vascular epithelium that leads to haemorrhages and petechiae on the surfaces of internal organs and external surfaces (Bragg and Broere, 1986; Eldar and Ghittino, 1999; Vendrell *et al.*, 2006). It is likely that these clinical findings are caused by toxin production, as Kusuda and Hamaguchi (1988) showed that symptoms could be reproduced in fish upon inoculation with extracellular products of *L. garvieae*.

1.2.2. Host range

The causative agent of lactococcosis, *L. garvieae* has been isolated from a wide range of fish species listed in Table 1.1. Apart from the reputation of *L. garvieae* as a fish pathogen contributing to economic losses, the involvement of *L. garvieae* in human clinical infections has been well documented (Chan *et al.*, 2011). An increasing number of human infections due to *L. garvieae* has been reported in recent years, giving rise to the status of an emerging zoonotic pathogen. A suggested source and route of infection in humans is handling and ingestion of raw fish followed by entry into the bloodstream when disturbances in the gastro-intestinal tract occur (Gibello *et al.*, 2016). Indeed, the majority of human cases reported presented with bacteraemia and had previously undergone gastrointestinal surgery or gastric acid suppressive therapy (Wang *et al.*, 2007; Gibello *et al.*, 2016).

Table 1.1: Aquatic hosts of *L. garvieae*

Host	Area	Reference
Japanese eel *Anguilla japonica* (**Temminck & Schlegel)**	Japan	Kusuda *et al.,* 1991
Red sea wrasse *Coris aygula* **(Lacépède)**	Israel	Colorni *et al.,* 2003
Brazil Nile tilapia *Oreochromis niloticus* **L.**	Brazil	Evans *et al.,* 2009
Pintado *Pseudoplathystoma corruscans* **(Spix & Agassiz)**		
Olive flounder *Paralichthys olivaceous* (**Temminck & Schlegel)**	Japan	Kawanishi *et al*., 2006
Amberjack *Seriola dumerili* **(Risso)**		
Kingfish *Seriola quinqueradiata* **(Temminck & Schlegel)**		
Rainbow trout *Oncorhynchus mykiss* **(Walbaum)**	South Africa Australia United Kingdom Taiwan France Bulgaria Israel Portugal Greece Iran Spain Italy Turkey	Boomker *et al.,* 1979 Bragg and Broere, 1986 Carson *et al.,* 1993 Bark and McGregor, 2001 Chang *et al.,* 2002 Eyngor *et al.,* 2004 Pereira *et al.,* 2004 Savvidis *et al.,* 2007 Sharifiyazdi *et al.,* 2010 Aguado-Urda *et al.,* 2011a Reimundo *et al.,* 2011 Didinen *et al.,* 2014
Grey mullet *Mugil cephalus* **L.**	Taiwan	Chen *et al.,* 2002
Catfish *Silurus glanis* **L.**	Italy	Ravelo *et al.,* 2003
Fresh water prawn	Taiwan	Chen *et al.,* 2001

***Macrobrachium rosenbergii* (De Man)**		
Bottlenose dolphin ***Tursiops truncatus* (Montagu)**	Kuwait	Evans *et al.,* 2006
Common octopus ***Octopus vulgaris* (Cuvier)**	Italy	Fichi *et al.,* 2015

1.3. *Lactococcus garvieae*

Lactococcus garvieae is a pathogen of importance in the aquaculture of freshwater and marine fish (Collins *et al.*, 1983; Bragg and Broere, 1986; Kusuda *et al.*, 1991; Eldar *et al.*, 1996). Initially named *Streptococcus garvieae*, it was originally isolated from a case of bovine mastitis in the United Kingdom and this isolate was selected as the reference strain (ATCC® 43921) for this species (Collins *et al.*, 1983). Lactic streptococci in the genus *Streptococcus* were assigned to a new genus *Lactococcus* in 1985 (Schleifer *et al.*, 1985). Gram-positive fish pathogens isolated from streptococcal disease outbreaks in Japanese yellowtail (*Seriola quinqueradiata*) were later unified under a new species*, Enterococcus seriolicida* (Kusuda *et al.*, 1991). In 1988, a bacterium isolated from the first Spanish lactococcosis outbreak in rainbow trout was described as an *Enterococcus* sp. (Palacios *et al.*, 1993), but was later identified as *Lactococcus garvieae* based on biochemical characteristics (Teixeira *et al.*, 1996). South African Gram-positive cocci, initially described as *Streptococcus* spp. (Bragg and Broere, 1986) were recently reclassified as *Enterococcus* spp. and *L. garvieae* based on 16S rDNA sequencing (Bekker *et al.*, 2011). Recent advances in next generation sequencing technologies have contributed to a steady increase in the numbers of publically available full and partial genome sequences of *L. garvieae* over the last decade, as described in Table 1.2.

1.3.1. Phenotypic and biochemical characteristics

Lactococcus garvieae is a Gram-positive, facultative anaerobic, non-motile bacterium that does not produce endospores. Growth occurs as cocci attached in short chains or pairs, at temperatures ranging from 4°C-45°C. Optimal growth occurs at 37°C (Boomker *et al.*, 1979; Kusuda *et al.*, 1991; Eldar *et al.*, 1996). The bacterium grows quickly in rich media such as trypticase-soy broth (TSB), bile-esculin agar (BEA) and brain-heart infusion (BHI) broth, but growth is inhibited on McConkey and *Enterococcus* agar (Toranzo *et al.*, 1994). It is generally described as an *α*-haemolytic

bacterium (Ravelo *et al.*, 2001), but has been noted as β-haemolytic (Teixeira *et al.*, 1996). The phenotypic, physiological and biochemical properties of *L. garvieae* are listed in Table 1.3.

Table 1.2: Available genome sequences of *L. garvieae* as listed on NCBI (https://www.ncbi.nlm.nih.gov/genome/genomes/699)

Strain	Source	Origin	Accession nr.	Reference
21881	Human blood	Spain	NZ_AFCF00000000	Aguado-Urda *et al.,* 2011b
8831	Rainbow trout	Spain	NZ_AFCD00000000	Aguado-Urda *et al.,* 2011a
ATCC® 49156	Yellowtail	Japan	NC_015930	Morita *et al.,* 2011
Lg2	Yellowtail	Japan	NC_017490	Morita *et al.,* 2011
UNIUD074	Rainbow trout	Italy	NZ_AFHF0000000	Reimundo *et al.,* 2011
DCC43	Mallard duck intestines	Norway	AMQS00000000	Gabrielsen *et al.*, 2012
IPLA 31405	Raw-milk cheese	Spain	NZ_AKFO00000000	Flórez *et al.,* 2012
LG9	Rainbow trout	Italy	NZ_AGQY00000000	Ricci *et al.,* 2012
TB25	Cheese	Italy	NZ_AGQX0000000	Ricci *et al.,* 2012
I113	Pork sausage	Italy	NZ_AMFD00000000	Ricci *et al*., 2013
Tac2	Turkey meat	Italy	NZ_AMFE00000000	Ricci *et al.,* 2013
Lg-ilsanpaik-gs201105	Human cholecystitis	South Korea	NZ_JPUJ00000000	Kim *et al.,* 2015
PAQ102015-99	Rainbow trout	United States of America	LXWL00000000	Nelson *et al.*, 2016
122061	Yellowtail	Japan	AP017373	Nishiki *et al.*, 2016
M14	Fermented milk	Algeria	NZ_CCXC00000000	Moumene *et al.*, 2016
A1	Soil	Turkey	NBBK00000000	Altın *et al.*, 2017

1.3.2. Isolation and identification

Methods for the selective isolation of *Streptococcus* spp. had not been succesfully applied to the isolation of fish-pathogenic streptococcal bacteria (Bragg *et al.*, 1989), therefore a biphasic procedure for the selective isolation of a fish-pathogenic *Streptococcus* sp. was developed by Bragg and co-workers (1989). During a selective enrichment phase, field samples were inoculated into nutrient broth (pH 9.6) supplemented with nalidixic acid (160 μg.mL^{-1}) followed by incubation at room temperature for 48 h (Bragg *et al.*, 1989). Nalidixic acid inhibits the growth Gram-negative bacteria, while the increased pH served to inhibit the growth of yeast. In the isolation phase, growth was plated onto tetrazolium agar (1,4% m/v agar, 1% m/v peptone, 1% m/v lablemco 0,5% m/v NaCl, 1% m/v glucose and 0,01% m/v tetrazolium salt) after which small red colonies were plated onto blood-tryptose agar (BTA). Colonies were further characterised by Gram-staining. Biochemical identification, slide agglutination and immunofluorescent antibody tests were performed on Gram-positive cocci. This procedure was shown to detect about 2 bacteria per mL (Bragg *et al.*, 1989).

A medium for differentiation between *L. garvieae* and other fish pathogens was recently developed (Chang *et al.*, 2014). The medium contains selective agents Difco™ Oxgall (3%) and potassium tellurite (10 ppm), which inhibits growth of most water-borne bacteria. A tetrazolium mixture (2,3,5-triphenyltetrazolium chloride/tetrazolium blue chloride = 9:1) at a concentration of 80 ppm was included to differentiate between capsulated and non-capsulated *L. garvieae* isolates. Differentiation is based on the conversion of TeO_3^{2-} to Te, which stains capsulated *L. garvieae* colonies metallic black, while reduction of triphenyltetrazolium to red triphenyl formazan results in a red halo. Colonies of capsulated isolates therefore appear metallic black with a red halo.

Molecular techniques based on PCR methods have shown to be useful in the identification of fish pathogens such as *L. garvieae* (Vendrell *et al.*, 2006) A PCR assay targeting a 1.1 kb region of the 16S rDNA failed to detect *L. garvieae* in environmental samples from ponds associated with outbreaks of lactococcosis (Zlotkin *et al.*, 1998). A similar assay targeting the dihydropteroate synthase gene proved to specifically detect *L. garvieae* in diseased yellowtail *Seriola quinqueradiata* (Temminck & Schlegel) kidney homogenates (Aoki *et al.*, 2000). An approach using PCR amplification of the 16S–23S RNA internal transcribed spacer (ITS) region was shown to be more specific than the previously published 16S rDNA-based approaches, in addition to showing the capacity to detect quantities as low as 2.63 pg DNA (Dang *et al.*, 2012). Analysis of ITS sequence data from lactococci, streptococci and enterococci indicated a high degree of polymorphism, thus

qualifying the ITS region as a valuable target for reliable differentiation of lactococci (Blaiotta *et al.*, 2002; Dang *et al.*, 2012).

Table 1.3: Phenotypic characteristics of *L. garvieae* (Vendrell *et al*. 2006)

Property	Reaction	Property	Reaction
Cell morphology	Ovoid cocci	*Production of*:	
Gram stain	+	Arginine	+
Motility	-	Ornithine	-
Growth:		Lysine	-
4 °C	+	*Acid from:*	
20 °C	+	Glycerol	-
37 °C	+	Raffinose	-
45 °C	+	Arabinose	-
pH 9.6	+	Sorbitol	+
6.5% NaCl	+	Mannitol	+
Haemolysis	α-	Cellobiose	+
Catalase	-	Galactose	+
Oxidase	-	D-Glucose	+
TSI	A/A-	Maltose	+
Oxidative/fermentative	F	Trehalose	+
Nitrate reduction	-	D-Mannose	+
Citrate	-	Inositol	-
Urea	-	Lactose	+
Indole production	-	Ribose	V
Esculin	+	Sucrose	V
Voges-Proskauer	+	Adonitol	-
H_2S production	-	Glycogen	-
Arginine dihydrolase	+	Melibiose	-
Pyrrolidonyl arylamidase	+	Melezitose	-
Alkaline phosphatase	-	Starch	-
β –Glucuronidase	V	Tagatose	V
Leucine arylamidase	+	L-Rhamnose	-
Sodium hippurate hydrolysis	-	D-Xylose	-
		Salicin	+

v: variable reaction/( ): weak or slow reaction; A/A–: acidification of medium

1.3.3. Antigenic characteristics

Lactococcus garvieae isolates are divided into two serotypes indistinguishable by biochemical tests (Kitao, 1982). Early work on *L. garvieae* revealed a high degree of variability in surface structure of fresh isolates subcultured on an artificial medium containing 2, 3, 5-triphenyltetrazolium chloride or subcultured repeatedly on Todd Hewitt agar. Surface antigen variability is evidenced by the inability of antiserum raised against subcultured isolates to agglutinate wild-type isolates from diseased fish (Kitao, 1982). The serotypes are distinguished by their ability to agglutinate serum raised against *L. garvieae.* Non-agglutinating phenotypes are designated KG- and agglutinating phenotypes KG+ (Hirono *et al.*, 1999). Immunofluorescent staining techniques have been applied on isolates from yellowtail to show that KG+ antigens are concentrated on the cell surface only, while KG- antigens were located across the cell capsule (Okada *et al.*, 2000). Transmission electron microscopy (TEM) revealed the presence of fimbriae on the surface of *L. garvieae* cells accompanied by capsular disruption following opsonization with yellowtail immune serum (Ooyama *et al.*, 1999). The antigenicity of these fimbriae has not yet been investigated.

Capsular variation is often the basis of serological differences in pathogens (Yother, 2011). However, few reports on serological variation between capsulated *L. garvieae* isolates exist. Using dot blot assays with specific group polysaccharides as antigen, Eyngor and colleagues (2004) determined that among Mediterranean isolates, two serovars or groups (SGT) can be distinguished: SGT I, inclusive of Italian and Israeli isolates, and SGT II, including Spanish, Greek and Bulgarian isolates (Eyngor *et al.*, 2004). Heterogeneity among French isolates was observed, with isolates grouping with both SGT I and II. The study combined restriction fragment length polymorphism ribotyping with the serological data, generating clear correlation between ribotypes and serovars. Molecular typing displayed discriminatory ability superior to serotyping because related ribotypes could group into a single serotype (Eyngor *et al.*, 2004). This phenomenon may be caused by strains possessing similarities in sections of their genomes encoding serotype specific antigens while displaying a greater degree of heterogeneity in genome portions subjected to ribotyping. Changes in serovar prevalence are usually attributed to immune pressure and population dynamics, with farmed fish populations comparable to semi-closed communities. Limited selective pressure in the form of vaccination against *L. garvieae* had been imposed on studied bacterial populations since currently available vaccines are not wholly effective with the result that only a fraction of the host population is vaccinated (Eyngor *et al.*, 2004). It can therefore be expected that capsular

stability had been preserved in the endemic sites (Israel, Italy, Spain, Greece and Bulgaria). Serotypic diversity, correlating to clonal diversity, observed in French isolates is typical of areas where the pathogen has been introduced recently and disease outbreaks are infrequent (Eyngor *et al.*, 2004). A comparative serological study of Japanese and European capsulated and non-capsulated isolates from rainbow trout indicated that antisera against all capsulated isolates strongly cross-reacted with all non-capsulated isolates, regardless of geographical origin (Barnes and Ellis, 2004). Conversely, antisera against non-capsulated isolates did not react with any capsulated isolates. No cross-reaction of antisera against Japanese and European isolates were observed (Barnes and Ellis, 2004). These serological differences could be attributed to variations in surface polysaccharide composition, assayed by agglutination with a panel of fifteen lectins. Capsulated European isolates were agglutinated by concanavalin A, which specifically binds to α-D-mannose and α-D-glucose moieties, while Japanese isolates were not agglutinated by any lectins used in the study. Non-capsulated isolates were agglutinated by more lectins compared to the capsulated European isolates, perhaps revealing the carbohydrate diversity of the cell wall (Barnes and Ellis, 2004).

Cell wall proteins are targets of immune surveillance, thereby contributing to serological differences between strains and forming the focus of vaccine development studies. A study by Hirono and colleagues (1999) identified antigenic proteins in a KG- strain, immunologically detected by anti-KG- rabbit serum. The detected proteins include enzymes involved in various cellular processes. A protein with 37.1% homology to an *N*-acetylglucosamine-6-phosphate deacetylase of *Vibrio furnissi* was detected in KG- cells, but not KG+ cells (Hirono *et al.*, 1999). Taking into account that *N*-acetylglucosamine-6-phosphate deacetylase plays a role in peptidoglycan and lipopolysaccharide synthesis in Gram-negative bacteria it can be speculated that this protein plays a role in capsule synthesis in *L. garvieae.* Other proteins found reacting with anti-KG- serum includes proteins with 47.7% and 45.8% sequence homology to processing protease of *Bacillus subtilis* and a trigger factor of *Escherichia coli,* respectively (Hirono *et al.*, 1999). The trigger factor of *E. coli*, a peptidyl-prolyl-cis/trans-isomerase, is induced by cold-shock and enhances cell viability at low temperatures (Hesterkamp and Bukau, 1996). A further investigation into the immunogenicity of KG+ and KG- cells using two-dimensional gel electrophoresis (2-DE) and immunoblotting assays revealed that elongation factor G, guanine monophosphate synthetase, elongation factor thermo-unstable (EF-Tu) and adenosine tri-phosphate synthase reacted more intensely with rabbit anti-KG+ sera in comparison to rabbit anti-KG- sera, suggesting that these may be major specific antigens for the KG+ strain. Results also identified glyceraldehyde-3-phosphate

dehydrogenase, phosphoglycerate kinase, arginine deaminase and ornithine carbamoyltransferase as common antigens in the two serotypes (Shin *et al.*, 2007). A repeat of the study by Shin and colleagues (2007) used olive flounder, *Paralichthys olivaceus* (Temminck & Schlegel), sera instead of rabbit sera and identified eight antigenic protein spots reacting specifically with anti-KG- sera. However, these proteins could not be identified by MALDI-TOF MS (Shin *et al.*, 2009).

1.4. DISEASE CONTROL OPTIONS

1.4.1. Chemotherapeutic administration

Antibiotics have been widely used to control streptococcal infections in various fish (Aoki *et al.*, 1990). Administration occurs generally *via* the oral route by combining antibiotics with specially formulated feed. Antimicrobial agents show strong *in vitro* activity against *L. garvieae,* but perform poorly under field conditions due to anorexia of infected fish (Bercovier *et al.*, 1997) and possibly the ineffective metabolism of antibiotics in fish (Romero *et al.*, 2012). Lincomycin, oxytetracycline and macrolide antibiotics (e.g. erythromycin, spiramycin, kitasamycin and josamycin) have been widely used to treat lactococcosis in cultured fish (Aoki *et al.*, 1990; Kawanishi *et al.*, 2005). In rainbow trout, erythromycin, oxytetracycline, amoxicillin and low-level doxycycline are mostly used to treat outbreaks of lactococcosis (Vendrell *et al.*, 2006).

1.4.1.1. Antibiotic resistance

Dissemination of antibiotic resistance in bacteria has grown into a global public health concern, accelerated by the unregulated and injudicious administration of antibiotics in humans and animals (Heuer *et al.*, 2009). In aquaculture, chemotherapeutic treatment has led to the emergence of resistance streptococcal fish pathogens (Aoki *et al.*, 1990; Austin and Austin, 2012). Multiple resistance is frequently encountered, referring to the occurrence of resistance to more than one chemotherapeutic agent in one isolate. The spread of antibiotic resistance genes in bacterial populations is aided by various mechanisms of horizontal gene transfer, of which plasmid mediated transfer is the most widely documented in streptococcal fish pathogens. The first report describing antibiotic resistance in aquatic *Streptococcus* spp. grouped resistance in isolates from cultured yellowtail (*Seriola quenqueradiata*) from various locations in Japan into two categories: intermediate-level resistance to macrolides, lincomycin and tetracycline, in which resistance genes

were constitutively expressed and non-transferable; and high-level resistance to macrolides, lincomycin and either tetracycline or chloramphenicol whose resistance genes were inducible and transferable (Aoki *et al.*, 1990). The authors surmised that the antibiotic resistance determinants were located either on resistance (R) plasmids or transposons. These findings led to the characterisation of R plasmids isolated from erythromycin-, lincomycin- and oxytetracycline-resistant *L. garvieae* isolates, revealing the presence of resistance genes *ermB* and *tet*(S) (Hirono and Aoki, 2001). The gene *ermB* contributes to erythromycin resistance by target modification mediated by the production of a 23S rRNA methylase (Leclerq and Courvalin, 1991) while the gene product of *tet*(S) is a ribosomal protection protein that confers tetracycline resistance (Chopra and Roberts, 2001). A study by Kawanishi and co-workers (2005) corroborated the findings of Aoki and co-workers (1990) as well as Hirono and Aoki (2001) by reporting high incidence of multiple resistance to erythromycin, lincomycin and oxytetracycline in Japanese *L. garvieae* aquatic isolates. Antimicrobial susceptibility determination of 170 *L. garvieae* isolates revealed that nearly half were simultaneously resistant to erythromycin (MIC ≥ 2 μg.mL^{-1}), lincomycin (MIC ≥ 128 μg.mL^{-1}) and oxytetracycline (MIC ≥ 4 μg.mL^{-1}) (Kawanishi *et al.,* 2005). Additionally, all resistant isolates harbours the resistance genes *ermB* and *tet*(S). A study on resistance to chemotherapeutic substances in Japanese *L. garvieae* isolates by Maki and colleauges (2008) revealed that 31.5% of tested isolates were highly resistant (MIC >400 μg.mL^{-1}) to erythromycin, tetracycline and lincomycin. Of the highly resistant isolates, 26% carried R plasmids transferable to *Enterococcus faecalis* by conjugation (Maki *et al.*, 2008). The remaining 74% of highly resistant isolates were shown to carry the same resistance genes present on the R plasmid, suggesting carriage of either an integrated R plasmid or transferable low frequency plasmids. Further characterisation of the R plasmid, pKL0018, described in the study by Maki and colleauges (2008), revealed high sequence homology to pRE25, a plasmid found in *E. faecalis* isolated from dried sausage (Maki *et al.*, 2009). Genes related to multiple drug resistance carried on pKL0018 were identified as a tetracycline resistance gene *tet*(S) and macrolide resistance genes encoding 23S rRNA methyltransferases (*ermB1* and *ermB2)*. The presence of *tet*(S) and another ribosomal protection protein gene, *tet*(M), was simultaneously detected in Japanese *L. garvieae* marine isolates (Kim *et al.*, 2004). All but one of these isolates additionally harboured the integrase gene of the Tn1545–Tn916 conjugative transposon family, a first indication of horizontal gene transfer of resistance genes in *L. garvieae*. The presence of transferable R plasmids and conjugative transposon-associated integrase genes in aquatic *L. garvieae* suggests that these isolates can function as antibiotic resistance vectors between clinical, terrestrial and marine environments (Kim *et al.*, 2004).

1.4.2. Vaccination

Vaccination is considered the best option to control lactococcosis, due to the poor efficiency of chemotherapeutic agents under field conditions and the risks associated with the spread of antibiotic resistance determinants. Practices include intraperitoneal injection one month prior to water temperature increasing over 15°C, with care being taken to maintain fish in optimum health and reducing stress. Vaccination is performed when fish weigh approximately 50 g and when water temperature measures between 12-14°C (Vendrell *et al.*, 2006). Autogenous formalin-inactivated vaccines against *L. garvieae* are commonly implemented, with protection of 80-90% observed upon intraperitoneal injection (Bercovier *et al.*, 1997) protection persisting for up to 5 months with adjuvant vaccines (Vendrell *et al.*, 2006). The safety and efficacy of an inactivated vaccine Ichtiovac-Lg, emulsified with an adjuvant (Aquamun), was assessed in rainbow trout (Vendrell *et al.*, 2007). An intraperitoneal injection of a double dose of vaccine (0.2 mL) resulted in 100% survival in treatment and control groups. Side effects observed during necropsy in the vaccinated group are considered acceptable by the European Pharmacopoeia. The side effects recorded were mild, localised adhesions and minor pigmentation of the visceral peritoneum and moderate adhesions between viscera (Vendrell *et al.*, 2007). To test the efficacy of Ichtiovac-Lg, rainbow trout were injected intraperitoneally with the recommended dosage (0.1 mL) and challenged with a capsulated strain of *L. garvieae* (CLFP LG1) 29 days post-vaccination. A cumulative survival rate of 94% was reported for the vaccinated group, while 4% cumulative survival rate was reported for the control group (Vendrell *et al.*, 2007). The authors believe that the observed efficacy of the vaccine might have been attributed to the immuno-stimulatory effect of the mineral oil adjuvant, which was demonstrated in Atlantic salmon (*Salmo salar* L.) vaccinated against furunculosis (Midtlyng *et al.*, 1996). Several studies have shown that subunit vaccines (i.e. vaccines consisting of immunogenic fractions) are capable of eliciting higher levels of protection in comparison to whole cell vaccines in fish (Ra *et al.*, 2009; Zhou *et al.*, 2010). Bacterial outer membrane proteins (OMPs) are often targets of subunit vaccine development, because their exposure on the cell surface promotes recognition by the host's immune system (Kawai *et al.*, 2004). Despite promising results obtained with bacterial subunit vaccines in fish (Liu *et al.*, 2005; Ra *et al.*, 2009) reports on the development of subunit vaccines against lactococcosis are rare. In a study by Tsai and colleagues (2013) an antigen common to both KG+ and KG- *L. garvieae* serotypes, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Shin *et al.*, 2009), was cloned and expressed in *Escherichia coli* BL21 (DE3). Western blotting analysis was used to show that both rabbit and tilapia antiserum reacted strongly with recombinant GAPDH. In addition, higher GAPDH-specific antibody titres were

reported in tilapia immunized with recombinant GAPDH as well as whole cells in comparison to tilapia immunized with whole cells only. However, fish immunized only with recombinant GAPDH showed higher percentage cumulative mortality over a period of 14 days post-challenge than fish immunised with whole cells only and fish immunised with whole cells and recombinant GAPDH. Tsai and colleagues (2013) partially attributed this observation to the immunostimmulatory effect of peptidoglycan in the whole cell vaccine. The duration of protection afforded by the GAPDH vaccine in comparison to a whole cell vaccine has not been investigated.

1.5. VIRULENCE FACTORS

1.5.1. Toxins

Toxigenesis plays a crucial role in the pathologic processes of various Gram-positive bacteria (Barnett *et al.*, 2015). Early studies on toxins of a non-Lancefield *Streptococcus* sp. isolated from yellowtail (presumably *L. garvieae)* showed the presence of a haemolytic toxin in culture supernatant (Kusuda and Hamaguchi, 1988). Mortalities caused by intramuscular injection of this toxin were low (<20%), but characteristic symptoms of streptococcosis (i.e. exophthalmus, ocular haemorrhaging and reddish fin base) were elicited. An intracellular toxin showing weak leukocidal activity (<44%) was responsible for higer mortaliy rates of up to 60% upon intramuscular injection (Kusuda and Hamaguchi, 1988).

A study by Aguado-Urda and colleagues (2012) identified and characterized five circular plasmids in a clinical isolate of *L. garvieae* strain 21881. The largest of these plasmids, pGL5 (68 798 bp), was shown to encode putative virulence factors, including a protein that posesses the enzymatic domain corresponding to the family of actin-ADP-ribosyltransferases (Aguado-Urda *et al.*, 2012). Bacterial ADP-ribosyltransferase toxins kill eukaryotic cells by transferring ADP-ribose to essential proteins, contributing to virulence in a range of pathogens (Holbourn *et al.*, 2006).

1.5.2. Immune evasion mechanisms

For a long time it has been known that virulence of *L. garvieae* is influenced by capsule formation (Vendrell *et al.*, 2006). Encapsulation contributes to virulence in both Gram-positive and Gram-negative bacteria in a number of ways, for example by conferring resistance to phagocytosis (Musher, 1992) and exhibiting molecular mimicry to host tissue (Johnson, 1991). In Gram-positive

pathogens, the structure of capsular polysaccharides (CPSs) vary between serotypes (Hammerschmidt *et al.*, 2005; Eyngor *et al.*, 2010). A comparative genome analysis of a virulent strain Lg2 and a non-virulent strain ATCC® 49156 of *L. garvieae* identified a 16.5 kb capsule gene cluster that is present in Lg2, but absent in ATCC® 49156 (Morita *et al.*, 2011). The capsular gene cluster consists of 15 genes, of which eight (*eps-R, X, A, B, C, D* and *cps-L, W*) are conserved in the exopolysaccharide (EPS) biosynthesis gene cluster of four *Lactococcus lactis* strains isolated from human faecal samples (Morita *et al.*, 2011). Analyses indicate that the capsular gene cluster is a genomic island, due to the presence of insertion sequences (IS) on both ends of the capsular gene cluster.

The KG- phenotype of *L. garvieae* was shown to possess a capsule rich in hydrophilic monosaccharides, possibly contributing to the observed increase in resistance to phagocytosis by *S. quinqueradiata* phagocytes in comparison to KG+ cells. Respiratory burst in phagocytic cells was shown to be suppressed in response to the KG- phenotype, indicating inhibition of binding of phagocytes to the encapsulated strain (Yoshida *et al.*, 1996). The *in vitro* findings are supported by challenge studies that indicate lower serum agglutinating antibody titres in fish challenged with KG- cells in comparison to KG+ cells (Yoshida *et al.*, 1996). Noncapsulated *L. garvieae* isolated from radish and broccoli sprouts were nonpathogenic toward mice and yellowtail, again highlighting the involvement of encapsulation in virulence towards fish. In addition, the avirulence of non-capsulated isolates correlated with their susceptibility to rainbow trout normal serum, while capsulated isolates were not susceptible to either normal or immune trainbow trout serum (Barnes *et al.*, 2002a). Unexpectedly, protection against capsulated isolates was afforded by passive immunization of rainbow trout with specific antiserum against *L. garvieae,* leading the authors to speculate that specific antibodies enhance phagocytosis and bactericidal activity by macrophages (Barnes *et al.*, 2002a). It was indeed proven that the antiphagocytic properties of the polysaccharide capsule can be overcome in the presence of specific antibodies (Barnes *et al.*, 2002b). The observed increased bactericidal activity of immune serum was most likely not due to complement, as the serum was first heat-treated. Fluorescence microscopy of fluorescein-isothiocyanate (FITC) labelled bacteria incubated with macrophages indicated that 90% of macrophages contained internalised bacteria that had been treated with immune serum, while only between 0 and 2% of macrophages contained internalised bacteria that had been treated with non-immune serum (Barnes *et al.*, 2002b). Many Gram-positive bacteria employ binding of immunoglobulins non-specifically by the Fc region as a virulence factor (Agniswamy *et al.*, 2004). Binding of antibodies by non-immune mechanisms inhibits activation of complement by the

classical pathway and allows the bacterium to shield itself from specific antibodies and evade phagocytosis. Barnes and co-workers (2002a) showed that non-capsulated *L. garvieae* is capable of non-specifically binding immunoglobulin more efficiently than capsulated isolates, an observation seemingly inconsistent with the avirulence of non-capsulated isolates. However, it needs to be considered that surface proteins play integral roles in adhesion and colonisation of host tissues, and that non-specific binding to host serum proteins might therefore inhibit adhesion of non-capsulated isolates to host cells (Barnes *et al.*, 2002a). Interestingly, capsulated *L. garvieae* Lg2 was shown to be avirulent toward mice (Kawanishi *et al.*, 2007) and *L. garvieae* 21881 (isolated from blood of a septicaemic patient) lacked a capsule gene cluster (Miyauchi *et al.*, 2012) perhaps indicating that encapsulation is not a prerequisite for virulence in mammals.

Even though the polysaccharide capsule is widely regarded as a major virulence factor of *L. garvieae,* it has been shown that non-capsulated strains Lgper and ATCC® 49156 are pathogenic towards rainbow trout, causing 89% and 98% mortality respectively (Türe *et al.*, 2014). Detection of putative virulence genes in 34 *L. garvieae* isolates pathogenic to fish revealed that the capsule gene cluster could only be amplified by multiplex PCR in the strain Lg2 (Türe and Altinok, 2016). These results suggest that the presence of the polysaccharide capsule cannot be directly correlated to pathogenicity in fish.

Internalisation of bacteria by non-phagocytic host cells represents another widely utilised immune evasion tactic. Immunofluorescence studies have demonstrated the ability of *L. garvieae* Lg8831 to be internalised by non-phagocytic zebrafish (*Danio rerio*) kidney cells following experimental infection (Aguado-Urda *et al.*, 2014). Intracellular localisation and proliferation is a survival mechanism employed by other piscine pathogens as well (Acosta *et al.*, 2009).

1.5.3. Adhesion

Both commensal and pathogenic bacteria express adhesins to facilitate binding to host cell receptors (Kline *et al.*, 2009). Genes encoding two putative surface proteins that contain a cell wall sorting motif associated with covalent binding to peptidoglycan, LPXTG (Leu-Pro-any-Thr-Gly), were identified on the plasmid pGL5 of a clinical *L. garvieae* isolate (21881). The gene *orf5* encodes a protein containing three mucin-binding protein domains in addition to a cell wall sorting motif (LPQTG) at the carboxy terminal, suggesting that protein Orf5 might aid in adhesion of *L. garvieae* to mucosa by interaction with mucosal receptors (Aguado-Urda *et al.*, 2012). Another putative cell

surface protein encoded by *orf25* contains a collagen-binding domain, which could allow adhesion of the cell to collagenous host tissues.

Based on the work of Miyauchi and co-workers (2012), Türe and Altinok (2016) determined the prevalence of a variety of putative virulence genes among 34 *L. garvieae* isolates pathogenic to fish *via* PCR. The adhesin encoding genes adhesion Pav (*adh*Pav), adhesin PsaA (*adh*PsaA), LPxTG-containing surface proteins 2 and 3 (LPxTG-2, -3) and adhesin clusters 1 and 2 (*adh*CI, *adh*CII) were present in all isolates tested.

Cell surface carbohydrates of various tissues are an important adhesion target for bacteria, especially pathogens that colonise mucosal tissues. Gangliosides, which are glycosphingolipids that contain sialic acid, of yellow tail brain and intestine have been shown to be receptors for *L. garvieae* (Shima *et al.*, 2006), although the molecular basis of ganglioside recognition by the bacterial cell surface is not yet known.

1.5.4. Diversification of virulence factor content

Insertion sequences, plasmids and lysogenic bacteriophages are mobile genetic elements that play an important evolutionary role by promoting adaptability in prokaryotic genomes (Eraclio *et al.*, 2015). Insertion sequences, broadly defined as short DNA segments capable of insertion at multiple sites in a target molecule represent the smallest and simplest mobile genetic elements. These compact, non-coding DNA segments typically contain terminal inverted repeat sequences involved in transposase binding and strand cleavage during sequence transposition, and generate short direct repeat sequences of the target DNA upon insertion (Mahillon and Chandler, 1998). During horizontal gene transfer, insertion sequences can play an important role in bacterial pathogenesis and exchange of virulence factors. Fifteen insertion sequences have been identified in the publically available genomes of *L. garvieae* (Eraclio *et al.,* 2015). The close relatedness between insertion sequences in *L. garvieae* and *L. lactis* described may suggest genetic exchange between the species.

Bacteriophages are viruses that infect and kill bacterial cells with great efficacy and are present in all ecosystems that support the growth of bacteria (Elbreki *et al.*, 2014). Replication of bacteriophages occurs *via* one of two cycles, the lytic or lysogenic cycle, of which the latter is of particular interest to bacterial virulence. During the lysogenic cycle, bacteriophage genetic material is integrated into the host genome, introducing genes that may encode miscellaneous virulence factors, such as toxins (Wagner and Waldor, 2002), regulatory factors that enhance the production

of virulence genes (Spanier and Cleary, 1980) and enzymes capable of altering virulence properties (Guan *et al.*, 1997). The presence of a lysogenic bacteriophage (PLgT-1) in the genomes of *L. garvieae* strains isolated from Japanese marine fish species was recently discovered (Hoai and Yoshida, 2016). Prophages were induced by mitomycin C treatment, integrated genomes of bacteriophages (prophages) were detected by a PCR assay and morphological study of phage particles by transmission electron microscopy revealed characteristics congruent with the morphology of phages from the family *Siphoviridae.* Considering the high incidence of prophages in strains isolated from Japanese marine fish as revealed by this study, in conjunction with the high virulence of marine isolates compared to trout and terrestrial mammalian isolates (Kawanishi *et al.*, 2006), it is probable that prophages contribute to the virulence of *L. garvieae. In silico* analyses of 16 *L. garvieae* genomes revealed eight complete prophages in dairy and aquatic isolates, although no known virulence factors were present in these prophage genomes suggesting that virulence factors of these isolates are encoded elsewhere in the genome (Eraclio *et al.*, 2017).

1.6. MOONLIGHTING PROTEINS IN BACTERIAL VIRULENCE

Until the late 1980s, pervasive doctrine stated that each gene product performs a sole biochemical function (Henderson and Martin, 2011). The first evidence for an alternative hypothesis arose when Piatigorsky and colleagues (1988) demonstrated that duck lens crystalline protein was the metabolic enzyme argininosuccinate lyase (Piatigorsky *et al.*, 1988); a phenomenon then dubbed “gene sharing”. Introduction of the term “moonlighting” (colloquially describing to hold an additional nocturnal occupation of disreputable nature) is traced to Campbell and Scanes (1995), who showed that somatostatin and growth hormone releasing hormone also display immunomodulatory activity (Campbell and Scanes, 1995). The publicising of the then novel concept in protein biology is attributed to Constance Jeffery, who attempted to delineate the definition of moonlighting proteins. The accepted definition states that proteins generated by gene fusions, homologous but non-identical proteins, splice variants, protein decoration variants, protein fragments and proteins operating in different locations or utilising different substrates are not considered to be moonlighting proteins (Jeffery, 1999).

Since the initial description of protein moonlighting, an increasing range of multifunctional proteins have been described. The role of glycolytic enzymes in bacterial virulence has been

investigated in numerous studies (Pancholi and Chhatwal, 2003; Brassard *et al.*, 2004; Ling *et al.*, 2004; Madureira *et al.*, 2007). The first observations on the versatility of bacterial glycolytic enzymes were made by Pancholi and Fischetti (1992), who demonstrated the binding of *S. pyogenes* glyceralaldehyde-3-phosphate dehydrogenase (GAPDH) to lysozyme, cytosolic proteins and fibronectin (Fn). This surface-localised GAPDH was termed streptococcal surface dehydrogenase (SDH) (Pancholi and Fischetti, 1992). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a cytosolic protein, is the most widely characterized glycolytic enzyme in terms of its role in bacterial pathogenesis.

1.6.1. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a bacterial virulence factor

Glyceraldehyde 3-phosphate dehydrogenase is a highly conserved metabolic enzyme found in all living cells, usually in tetrameric form (Seidler and Seidler, 2013). As illustrated in Fig. 1.1, oxidative phosphorylation of D-glyceraldehyde 3-phosphate to 1,3-*bis*phospho-D-glycerate with the concomitant reduction of NAD^+ to NADH is catalysed by GAPDH in the first step of the "pay-off" phase in glycolysis, characterised by the net gain of ATP and NADH. The reaction involves dehydrogenation of the two triose sugars generated in the preparatory phase of glycolysis and addition of an inorganic phosphate molecule, yielding the intermediate 1,3-*bis*phospho-D-glycerate. NAD^+ is reduced by hydrogen to yield one NADH per triose sugar.

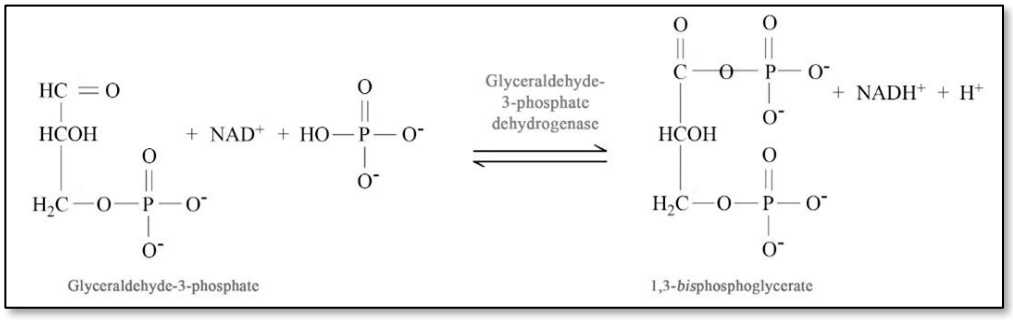


Figure 1.1: The glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase catalyses the conversion of D-glyceraldehyde 3-phosphate to 1,3-bisphospho-D-glycerate using NAD+ as cofactor.

Generally, GAPDH is regarded as a cytosolic enzyme lacking signal sequences or sorting motifs. Interestingly, GAPDH was found to be present in extracellular fractions of various bacteria (Deng *et al.*, 2012; Vanden Bergh *et al.*, 2013; Whitworth and Morgan, 2015) suggesting export or release in

an unkown fashion. Release by cellular autolysis presents a plausible hypothesis, as shown by Terrasse and co-workers (2015) who reported that GAPDH release in *S. pneumoniae* depends on autolysis mediated by autolysin LytA, followed by binding of GAPDH to peptidoglycan of unlysed cells. From this extracellular vantage point, GAPDH contributes to bacterial virulence by interaction with extracellular matrix components of the host, qualifying GAPDH as a microbial surface component recognising adhesive matrix molecules (MSCRAMM) (Terrasse *et al.*, 2015). Extracellular matrix (ECM) proteins of the vertebrate host include the fibrous proteins, collagens, elastins, fibronectins and laminins that mediate cell attachment, chemotaxis, cell migration, provide flexibility and direct tissue development (Frantz *et al.*, 2010). Binding of host ECM components by adhesins is a crucial step in bacterial colonisation for pathogenic and commensal species. A variety of Gram-positive bacteria employ GAPDH as an adhesin as outlined in Table 1.4.

Table 1.4: Ligands of the moonlighting glycolytic enzyme, GAPDH, in various Gram-positive bacterial species.

Ligand	Bacterial species	Reference
Plasminogen	*Bacillus anthracis*	Matta, Agarwal and Bhatnagar, 2010
	Lactobacillus crispatus	Hurmalainen *et al.*, 2007
	Lactobacillus plantarum	Glenting *et al.,* 2013
	Listeria monocytogenes	Schaumburg *et al.,* 2004
	Streptococcus equisimilis	Gase *et al.,* 1996
	Streptococcus pneumoniae	Bergmann, Rohde and Hammerschmidt, 2004
	Streptococcus pyogenes	Pancholi and Fischetti, 1992
Fibronectin	*L. plantarum*	Glenting *et al.*, 2013
	S. pyogenes	Pancholi and Fischetti, 1992
Mucin	*L. plantarum*	Glenting *et al.*, 2013
Actin	*S. pyogenes*	Pancholi and Fischetti, 1992
	S. agalactiae	Seifert *et al.*, 2003
Lysozyme Myosin	*S. pyogenes*	Pancholi and Fischetti, 1992
Complement C1q	*S. pneumoniae*	Terrasse *et al.*, 2012
Complement C5a	*S. pyogenes*	Terao *et al.*, 2006

Human pharyngeal urokinase receptor	*S. pyogenes*	Jin *et al.*, 2005
Fibrinogen	*S. agalactiae*	Seifert *et al.*, 2003
Blood group antigens A & B	*L. plantarum*	Kinoshita *et al.*, 2008
Peptidoglycan	*S. pneumoniae*	Terrasse *et al.*, 2015

Innate immune response defeat or evasion is another process that promotes virulence where GAPDH participates (Terao *et al.*, 2006). In cooperation with *S. pyogenes* cell-associated streptococcal C5a peptidase (ScpA), GAPDH contributes to evasion of complement by binding to, and by proteolytic degradation of human complement C5a (Terao *et al.*, 2006). GAPDH may contribute to virulence in an immunomodulatory capacity by polyclonal B-cell activation and enhancing immune evasion *via* suppression of the specific immune response. Additionally, GAPDH proved to stimulate production of the immunosuppressive cytokine, interleukin-10, which was shown to play a crucial role in host susceptibility to *S. agalactiae* infection (Madureira *et al.*, 2007). Another study identified complement C1q as a ligand of both pneumococcal and human GAPDH, indicating that *S. pneumoniae* GAPDH activates complement cascade *via* the classical pathway, a finding inconsistent with its widely described roles in virulence enhancement (Terrasse *et al.*, 2012). The study further showed an increase in extracellular eukaryotic GAPDH following induction of apoptosis in HeLa cells. Recognition of human GAPDH on the apoptotic cell surface by C1q was associated with detection and uptake of noxious altered-self substances. These findings could suggest a novel immune system subversion mechanism by bacterial mimicry of apoptotic cells (Terrasse *et al.*, 2012).

1.7. APPLICATION OF PHAGE DISPLAY IN PROTEIN INTERACTION ANALYSIS

Unravelling the interactions between proteins and other compounds plays a pivotal role in the study of biological systems, especially considering the ability of proteins like GAPDH to perform more than one function. Experimental approaches to studying protein binding on the atomic and residue levels, such as X-ray diffraction, site-directed mutagenesis and binding tests, are well established (Moreira *et al.*, 2007; Bickerton *et al.*, 2011). However, such experimental approaches are not amenable to large scale studies and are laborious and expensive. Computer-based analyses have progressively gained popularity as integrative techniques in prediction of protein interactions

(Huang *et al.*, 2011). These analyses may rely on amino acid sequence or structural data (molecular docking and simulation) as input. Phage display of peptides offers the possibility of using mimotope motifs in conjunction with sequential and structural data for elucidation of protein interactions (Huang *et al.*, 2011). Phagotopes are peptides displayed on a phage virion that display affinity toward the target. Readily obtainable by affinity selection of a peptide library against a defined target, phagotopes are instinctively similar to binding sites of natural ligands (known as the template) of the target at sequential or structural level. The affinity of phagotopes capable of binding to the target molecule (obtained by biopanning as indicated in Fig 1.4) can be measured by surface plasmon resonance, competitive ELISAs or titration of plaque forming units (Dyson *et al.*, 1995).

Prediction of protein interaction sites based on phagotopes depends on either the sequence or the structure of the template (Huang *et al.*, 2011). General purpose sequence analysis tools for sequence alignment, local alignment or pattern search can be utilised to align phagotopes, or consensus sequences of the phagotopes, to template sequences to locate the interaction site of the template protein. If a template is unknown, a local alignment search may be performed to predict candidate templates and binding sites. BLAST against protein databases has been used to identify putative ligands of various target molecules. For instance, a random heptapeptide library was screened against LipL32 of the pathogenic spirochete *Leptospira interrogans* in order to elucidate the role of this outer membrane protein in virulence (Chaemchuen *et al.*, 2011). Six affinity selected peptide sequences were identified, and were used to search a database of host proteins for at least four exact amino acid matches. Host proteins with the highest similarity were chloride channel accessory 2, glycoprotein VI, scavenger receptor expressed by endothelial cell isoform I, laminin alpha 5, collagen XX, coronin 2A, and prostaglandin E receptor 1, but further confirmation of interaction between these proteins and LipL32 *in vivo* and *in vitro* is required (Chaemchuen *et al.*, 2011). Pattern or motif search against protein databases has also been employed to identify ligands. This approach was used to identify a ligand for the cell wall anchored MSCRAMM, serine-aspartate repeat-containing protein C (SdrC), of *Staphylococcus aureus* (Barbu *et al.*, 2010). Using immobilised SdrC as bait to screen a 12-mer random peptide library (New England Biolabs Ph.D.™ - 12 Phage Display Peptide Library), a degenerate consensus amino acid sequence was identified after three successive rounds of biopanning. A pattern search of the human protein database returned a synaptic cell surface protein, β-neurexin. Furthermore, it was shown that intact β-neurexin is a functional ligand of SdrC (Barbu *et al.*, 2010).

1.7.1. Filamentous phage M13 display

The basis of phage display technology was created in 1985 when it was illustrated that a foreign gene sequence could be fused to the coat protein of filamentous phage M13, resulting in the display of the foreign protein on virions produced in *E. coli* (Smith, 1985). Soon after, the concept of affinity selection of phage displayed peptides was introduced and the first random peptide display libraries were developed, allowing a vast variety of random peptide sequences each to be displayed on a phage virion (Cwirla *et al.*, 1990; Devlin, Panganiban *et al.*, 1990; Scott and Smith, 1990). Presently, phage display is one of the most widely used research tools in biological sciences (Wang and Yu, 2004). Phage T7 (Danner and Belasco, 2001), phage Lambda (Beghetto and Gargano, 2011) and filamentous phages (f1, fd and M13) display systems have been developed utilising insertion of peptide or antibody genes, but filamentous phage M13 is the favoured system used in random peptide display.

1.7.1.1. Filamentous phage biology

The genus Inovirus is considered contra the archetypal head-and-tail, lytic *E. coli* bacteriophage. Filamentous phage M13 is 1 μm in length and contains a circular single stranded genome of ~6400 nucleotides (Russel, 1995). The genome is encased by thousands of copies of the major coat protein, pVIII, while other structural proteins pIX and pVI adorn the "head" region of the virion. The "tail" region is formed by pVI and pIII. Its dissimilarity to typical *E. coli* phages makes it amenable to utilisation in phage display techniques. The filamentous phage genome can accommodate large foreign sequence inserts without detriment to packaging and replication, and the mature phage particle can withstand harsh physiochemical conditions (Wang and Yu, 2004).

In contrast to the typical *E. coli* phages, filamentous phage dissemination occurs without lysis of the host. Filamentous phage infection, replication and dissemination proceeds according to the general steps of phage replication, schematically summarised in Fig. 1.2:

1. The virion adsorbs to the host cell *via* interaction between the D2 domain of pIII and the host's F-pilus. Only F-plus cells are susceptible to infection. The membrane penetration domain of pIII, D1, attaches to the bacterial co-receptor TolA (Marvin, 1998).
2. After injection of the single-stranded (+ sense) genome, a host polymerase synthesises a complementary (- sense) strand to generate the double stranded replicative form (RF) (Wang and Yu, 2004).
3. Non-structural protein pII nicks the + sense strand at the origin of replication (ori^{+}) and the free 3' end acts as a primer for rolling circle replication. Circular progeny is formed by the

ligase activity of pII (Wang and Yu, 2004). Single stranded progeny forms a complex with pV that blocks complementary strand synthesis and pre-packages the progeny DNA to a linear form to be inserted into virions (Marvin, 1998).

4. Structural (pIII, pVI, pVII, pVIII and pIX), replication (pII, pV and pX) and export and assembly (pI and pIV) proteins are produced by host-mediated protein synthesis.
5. Virion assembly and export requires co-operation between host membranes and phage structural and assembly proteins. Assembly of the virion requires three non-structural proteins (pI, pIV and pXI) and one host protein, thioredoxin.

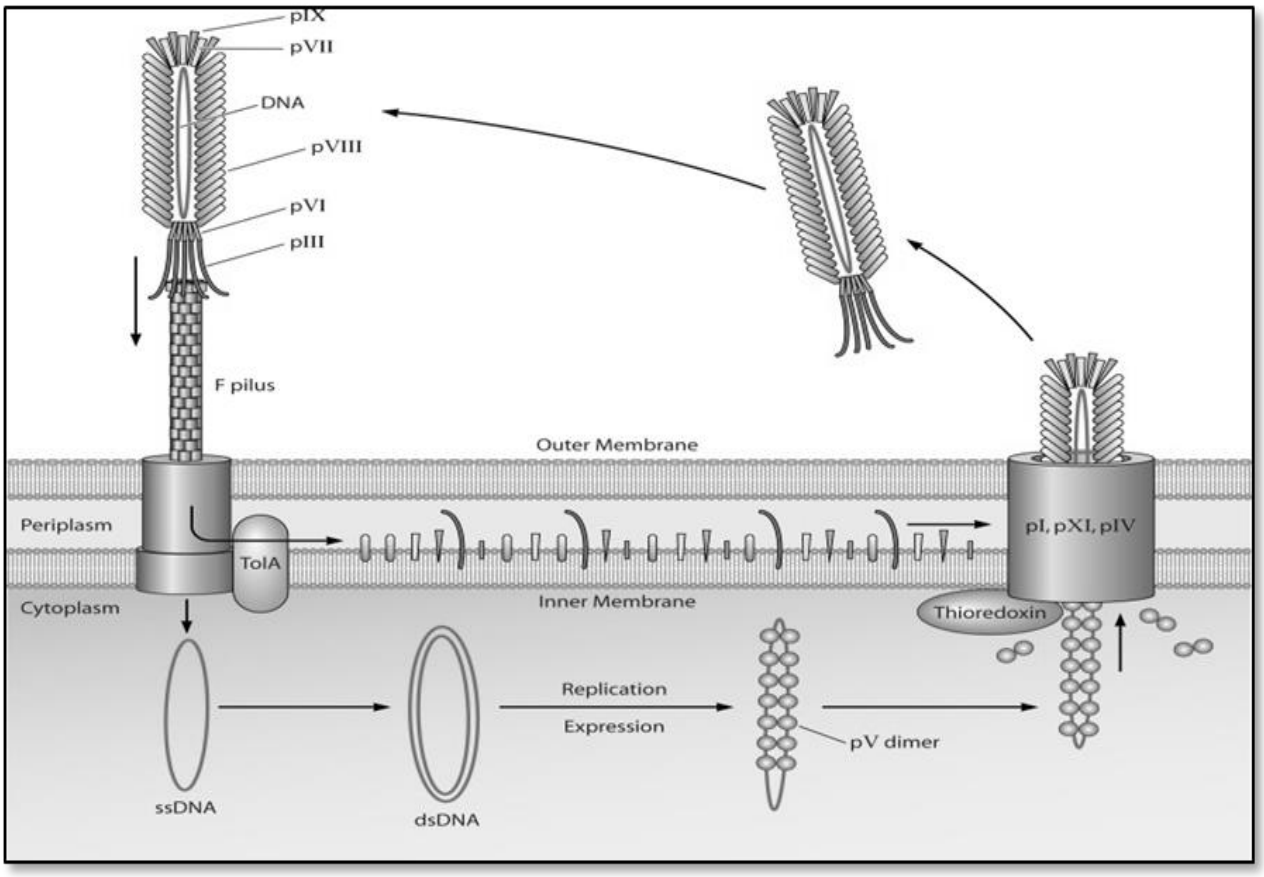


Figure 1.2: Infection cycle of the filamentous phage.

1.7.2. Classification of phage display libraries

Display systems are generally grouped according to the type of vector used for production of virions. Three types of phage display libraries are recognised, based on the method of fusion library construction illustrated in Fig. 1.3 (Smith and Petrenko, 1997; Huang *et al.*, 2012):

1. Foreign DNA inserted into the natural filamentous phage genome, resulting in the display of the fusion gene product on all coat proteins. In this type of library a phage genome carries a single coat protein gene to which fusions are made, resulting in display of foreign peptides on all copies of the specific coat protein.
2. A plasmid vector derived from the phage genome, termed a phagemid, containing both viral and bacterial origins of replication, an antibiotic resistance gene and a fusion gene with a weak promoter. Two coat protein genes are thus located on two separate genomes: a wild-type gene on the phage genome (a "helper" phage) and a fusion gene on the phagemid.
3. A hybrid system, utilising a phage genome containing a wild-type coat protein in addition to a fusion coat protein. The coat of the resulting virion contains both wild-type proteins and coat proteins fused to a foreign protein.

Smith and Petrenko (1997) formulated the terms "3", "3+3" and "33" to distinguish between the types of libraries, where the number indicates the coat protein to which the foreign peptide is fused, visually explained in Fig. 1.3. Peptide fusions have been created with proteins pIII, pVI, pVII (Løset *et al.*, 2011a), pVIII and pIX (Løset *et al.*, 2011b).

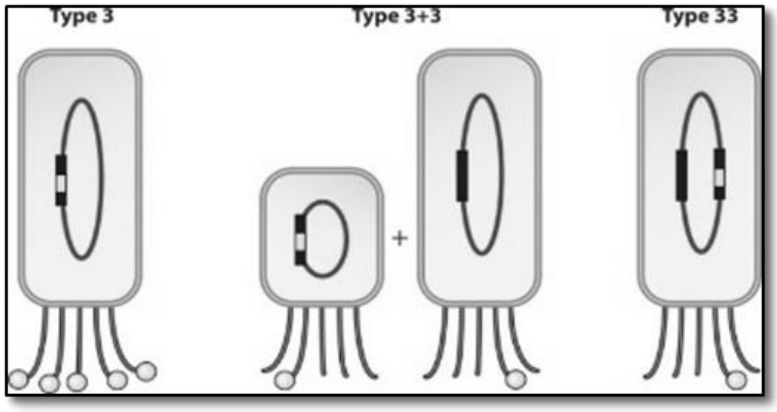


Figure 1.3: A graphic representation of the three types of phage display libraries. Black boxes represent pIII genes and colourless boxes represent foreign genes fused to the coat protein gene. Circles show foreign proteins fused to the N-termini of pIII (Huang *et al.,* 2012)

1.7.3. Affinity selection

During this iterative process (also termed biopanning), phage-displayed peptides with affinity for an immobilised target molecule are enriched and selected. Soluble targets are typically immobilised on a solid surface, such as polystyrene microtitre plates, or may be biotinylated and captured on a streptavidin-coated plate or streptavidin-agarose beads (Huang *et al.,* 2012). The

basic steps of an affinity selection experiment are graphically represented in Fig. 1.4. Selection of target unrelated peptides (TUPSs), capable of binding materials and reagents used in the affinity selection experiment, can be avoided by negative screening steps (Menendez and Scott, 2005) or can be excluded using the web application, Scanner And Reporter Of Target Unrelated Peptides (SAROTUP), that identifies TUP motifs within input peptide sequences. Target unrelated peptides include motifs capable of binding capturing agents, constant regions of antibodies, solid phase, contaminants in the target sample (Huang *et al.*, 2010) and advantageous mutations in the phage library (Brammer *et al.*, 2008).

While general-purpose tools for sequence alignment, local alignment and pattern search are undoubtedly popular and convenient in the study of protein interaction, certain limitations require mentioning. These tools are often unfit for aligning short input sequences (<15 aa) such as those identified by affinity selection experiments to longer sequences of templates. They are also not capable of identifying non-linear binding sites, i.e. conformational binding sites composed of residues brought into proximity by tertiary structures within the template (Huang *et al.*, 2012). Specialised analysis tools, such as FINDMAP, presents a novel method to align non-linear binding sites to discontinuous regions of the primary amino acid sequence of the target protein (Mumey *et al.*, 2003).

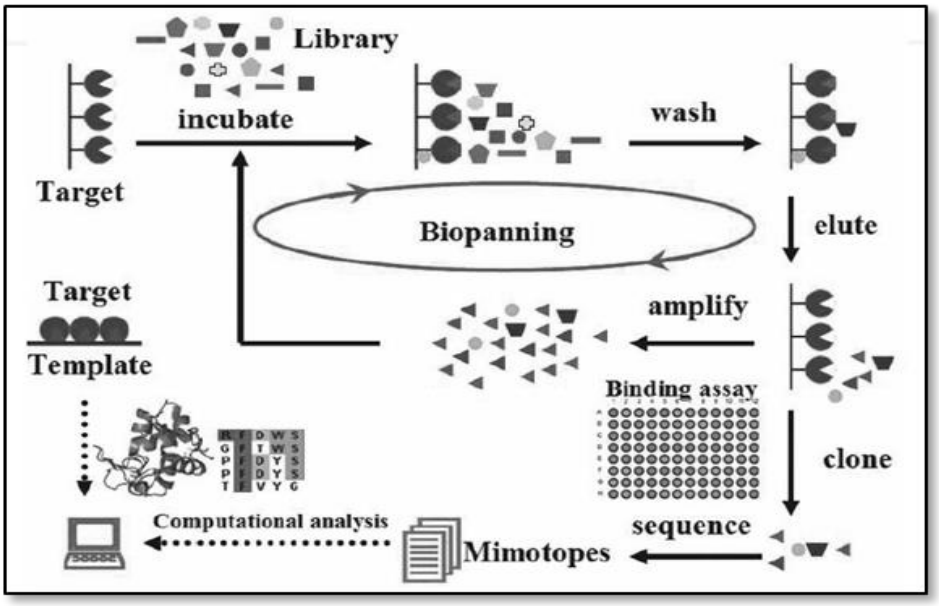


Figure 1.4: Phages expressing peptides with affinity to a defined target are obtained by consecutive rounds of affinity selection. Sequences are obtained and analysed by various computational methods, either based on template (natural ligand of the target molecule).

1.8. CONCLUSION

Lactococcosis is a globally occurring disease of cultured fish caused by a Gram-positive bacterium *Lactococcus garvieae*. In addition to causing monetary losses to the ever-growing aquaculture industry globally, it is considered an emerging zoonotic agent placing immunocompromised individuals at risk. Application of chemotherapeutic agents is effective under experimental conditions, but is ultimately an unsustainable strategy in the control of lactococcosis due to the development and spread of antibiotic resistance. Many reports concerning high levels of multiple resistance to frequently used antibiotics (macrolides, lincomycin and tetracycline) exist, creating the need to investigate alternatives for disease prevention and control. Preventative measures in the form of biosecurity and vaccination could therefore be investigated as alternatives to antibiotic dependency. The possible application of new generation vaccines for the prevention of lactococcosis is yet to be fully investigated as well.

Although putative adhesion factors have been identified in a clinical *L. garvieae* isolate, this integral step in the infection process has not been further elucidated. Similar to other Gram-positive pathogens, the polysaccharide capsule of *L. garvieae* is a major virulence factor involved mainly in the evasion of the host immune response. Despite this integral role of the polysaccharide capsule in pathogenesis, none has been structurally characterized. Serological differing, capsulated, geographically distinct isolates have been shown to possess differences in capsular carbohydrate composition, suggesting that EPS structure may form the basis of serological variability as observed in various Gram-positive pathogens. Understanding of the underlying genetic basis of variability in EPS structure and its likely interrelationship with serological variability in *L. garvieae* is lacking presently. Recent findings suggest that the EPS capsule is not the sole determinant of pathogenicity but that other various virulence factors are involved in the pathogenesis of *L. garvieae.* Lack of knowledge of virulence factors, pathogenesis and serology of *L. garvieae* is an impediment to the development of effective typing methods and control measures.

The aims of this study are:

- Detection and identification of putative virulence factors of South African *L. garvieae* isolates from rainbow trout
- Investigation of the role of GAPDH in *L. garvieae* pathogenesis

The aims will be fulfilled by the following objectives:

- Identify putative virulence factor genes of South African *L. garvieae* isolates by PCR and sequencing
- Identify extracellular proteins produced by South African *L. garvieae* isolates
- Clone, express and purify *L. garvieae* GAPDH
- Identify putative ligands to recombinant GAPDH using a commercially available random peptide phage display library

2. Detection of virulence factors of South African *Lactococcus garvieae* isolates

2.1 INTRODUCTION

The polysaccharide composition of capsules is highly polymorphic among strains of pathogenic streptococcal species, manifesting as a high degree of serological diversity typically observed within streptococci (Millard *et al.*, 2012). Two main serotypes of *L. garvieae* are recognised at present: capsulated (KG-) and non-capsulated (KG+).

Barnes and Ellis (2004) presented the first indication of capsular heterogeneity among *L. garvieae* isolates. The authors studied serological cross-reactivity in European and Japanese isolates and determined that anti-KG- serum, regardless of geographical origin, cross-reacted with KG+ isolates but did not cross-react with any capsulated isolates. It was concluded that cross-reaction between the two serotypes is due to the presence of common cell wall antigens, since the two serotypes are known to have identical protein cell wall components (Barnes *et al.*, 2002b). Antisera raised against KG+ isolates did not react with any of the KG- isolates. These observations suggest concealment of cell wall antigens by the capsule and that capsular variation is, at least partially, implicated in serological diversity observed between European and Japanese isolates. The results of this study led to the establishment of three distinct serotypes; a Japanese capsulated serotype, a European capsulated serotype, and a non-capsulated serotype from both regions (Barnes and Ellis, 2004). An unrelated study further suggested the existence of two European serotypes: serotype I (Italian and Israeli isolates) and serotype II (Spanish, Greek and Bulgarian isolates) (Eyngor *et al.*, 2004). No further reports on capsular variation or the genetic basis thereof in *L. garvieae* exist at present; although considering its widespread occurrence, existence of serological diversity is highly probable.

Capsular serotypic diversity, as a mechanism of immune evasion, is believed to be the result of selective pressure imposed by the host's adaptive immune system (Bentley *et al.*, 2006). In a long term study by Millard and co-workers (2012), comparison of the capsular biosynthesis operon between *Streptococcus iniae* autogenous vaccine isolates and isolates from fish that became infected

following vaccination revealed mutations in a limited amount of capsular operon genes. Thus, it was observed that vaccination imposes selective pressure on the species resulting in the emergence of novel serotypes not recognised by the specific immune response elicited by the original vaccine, leading to increased mortalities (Millard *et al.*, 2012). Knowledge concerning serological diversity and the underlying genetic mechanisms will therefore prove invaluable to the development of effective vaccines and accurate serological methods for detection and sero-surveillance.

This chapter aims to investigate the prevalence of various putative virulence factors in South African *L. garvieae* isolates and to speculate on their role in the process of infection in order to further elucidate the pathogenesis of lactococcosis in rainbow trout, and identify possible targets for recombinant vaccine development.

The objectives of this chapter are to:

- Identify South African *L. garvieae* isolates using molecular techniques;
- Characterise exopolysaccharides produced by South African *L. garvieae* isolates at both the genotypic and phenotypic levels by sequencing capsular biosynthesis genes and examination by light microscopy respectively;
- Detect and identify extracellular virulence factors of South African *L. garvieae* isolates, and
- Detect and identify previously identified virulence factor genes in South African *L. garvieae* isolates by PCR assays and Sanger sequencing.

2.2 MATERIALS AND METHODS

2.2.1 Isolates used in this study

Cultures used in this study were obtained from Bragg and Broere (1986) during whose study these bacteria were isolated from diseased rainbow trout, *Oncorhynchus mykiss* (Walbaum), from the former Eastern Transvaal (currently Mpumalanga area) and Johannesburg, Gauteng areas. Symptoms observed in diseased rainbow trout included extreme exophthalmos and rupture of one or both eyes, haemorrhaging of the ocular chamber, melanosis, enlargement of the spleen and haemorrhaging of intestines (Bragg and Broere, 1986). Geographic origins and isolate numbers (A1-12), which will hereafter be used to refer to isolates, are disclosed in Table 2.1. *Pseudomonas aeruginosa* and a KG+ reference strain, *L. garvieae* NCFB 657, were included as positive and negative controls respectively in the phenotypic characterisation of exopolysaccharides. Freeze

dried isolates (A1-12) were revived in BHI broth (Merck, 1.10493) and were incubated under aerobic conditions at 30°C (A1-3, A5, A6, A11, A12) for 48 h or anaerobic conditions at 37°C (A4, A7-10) for 48 h using an anaerobic jar with a gas generating kit (Oxoid BR0038). Culture purity was confirmed by Gram-staining. Revived isolates were stored in commercially available cryogenic vials (Microbank™, Pro-Lab Diagnostics) at -20°C.

Table 2.1: Isolate numbers and geographic origins of isolates used in the current study.

Isolate code	UFS number	Isolate number	Geographic origin
88-3425-14 strep 1	UFSBC574	A1	TPA Lydenburg, Mpumalanga
88-1039-1 vict	UFSBC575	A2	Farm near Lydenburg, Mpumalanga
88/1036 strep1	UFSBC576	A3	Farm near Lydenburg, Mpumalanga
F1/88 B	UFSBC577	A4	Malony's eye – Johannesburg, Gauteng
88-598 fish strep	UFSBC578	A5	G. Coetzee – Lydenburg
F21/86	UFSBC579	A6	Lunsklip Fisheries – Lydenburg, Mpumalanga
GC	UFSBC580	A7	Exact location unknown
F2/88	UFSBC581	A8	Exact location unknown
8002	UFSBC582	A9	Exact location unknown
VDM	UFSBC583	A10	Lunsklip Fisheries – Lydenburg, Mpumalanga
Crab int	UFSBC548	A11	Freshwater crab – Malony's eye, Gauteng
45/87	UFSBC547	A12	Pleasant Ways Trout Farm - Dullstroom, Mpumalanga

2.2.2. Identification of isolates by 16S rDNA sequencing

2.2.2.1. Cultivation and DNA extraction

Tryptic soy broth (Merck, 1.05459) was inoculated with isolates A1-12 by transferring three beads directly from stored Microbank™ vials into 5 mL broth. After incubation under aerobic conditions at either 30°C or 37°C for 18 h with no agitation, DNA was extracted according to the method described by Thanh (2006), with slight modifications. Briefly, 1 mL culture was transferred

to a microcentrifuge tube and cells were harvested by centrifugation at 20 000 x*g* for 2 min (Eppendorf® Centrifuge 5417R). Cells were resuspended in 1 mL 2x SSC buffer (Table A.1, Appendix I) and incubated in a boiling water bath for 10 min. Following another centrifugation step at 20 000 x*g* for 2 min, the pellet was resuspended in 1 mL dH_2O. One millilitre chloroform, 200 μL Milli-Q® H_2O and 200 μL glass beads (0.2–0.5 mm in diameter) were added and tubes were vortexed for 10 min followed by centrifugation at 10 000 x*g* for 10 min. The top layer was transferred to clean microcentrifuge tubes and stored at -20 °C until subsequent analyses.

2.2.2.2. Amplification of 16S rDNA by PCR

Universal sequence primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1525R (5'-AAGGAGGTGATCCAGCC-3') were used to amplify a 1 517 bp sequence of the 16S rDNA gene by PCR (Beumer and Robinson, 2005). Each 50 μL PCR mixture contained the following reagents at final concentrations parenthesised: dNTPs (0.2 mM), ThermoPol reaction buffer (1x) (New England BioLabs #B9004S), *Taq* DNA polymerase (1.25 U) (New England BioLabs #M0267S), forward and reverse primers (0.2 μM) (Integrated DNA Technologies), $MgCl_2$ (0.5 mM), genomic DNA (1 ng.μL^{-1}) and Milli-Q® H_2O up to a final reaction volume of 50 μL. Reactions were performed in a Vacutec® G-Storm™ Thermocycler under the following conditions: initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 53°C for 30 s, elonagation at 72°C for 90 s and a final elongation step at 72°C for 2 min. Amplicons were visualised by gel electrophoresis (Sambrook and Russell, 2001). An agarose gel (1% w/v) (SeaKem® LE Agarose, Lonza) containing ethidium bromide (10 mg.mL^{-1}) was prepared. Each sample (2 μL) of the PCR amplicon was mixed with 6x loading dye solution (Fermentas #R0611) before loading. Electrophoresis was performed in TAE buffer at a constant voltage of 90 $V.cm^{-1}$ for 30 min followed by visualisation under UV light in a Bio-Rad Gel Doc™ EZ Imager system. A commercial DNA size marker, O'GeneRuler™ DNA Ladder Mix (Fermentas #SM1173), was included for reference. For purposes of purification of the PCR product, a 2% (w/v) low-melt agarose (NuSieve® GTG® Agarose) gel was made and the entire PCR reaction (~48 μL) was mixed with 6x loading dye before loading. Samples were electrophoresed for 45 min at constant voltage of 70 $V.cm^{-1}$. Bands corresponding to the expected product size were excised with a sterile razor blade under UV illumination. The PCR product was purified from the gel slice using a Wizard® SV Gel and PCR Clean-up System (Promega, USA) according to manufacturer's instructions. This purification step was included to remove enzyme, unincorporated dNTPs and primers from the reaction mixture that could interfere with downstream sequencing procedures.

2.2.2.3. Sanger sequencing

Sequencing reactions were prepared using the Applied Biosystems™ BigDye® terminator v.3.1 sequencing kit. Each reaction consisted of 1 μL premix, 2 μL buffer, 1 μL of either 8F or 1525R universal sequence primers (3.2 pmol.μL^{-1}), 10-40 ng purified 16S PCR product and sufficient Milli-Q® water up to a final volume of 10 μL. Reactions were performed in a Vacutec® G-Storm™ Thermocycler under the following conditions: initial denaturation at 96°C for 1 min, followed by 25 cycles of denaturation at 96°C for 10 s, annealing at 50°C for 5 s and elongation at 60°C for 4 min. Post-reaction cleanup was performed to remove unincorporated primers and dye-labelled nucleotides using EDTA/ethanol precipitation, as recommended by the manufacturer. The sequencing reaction volume was adjusted to 20 μL with Milli-Q® water and transferred to a 1.5 mL microcentrifuge tube containing 5 μL 125 mM EDTA and 60 μL absolute ethanol. After vortexing for 5 s and precipitating at room temperature for 15 min, the tubes were centrifuged at 4°C for 10 min at 20 000 x *g* (Eppendorf® Centrifuge 5417 R). While ensuring not to disturb the pellet, the supernatant was completely aspirated. Samples were dried in a Concentrator Plus (Eppendorf®) vacuum concentrator for 5 min at 45 °C. Samples were submitted for Sanger sequencing at an in-house facility at the University of the Free State. Sequence data was viewed using Geneious version 9 (http://www.geneious.com, Kearse *et al*., 2012) and sequences were used to compare against the NCBI database sequences using the nucleotide-nucleotide BLAST (blastn) algorithm (Altschul *et al.*, 1990).

2.2.3. Phenotypic characterisation of exopolysaccharides

2.2.3.1. Light microscopy

Negative staining with nigrosine was performed on isolates A1-12 to determine the presence of exopolysaccharide capsules. A drop of nigrosine was placed on a clean microscope slide and 5 μL of culture (cultured as described in Section 2.2.2.1) was suspended therein. The suspension was smeared over the slide and allowed to air-dry before examination by light microscopy. Encapsulated *Pseudomonas aeruginosa* and a non-capsulated (KG+) reference strain of *L. garvieae,* NCFB 657, were included as positive and negative controls, respectively.

2.2.4. Genotypic characterisation of exopolysaccharides

2.2.4.1. Amplification of *L. garvieae* capsule gene cluster by long range PCR

The *L. garvieae* exopolysaccharide synthesis gene cluster, as described for *L. garvieae* Lg2 (Miyauchi *et al.*, 2012), was amplified in isolates A1-3, 5, 6, 11 and 12 using the TaKaRa LA PCR Kit (TaKaRa Bio Inc. #RR002A). Primers LgC-F (5'-TGCTGTCATCATATTGTGTCCA-3') and LgC-R (5'-

GGCTATGGCATTAGTCAGGAAG-3'), as described by Miyauchi and co-workers (2012), were obtained from Integrated DNA Technologies. Each reaction contained dNTPs (0.4 mM), LA PCR Buffer II (1x), TaKaRa LA *Taq*® DNA Polymerase (0.05 U.μL^{-1}), forward and reverse primers LgC-F and LgC-R (0.5 μM) (Integrated DNA Technologies), $MgCl_2$ (2.5 mM), genomic DNA (1 ng.μL^{-1}) and Milli-Q® H_2O up to a final volume of 25 μL. Reactions were performed in a Vacutec® G-Storm™ Thermocycler under the following conditions: initial denaturation at 95°C for 3 min followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 62°C for 30 s, elongation at 68°C for 5 min and a final elongation step at 72°C for 10 min. Products were visualised by gel electrophoresis as described in section 2.2.2.2. Primers for the amplification of the transcriptional regulator gene (*epsR*) within the EPS synthesis gene cluster were designed using Geneious version 9 (http://www.geneious.com, Kearse *et al*., 2012) and analysed using an online tool, Integrated DNA Technologies OligoAnalyzer 3.1 (http://eu.idtdna.com/calc/analyzer). Conditions for the amplification of *epsR* were optimised using pooled DNA of all *L. garvieae* isolates used in this study. Annealing temperatures between 46.7°C and 59.4°C and Mg^{2+} concentrations of 2-4 mM were tested under the following conditions: initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 46.7 – 59.4°C for 35 s, elongation at 64°C and final elongation at 64°C for 3 min. Each reaction contained dNTPs (0.2 mM), ThermoPol reaction buffer (1x) (New England BioLabs #B9004S), *Taq* DNA polymerase (1.25 U) (New England BioLabs #M0267S), forward and reverse primers (0.2 μM) (Integrated DNA Technologies), $MgCl_2$ (2-4 mM), genomic DNA and Milli-Q® H_2O up to a final reaction volume of 25 μL. Amplification products were visualised on a 1% (w/v) agarose gel as described in Section 2.2.2.2.

2.2.5. Detection of putative virulence factor genes by PCR

Full genome sequencing of *L. garvieae* strains ATCC 49156® and Lg2 strains by Morita and colleagues (2011) identified several genes encoding putative virulence factors. These gene products showed significant similarity to virulence factors of related species, as summarised in Table 2.2. In this study, the presence of some of these genes in South African isolates were investigated by PCR using primers designed by Türe and Altinok (2016). The presence of the following genes encoding candidate virulence factors in *L. garvieae* strains A1-3, A5, A6, A11 and A12 were investigated: haemolysins 1, 2, and 3 (*hly*1, -2, -3), NADH oxidase (*nox*), superoxide dismutase (*sod*), pneumococcal adherence and virulence factor A (*pavA*) and pneumococcal surface adhesin A (*psaA*). Oligonucleotide sequences, expected product sizes and annealing temperatures of primers are tabulated in Table 2.3. In order to obtain DNA template, DNA was extracted as described in

Section 2.2.2.1. Reactions were performed in a Vacutec® G-Storm™ Thermocycler under the following conditions: initial denaturation at 95°C for 30 s followed by 35 cycles of denaturation at 95°C for 30 s, annealing at primer-specific temperatures for 30 s, elongation at 68°C for 2 min and a final elongation step at 68°C for 5 min. Products were visualised on a 1% agarose gel as described in Section 2.2.2.2. Products were purified using a Wizard® SV Gel and PCR Clean-up System (Promega, USA) according to manufacturer's instructions. A sequencing PCR was performed using the Applied Biosystems™ BigDye® terminator v.3.1 sequencing kit and a post-reaction clean-up step was included as described in Section 2.2.2.3. Obtained sequences were viewed and edited with Geneious 9 (Kearse *et al.*, 2012) and used to query the NCBI database using the blastn algorithm (Altschul *et al.*, 1990).

Table 2.2: Genetic loci and predicted gene products of candidate virulence genes, previously identified in *L. garvieae* Lg2 (Morita *et al*. 2011), under investigation in this study.

Locus	Predicted gene product	Identity (%)	Species	Accession number	Features
LCGL_0323	Haemolysin	56	*Enterococcus faecalis*	AAO81463	Motif (PF03006) conserved in proteins with haemolytic activity
LCGL_0597	Haemolysin	72	*Streptococcus suis*	EEF64743	-
LCGL_0374	Haemolysin	59	*S. pyogenes*	AAK33420	Cleavable N-terminal signal sequence
LCGL_0664	NADH oxidase (SP1469)	51	*S. pneumoniae* TIGR4		-
LCGL_0285	Superoxide dismutase (SP0766)	68	*S. pneumoniae* TIGR4		-
LCGL_1330	PavA (SP0966)	62	*S. pneumoniae* TIGR4		Fibronectin-binding motif (PF05833)
LCGL_1533	PsaA (SP1650)	49	*S. pneumoniae* TIGR4		-

Table 2.3: Primer sequences, target genes, product sizes and annealing temperatures of primers used to amplify putative *L. garvieae* virulence genes (Türe and Altinok, 2016).

Primer	Sequence (5'-3')	Target gene	Annealing temperature(°C)	Product size (bp)
H1-F	CCTCCTCCGACTAGGAACCA	Haemolysin 1 (*hly1*)	54	521
H1-R	GAAAAGCCAGCTTCTCGTGC			
H2-F	TCTCGTGCACACCGATGAAA	Haemolysin 2 (*hly2*)	53	492
H2-R	TGAACTTCGGCTTCTGCGAT			
H3-F	AACGCGAGAACAGGCAAAAC	Haemolysin 3 (*hly3*)	56	291
H3-R	CCCACGTCGAGAGCATAGAC			
NADHO-F	TGCGATGGGTTCAAGACCAA	NADH oxidase (*nox*)	53	331
NADHO-R	GCCTTTAAAAGCCTCGGCAG			
SOD-F	GCAGCGATTGAAAAACACCCA	Superoxide dismutase (*sod*)	54	80
SOD-R	TCTTCTGGCAAACGGTCCAA			
AP-F	CCTGTCGGGCGCTTTTATTG	Adhesin Pav (*pavA*)	56	232
AP-R	TCCCGGAAGAAGAGTACGGT			
APSA-F	GTTGCAACAGCTGGACACAG	Adhesin PsaA (*psaA*)	54	180
APSA-R	ATACGGTTGAGTTGGGCTGG			

2.2.6. Detection of extracellular virulence factors

Lactococcus garvieae strains A1-A3, A5, A6, A11, A12 and NCFB657 were cultivated in TSB medium for 24 h at RT. A negative control, consisting of sterile TSB, was included. Cells were pelleted by centrifugation at 20 000 x*g* for 2 min, and the supernatants filtered with a 0.2 μm syringe filter unit to remove residual cellular components. Amicon® Ultra-4 10K Centrifugal Filter Units (Merck) with a Molecular Weight Cut-off (MWCO) of 10 kDa were used to concentrate extracellular proteins. A volume of 4 mL filtered supernatant was applied to the centrifugal filter and the unit was centrifuged at 7 197 x *g* for 20 min. The eluate was discarded, the centrifugal filter was inverted inside a 50 mL Falcon tube and again centrifuged at 7 197 x*g* for 20 min in order to collect the concentrated protein fraction.

2.2.6.1. Visualisation of extracellular proteins

Extracellular proteins, concentrated by Amicon® Ultra-4 10K Centrifugal Filter Units (Merck), were visualised by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Samples were prepared for electrophoresis by adding 5 μL 4x Laemmli sample buffer and 1 μL β-mercaptoethanol to 14 μL of sample. The samples were boiled for 25 min at 95 °C. A 12% SDS-

PAGE gel was prepared with the following reagents at final concentrations parenthesised: 1.4 mL polyacrylamide (12%), 1.19 mL dH_2O, 875 μL 1.5 M Tris pH 8.8 +0.4% SDS (0.375 M and 0.1%), 30 μL 10% APS (0.085%) and 6.5 μL TEMED (0.002%). A 4.2% stacking gel was prepared with the following reagents at final concentrations parenthesised: 280 μL polyacrylamide (4.2%), 1.2 mL dH_2O, 500 μL 0.5 M Tris pH 6.8 +0.4% SDS (0.125 M and 0.1%), 10 μL 10% APS (0.05%) and 8 μL TEMED (0.004%). For a molecular weight reference, 5 μL of Precision Plus Protein™ All Blue Standard (Bio-Rad) was included. Electrophoresis was performed in a Mini-PROTEAN® Tetra System (Bio-Rad) at 120 $V.cm^{-1}$ for 80 min. The polyacrylamide gel was stained and destained using the rapid Fairbanks Coomassie Blue protein staining and destaining method (Wong *et al.*, 2000), based on the original Fairbanks technique developed by Fairbanks and colleagues (Fairbanks, Steck and Wallach, 1971). Following electrophoresis, the gel was placed in a container suitable for microwaving, rinsed with dH_2O and covered in Fairbanks A solution (Table A.3, Appendix I). The solution containing the gel was heated in a conventional microwave oven at full power for approximately 2 min until boiling of the solution was observed. The Fairbanks A solution was discarded and the gel rinsed with dH_2O. Lastly, destaining solution, Fairbanks D (Table A.3, Appendix I), was used to cover the gel and the solution was again brought to boiling point in a microwave. In order to obtain a clear background, the gel was left overnight in the Fairbanks D solution on a Stuart® Mini Orbital Shaker SSM1 with gentle shaking (30 rpm). A Kim-wipe was immersed in the solution to absorb excess Coomassie Blue stain. The gel was viewed with a Gel Doc™ EZ Viewer (Bio-Rad).

2.2.6.2. Protein identification

2.2.6.2.1. In-gel digest of proteins

To prepare proteins for identification with mass spectrometry (MS), bands were excised from the polyacrylamide gel, cut into cubes (± 1 mm^3) and transferred to a clean 1.5 mL tube. Gel pieces were washed with 100 μL dH_2O for 15 min, followed by washing with 100 μL 50% acetonitrile (ACN) for 15 min. The washing step was repeated. In order to shrink gel pieces, 50 μL 100% ACN was added. Shrunken gel pieces became white and stuck together where after ACN was removed. Reduction and alkylation was performed by swelling gel particles in 50 μL dithiothreitol (DTT) solution with incubation at 56°C for 45 min. Tubes were chilled to RT and the DTT solution was replaced with 50 μL iodoacetamide solution, followed by incubation at RT in the dark for 30 min. Iodoacetamide solution was removed and the washing step described above was again performed twice. At this stage, all Coomassie Blue stain was removed and gel pieces appeared transparent. For trypsin digestion of proteins, gel particles were rehydrated in digestion solution (Table A.3,

Appendix I) on ice and then incubated at 37°C overnight. Peptides were extracted from the gel particles by transferring the digest solution from the previous step to a clean tube. A volume of 20 μL 5% formic acid was added to the gel particles, vortexed for 10 s and incubated for 15 min at RT. Before vortexing and incubating for 15 min at RT again, 20 μL 100% ACN was added. After centrifugation at maximum speed (20 000 *x*g), the supernatant was removed and added to the digest solution removed previously. This process, from addition of formic acid to gel pieces to centrifugation, was repeated and the supernatant was combined with the supernatant obtained above. The solution was dried in a SpeedyVac (Eppendorf®) for 60 min. Samples were submitted to the Facility for Genomics and Proteomics at the University of the Free State for identification by nano-scale liquid chromatography coupled to tandem MS (Nano LC-MS/MS) and by querying the SWISS-PROT annotated protein sequence database (Bateman *et al.*, 2015) using the Mascot search engine that uses a probability-based scoring algorithm based on the molecular weight search (MOWSE) algorithm (Perkins *et al.*, 1999).

2.3 RESULTS

2.3.1 Identification of isolates

Amplification of the 16S rDNA gene fragment by PCR yielded the expected ±1 500 bp product, visualised by gel electrophoresis in Fig. 2.1. Sequencing of the fragments and a nucleotide BLAST search was used to identify the strains. The search results, tabulated in Table 2.4, identified strains A1-3, A6, A11 and A12 as *L. garvieae,* A4 as *Enterococcus faecalis*, A5 as *Lactococcus* sp., A7 as *E. durans* and A8-10 as *E. faecium*. These results are consistent with the results of Bekker and colleagues (2011), who also identified 5 *Enterococcus* spp. isolates in this set of 12 strains using 16S rDNA sequencing (Bekker *et al.*, 2011). Strains A4 and A7-10, identified *Enterococcus* spp., were excluded from further experiments in this study.

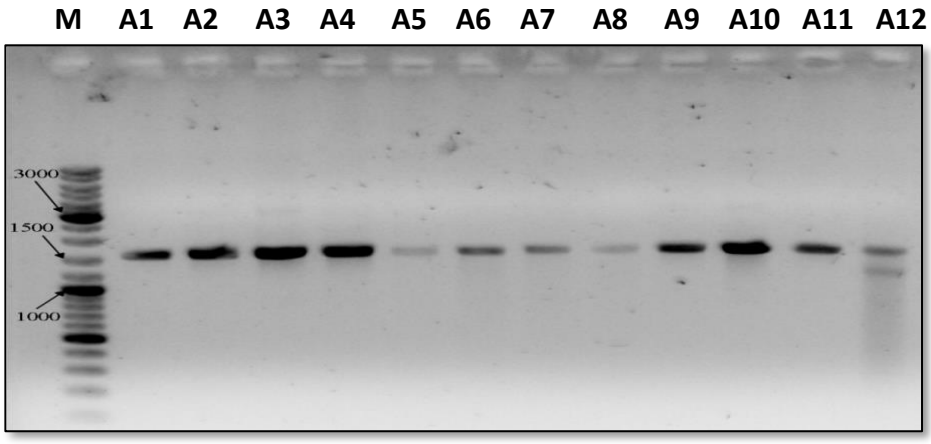


Figure 2.1: Visualisation of 16S PCR products of strains A1-12 on a 1% (w/v) agarose gel. Bands in the expected size range (±1500 bp) were observed. Fragment sizes are indicated in bp. M = marker.

2.3.2. Phenotypic characterisation of exopolysaccharides

2.3.2.1. Negative staining

Nigrosin is an acidic dye that is repelled by positively charged cell capsules, resulting in the appearance of capsules of clear halos when viewed under light microscopy. Strains A1-12 were stained with nigrosin and viewed with a light microscope using 100x oil immersion lens. As shown in Fig. 2.2, no halos were observed in any of the strains including the negative control NCFB657, in comparison with the positive control *P. aeruginosa*, where a clear halo is observed indicating the presence of a polysaccharide capsule.

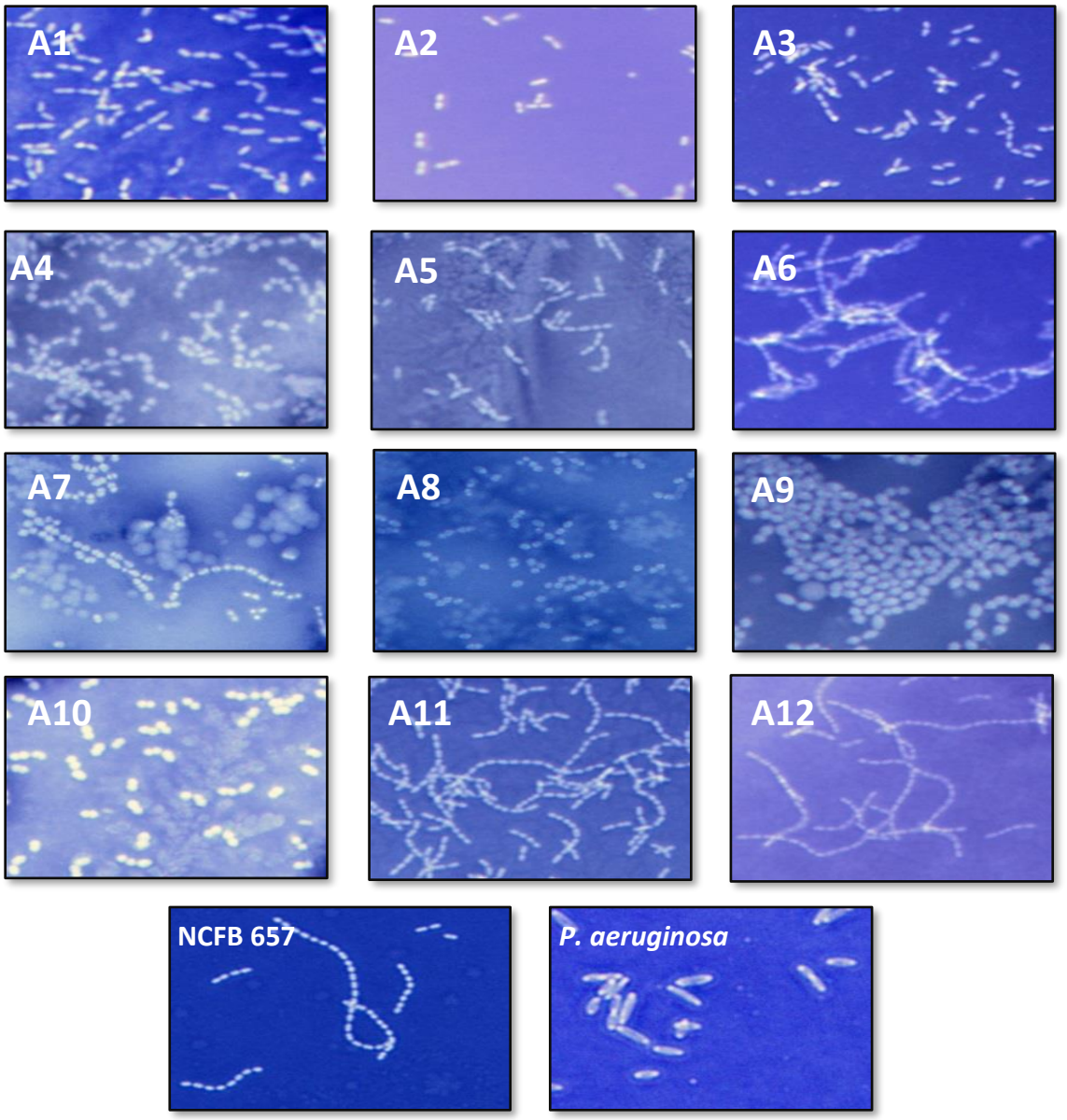


Figure 2.2: Negative staining of strains A1-12 using nigrosin, visualised using 100x magnification. *L. garvieae* NCFB657 and *P. aeruginosa* were included as negative and positive controls, respectively.

Table 2.4: Top BLAST search hits generated for 16S rDNA genes amplified from strains A1-12

Isolate	Nucleotide BLAST Result	Query length	Query cover	E value	Identity	Accession no
A1	*Lactococcus garvieae* strain A4 16S ribosomal RNA gene, partial sequence	619	100%	0.0	100%	KT924257.1
A2	*Lactococcus garvieae* strain A2 16S ribosomal RNA gene, partial sequence	783	100%	0.0	100%	KT924256.1
A3	*Lactococcus garvieae* strain RCB99 16S ribosomal RNA gene, partial sequence	742	100%	0.0	100%	KT260311.1
A4	*Enterococcus faecalis* strain RCB986 16S ribosomal RNA gene, partial sequence	466	100%	0.0	99%	KT261198.1
A5	*Lactococcus* sp. SI-Km(R)-3B 16S ribosomal RNA gene, partial sequence	101	100%	2e-39	97%	KR399992.1
A6	*Lactococcus garvieae* strain RCB131 16S ribosomal RNA gene, partial sequence	80	100%	8e-33	100%	KT260343.1
A7	*Enterococcus durans* strain R08-28 16S ribosomal RNA gene, partial sequence	44	100%	1e-14	100%	JF896442.1
A8	*Enterococcus faecium* strain 133 16S ribosomal RNA gene, partial sequence	166	98%	5e-54	91%	EU418442.1
A9	*Enterococcus faecium* strain SA-10 16S ribosomal RNA gene, partial sequence	574	100%	0.0	100%	KR265372.1
A10	*Enterococcus faecium* strain UW7606x64/3 TC1, complete genome	354	100%	0.0	99%	CP013009.1
A11	*Lactococcus garvieae* strain RCB99 16S ribosomal RNA gene, partial sequenc	405	100%	0.0	100%	KT260311.1
A12	*Lactococcus garvieae* strain RCB130 16S ribosomal RNA gene, partial sequence	96	98%	5e-31	94%	KT260343.1

2.3.3. Genotypic characterisation of exopolysaccharides

2.3.3.2. Amplification of *L. garvieae* EPS biosynthesis gene cluster and transcriptional regulator, *epsR*

The amplification of the EPS biosynthesis gene cluster by long range PCR was attempted as described by Miyauchi and co-workers (2012). Figure 2.3 shows a product of ~750 bp for all strains tested, consistent with the product sizes observed in the KG+ (non-capsulated) strains tested in the study by Miyauchi and colleagues (2012). The presence of insertion sequences flanking the EPS biosynthesis gene cluster in the KG- strain Lg2 used in the study by Miyauchi and colleagues (2012) may suggest that the EPS biosynthesis gene cluster could be inserted at different genomic loci in strains other than Lg2 strain. If this should happen, the primers used by Miyauchi and colleagues (2012) would fail to amplify this gene cluster in other strains since the primer sequences include the insertion sequences flanking the gene cluster. Therefore, primers that amplify the first gene in the cluster, a transcriptional regulator (*epsR*), were designed. However, PCR amplification of this gene yielded no product for any strain tested.

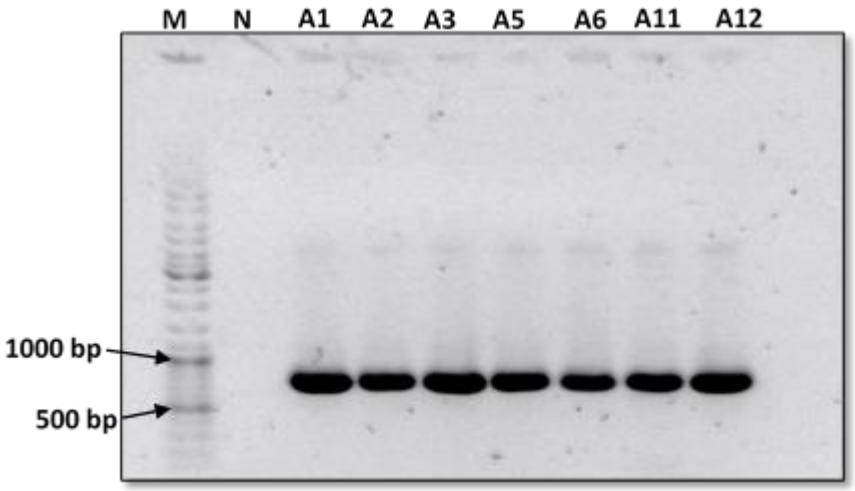


Figure 2.3: Visualisation of LR PCR products on a 1% agarose gel. A ~750 bp band was observed in all isolates tested, indicating the absence of an EPS gene cluster in the genomes of these isolates. M = marker; N = negative control.

2.3.4. Detection of extracellular virulence factors

Extracellular proteins of eight *L. garvieae* strains, purified and concentrated by Amicon® Ultra-4 10K Centrifugal Filter Units (Merck) were visualised on a 12% SDS-PAGE gel. More protein bands were observed in strain A3 in comparison to other strains, as can be seen in Fig.2. 4. No clear bands were observed in the negative control that consisted of sterile TSB medium, indicating that the

growth medium did not contribute any of the protein bands detected in the supernatants. The results of identification of selected bands are shown in Table 2.5. Two bands in the molecular weight range of 50 and 75 kDa respectively were observed in all isolates, including the reference strain NCFB657. In strain A3, the 50 kDa band was identified by nano-LC-MS/MS as enolase and the 75 kDa band as a chaperonin. A 37 kDa band present in all strains was identified as lactate dehydrogenase. This band appeared much fainter in the reference strain. A band present in A2 and A3 of approximately 100 kDa was identified as phosphoenolpyruvate-protein phosphotransferase.

The Mascot search engine employs probability-based scoring to describe the probability that the observed match between experimental data and protein database entries is a random event, expressed as a MOWSE score (Perkins *et al.*, 1999). The match with the lowest probability, with a significance threshold of 0.05 ($p < 0.05$), is reported as the best match. Good matches will inevitably yield small probability (P) values, which are expressed as $-10\text{Log}_{10}(P)$ for the sake of convenience, where P is the probability that the reported match is a random event (Perkins *et al.*, 1999). This implies that the highest MOWSE score is assigned to the best match, while significant matches display scores in the range of 70 (Perkins *et al.*, 1999). Individual ions scores upwards of 92 were obtained (Table 2.5), indicative of identity or extensive homology.

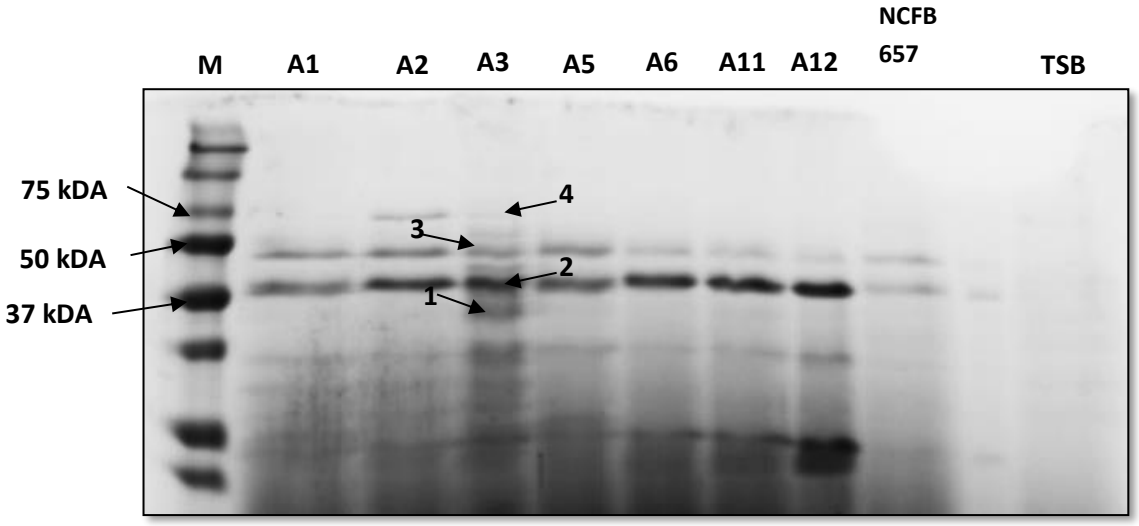


Figure 2.4: Extracellular proteins of eight *L. garvieae* strains, including the reference strain NCFB657, visualised on a 12% SDS-PAGE gel. A negative control (sterile TSB), indicated as TSB, was included. M = molecular weight marker (in kDa).

Table 2.5: Top protein scores generated by query of the SWISS-PROT protein sequence database with the search engine Mascot, following nano LC-MS/MS analysis of extracellular proteins from *L. garvieae* strain A3.

Band nr.	Top protein scores	MOWSE score	MW (kDa)
1	L-lactate dehydrogenase OS=*Lactobacillus helveticus*	174	35,089
2	Enolase OS=*Streptococcus suis* (strain 98HAH33)	234	47,066
3	60 kDa chaperonin OS=*Lactobacillus brevis* (strain ATCC® 367 / JCM 1170)	92	57,044
4	Phosphoenolpyruvate-protein phosphotransferase OS=*Lactococcus lactis* subsp. *lactis* (strain IL1403)	137	62,521

2.3.5. Detection of putative virulence factor genes by PCR

Genes encoding putative virulence factors in the virulent, capsulated strain Lg2 were identified by Morita and co-workers (2011). Primers designed by Türe and Altinok (2016) were used to detect these genes by PCR in South African isolates and an avirulent reference strain, NCFB657. Visualisation of PCR products by gel electrophoresis, as presented in Fig. 2.5, followed by Sanger sequencing and nucleotide database search confirmed the presence of seven candidate virulence genes in genomes of seven South African isolates as well as an avirulent reference strain, NCFB657.

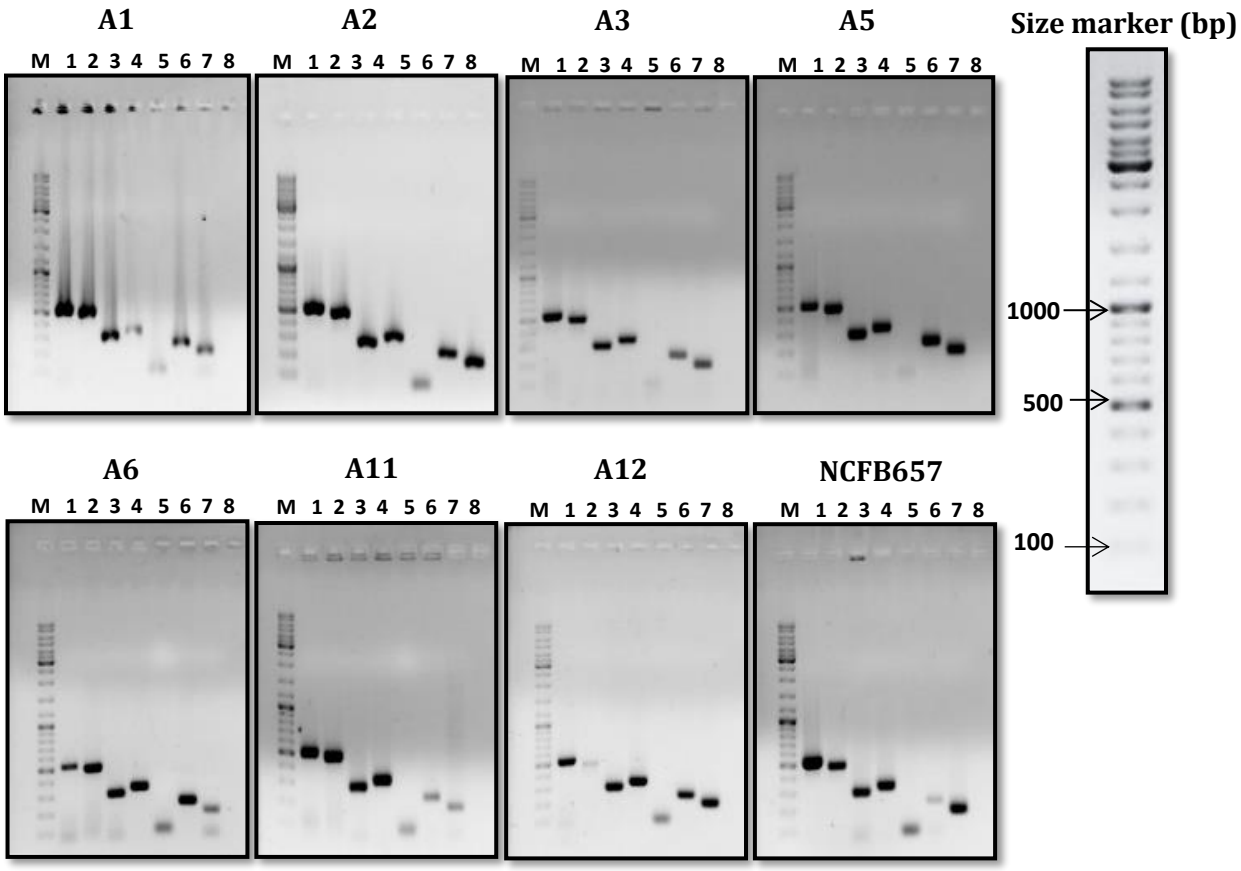


Figure 2.5: Putative virulence factor genes were detected by PCR assays. Products are visualised on 1% agarose gels. The first lanes on all gels are size markers, and the subsequent lanes represent virulence genes in the following order: 1. *hly1* (521 bp); 2. *hly2* (492 bp); 3. *hly3* (291 bp); 4. *nox* (331 bp); 5. *sod* (80 bp); 6. *pavA* (232 bp); 7. *psaA* (180 bp). Lane 8 is a negative control

2.4 DISCUSSION

The identification of isolates A4 and A7-A10 as *Enterococcus* spp. by 16S rDNA sequencing in this study corresponds to the results obtained by Bekker and colleagues (2011), whom identified isolate A4 as *E. faecalis* and isolates A7-A10 as *E. faecium* by 16S rDNA sequencing. Association of *Enterococcus* spp. with infected fish is not often reported. Austin and Austin (2012) reported the isolation of *E. faecalis* subsp. *liquefaciens* from farmed brown bullfish (*Amiurus nebulosus*) displaying symptoms of lactococcosis in Croatia, however the authors did question the accuracy of this identification.

All putative virulence genes under investigation were present in the genomes of all South African isolates used in this study, as well as the reference strain NCFB657. These results are in accordance with the findings of Türe and Altinok (2016), who reported the presence of this set of putative virulence factor genes in the genomes of a total of 34 *L. garvieae* strains, including the avirulent reference strains ATCC® 49156 and ATCC® 4392 and isolates from diseased rainbow trout in Turkey, France, Iran, Spain and Italy. Even though no phenotypic proof of the presence of a polysaccharide capsule was collected in this study using light microscopy, these observations alone should not be considered as definitive proof of the absence of capsules, as Yoshida and colleagues (1997) reported that neither negative staining using Muir's method or Indian ink, nor the Quellung reaction was successful in visualising *L. garvieae* KG- capsules. The authors successfully applied TEM to the visualisation of these capsules, using antiserum for stabilisation of capsules and ruthenium red for staining purposes (Yoshida *et al.*, 1997).

The EPS capsule influences a variety of host interactions, including adhesion, invasion and sensitivity to opsonophagocytosis, and is a well-recognised virulence factor of *L. garvieae.* The *eps* locus of *L. garvieae* Lg2 was reported to be a genomic island, attributed to the presence of insertion sequences IS982 on both ends of the EPS biosynthesis cluster, differences in GC content between the Lg2 chromosomal average and cluster (39% and 31% respectively) and demonstrates that this locus was probably inserted into the locus and is syntenic in the four sequenced *L. lactis* strains (Morita *et al.*, 2011). It is postulated that this gene cluster might have been present in the avirulent non-capsulated reference strain ATCC® 49156, originally isolated from diseased yellowtail, but was lost during repeated subculturing (Morita *et al.*, 2011). The negative results obtained using the PCR amplification of the EPS biosynthesis cluster as described by Miyauchi and co-workers (2012) may reflect a similar phenomenon as the attenuation of ATCC® 49156 by repeated subculturing. The

strains used in this study were isolated from diseased rainbow trout in 1986 and has been repeatedly subcultured under laboratory conditions that may have been selective for the non-capsulated phenotype since no selective pressure in the form of host immune mechanisms is applied. Presence of the capsule confers fitness only when growth occurs under *in vivo* conditions. Therefore, deletion of the EPS biosynthesis cluster and subsequently the loss of the capsulated KG- phenotype is a possible explanation for the results obtained. However, in light of the recent findings by Türe and Altinok (2016), it is possible that the strains investigated in this study were not capsulated at the time of isolation.

Immunoproteomic studies on *L. garvieae* did not identify enolase, 60 kDa chaperonin or phosphoenolpyruvate-protein phosphotransferase as antigens recognised by rabbit antisera (Shin *et al.*, 2007) or olive flounder (*Paralichthys olivaceus*) antisera (Shin *et al.*, 2009). However, L-lactate dehydrogenase was reported as an antigen common to both KG+ and KG- serotypes (Shin *et al.*, 2009). Although enolase, 60 kDa chaperonin and phosphoenolpyruvate-protein phosphotransferase has not been identified as antigens in *L. garvieae*, the identification of these extracellular proteins are of interest as they have been described as moonlighting proteins with involvement in virulence and regulation of virulence genes in Gram-positive bacteria (Bergmann *et al.*, 2001; Henderson and Martin, 2011; Mizrachi Nebenzahl *et al.*, 2016).

Enolase (EC 4.2.1.11) is a glycolytic enzyme responsible for catalysing the conversion of 2-phosphoglycerate to phosphoenolpyruvate in the second last step of glycolysis, and the reverse reaction in gluconeogenesis (Lee *et al.*, 2006). Additionally, this enzyme plays a role in virulence of Gram-positive and Gram-negative bacteria and is either secreted from the cell or displayed on the extracellular surface by mechanisms unclear to date (Pancholi and Fischetti, 1998; Bergmann *et al.*, 2001; Nogueira *et al.*, 2013). Numerous studies on the moonlighting activities of enolase in prokaryotes have highlighted its involvement in binding of host plasmin(ogen), a process of critical importance to invasion of host tissues (Pancholi and Fischetti, 1998; Bergmann *et al.*, 2001; Henderson and Martin, 2011). Plasminogen is the monomeric zymogen of plasmin, a serine protease involved in fibrinolysis and ECM degradation. Binding of host plasmin(-ogen) to its surface thus endows the bacterial cell with protease activity that facilitates invasion and dissemination in the host (Winram and Lottenberg, 1996). Surface associated enolase with plasmin(ogen) binding activity has been reported in human streptococcal pathogens *Streptococcus pyogenes* (Pancholi and Fischetti, 1998)*, S. pneumoniae* (Bergmann *et al.*, 2001) and piscine pathogen *S. iniae* (Kim *et al.*, 2007). Reassociation of extracellular α-enolase to the bacterial cell

surface may present a possible explanation for surface display (Bergmann *et al.*, 2001). A study by Wang and colleagues (2015) confirmed the immunogenicity of *S. iniae* recombinant α-enolase in mice, showing that immunisation with α-enolase elicited a significant increase in specific IgG in comparison with the control group and ultimately protected mice against systemic *S. iniae* infection (Wang *et al.*, 2015). The ability of *S. iniae* α-enolase to confer protection in Nile tilapia (LaFrentz *et al.*, 2011) and turbot *(Scophthalmus maximus)* (Zhang *et al.*, 2014) has been previously suggested.

The prokaryotic 60 kDa chaperonins, also designated Cpn60, heat shock protein 60 or GroEL, is a highly conserved group of proteins that mediate intracellular protein folding to ensure correct functioning (Henderson *et al.*, 2013). In addition to its intracellular function as molecular chaperone, Cpn60 is found on the cell surface of a variety of bacteria where it functions mainly as an adhesion protein (Henderson *et al.*, 2013). An additional moonlighting activity of Cpn60 is intercellular signalling, demonstrated by the ability of *Mycobacterium tuberculosis* Cpn60.2 to stimulate pro-inflammatory cytokine production by human monocytes (Friedland *et al.*, 1993). Among the members of the order Lactobacillales reported to produce extracellular Cpn60 are *Lactobacillus johnsonii* (Bergonzelli *et al.*, 2006), *Lactococcus lactis* (Katakura *et al.*, 2010), *S. agalactiae* (Hughes *et al.*, 2002) and *S. suis* (Wu *et al.*, 2008). Diverse ligands of bacterial cell-surface Cpn60 have been identified, ranging from mucin, lactoferrin, invertase and glycosphingolipids to integrin receptors CD11/CD18, $\alpha_v\beta_3$ and dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (Henderson *et al.*, 2013). Proteomic analysis of *S. iniae* ATCC® 29178 and *L. garvieae* KG9408 (capsulated) by 2-dimensional gel electrophoresis identified Cpn60 in both fish pathogens (Shin *et al.*, 2006), but subsequent immunoproteomic analyses by immunoblotting with rabbit and olive flounder antiserum did not identify this protein as an antigen of either capsulated or non-capsulated *L. garvieae* (Shin *et al.*, 2007, 2009).

Phosphoenolpyruvate (PEP)-protein phosphotransferase (EC 2.7.3.9) is a key enzyme in the bacterial phosphotransferase system (PTS) that is mainly responsible for the detection, transmembrane import and concurrent phosphorylation of monosaccharides, but also participates in regulatory processes concerning carbon, nitrogen and phosphate metabolism through a phosphorylation cascade (Deutscher *et al.*, 2014). In the cytoplasm, PEP-protein phosphotransferase acts as the first enzyme (EI) in the PTS phosphorylation cascade by transferring phosphate from PEP *via* a histidine-containing phosphocarrier protein (HPr) to a histidine residue of the next enzyme (EII) in the cascade sequence (Deutscher *et al.*, 2014). The multifunctionality of *S. pneumoniae* PEP-protein phosphotransferase (PtsA) has been investigated

by demonstrating its presence on the cell surface of *S. pneumoniae* using immunofluorescence techniques and using recombinantly expressed PtsA (rPtsA) to screen a filamentous phage display library (random 12-mer peptide library) to identify peptides capable of inhibiting adhesion of *S. pneumoniae* to human lung adenocarcinoma cells (Mizrachi Nebenzahl *et al.*, 2016). These peptides showed homology to various human ECM proteins, including multimerin I, protocadherin 19 and collagen type VIIα1, suggesting that PtsA acts as an adhesin of *S. pneumonia*e (Mizrachi Nebenzahl *et al.*, 2016). Furthermore, rPtsA was identified as a candidate target for vaccine development by showing that immunisation of mice with rPtsA offered protection against pneumococcal challenge *via* different routes (Mizrachi Nebenzahl *et al.*, 2016).

Pore-forming toxins are secreted by a variety of Gram-negative and –positive pathogenic bacteria to disrupt lipid bilayers of host cells (Iacovache *et al.*, 2008). *Lactococcus garvieae* is known to be α-haemolytic (Kawanishi *et al.*, 2007), referring to its ability to produce an exotoxin that forms pores in membranes of host cells resulting in the passage of vital molecules out of the cell and ultimate loss of viability (Bhakdil and Tranum-Jensen, 1991). Alpha haemolytic activity can be observed as greenish discolouration surrounding growth on blood agar plates. The putative virulence gene *hly1* was reported to display 56% amino acid sequence homology to a protein in *E. faecalis* containing motif PF03006 conserved in proteins with haemolytic activity (Morita *et al.*, 2011). Proteins containing this motif are classified in the haemolysin-III related family according to the Pfam protein families database (http://pfam.xfam.org/; Finn *et al.*, 2014). Haemolysin-III related proteins are integral membrane proteins, characterised by type 1 topology (i.e. extracellular N-terminus and intracellular C-terminal domain) and seven transmembrane-spanning α-helices (Tang *et al.*, 2005). Based on the structural features of Hly1, it is classified as a member of the α-pore forming toxins that include colicins of *E. coli* (Iacovache *et al.*, 2008). Another putative haemolysin under investigation in this study, encoded by *hly2*, was reported to display 72% amino acid homology to a haemolysin in *S. suis* (Morita *et al.*, 2011). The gene product of *hly3* was reported to display 59% amino acid sequence similarity to a haemolysin in *S. pyogenes* (Morita *et al.*, 2011), an organism associated with β-haemolytic activity.

If full reduction (four electron reduction) of molecular oxygen (O_2) to H_2O does not occur, one- or two-electron reduction of O_2 results in the formation of reactive oxygen intermediates such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) (Yu *et al.*, 2001). Reactive oxygen species (ROS) have various noxious effects on cells, including damage of nucleic acids and peroxidation of phospholipids (Yesilkaya *et al.*, 2000). Protection against oxidative stress is afforded by the

enzymatic removal of ROS by catalase and superoxide dismutase (SOD). Superoxide dismutase (EC 1.15.1.1) is a metallo-enzyme that converts O_2^- to O_2 and H_2O_2, followed by the metabolism of the latter by catalase (Tang *et al.*, 2012). The LAB are catalase negative (Collins *et al.*, 1983), therefore SOD represents a major defence mechanisms against oxidative stress in *L. garvieae*. The detoxifying properties of SOD can also enhance intraphagocytic survival of bacteria upon generation of bactericidal ROS as a result of respiratory burst (Fang *et al.*, 2015). The enzyme NADH oxidase (NOX) reduces molecular oxygen to H_2O or H_2O_2 and was proposed to perform an important role in defence against O_2 toxicity and O_2 sensing that are essential functions during aerobic growth of LAB (Muchnik *et al.*, 2013). The importance of NADH oxidase in the virulence of *S. pneumoniae* has been established by Yu and co-workers (2001) by demonstrating significant attenuation of a *nox*-deficient mutant, leading to the conclusion that NOX is required for *in vivo* proliferation in O_2-rich environments. Further investigation into the involvement of NOX in the virulence of *S. pneumoniae* revealed that the enzyme is localised to the cell wall, where it may be fit for other functions relating to virulence. Random peptide library phage display was used to identify several ECM proteins as ligands to recombinant NOX, suggesting that the enzyme may play a multifunctional role in virulence by additionally acting as an adhesin (Muchnik *et al.*, 2013).

Adherence to host cells is a prerequisite step in the colonisation processes of pathogenic and commensal microbes (Kline *et al.*, 2009). Morita and co-workers (2011) reported that protein LCGL_1330 of *L. garvieae* Lg2 shared 62% amino acid identity with *S. pneumoniae* TIGR4 PavA and contained a fibronectin-binding motif (PF05833). Pneumococcal adherence and virulence factor A (PavA) is a cell surface-localised fibronectin-binding protein that lacks both typical signal sequences for secretion as well as the LPXTG anchorage motif characteristic of cell surface proteins (Holmes *et al.*, 2001). Another protein with amino acid sequence homology (49%) to a *S. pneumoniae* cell surface-exposed virulence determinant, pneumococcal surface antigen A (PsaA), was identified in Lg2 by Morita and coworkers (2011). This protein was originally identified as a putative adhesin (Berry and Paton, 1996), however its structure was not found to be consistent with the function of adhesion (Johnston *et al.*, 2004). Its function was revealed to be a divalent metal ion-binding lipoprotein component of an ATP-binding cassette (ABC) transporter for Mn^{2+}, a metal of vital importance to pathogenesis (Johnston *et al.*, 2004). Manganese acts as a cofactor of enzymes involved in a variety of cellular processes. These include central metabolic pathways like glycolysis and homolactic fermentation, where pyruvate kinase and lactate dehydrogenase require the metal cofactor Mn^{2+} (Jakubovics and Jenkinson, 2001).

2.5. CONCLUSION

The major virulence factor of *L. garvieae* defined thus far is the antiphagocytic polysaccharide capsule. With the aid of next generation sequencing of full genomes, several other putative virulence factors have been identified. Despite rapid advances in the areas of molecular biology and bioinformatics, its pathogenic processes are inadequately understood. Unexpectedly, no polysaccharide capsule was detected in any of the strains studied. Therefore, the presence of other virulence factors in these isolates was investigated. In this chapter, putative virulence factors with roles in adhesion, cytolytic activity, oxidative stress tolerance and metal homeostasis were identified in a set of seven South African fish pathogenic *L. garvieae* isolates, as well as an avirulent isolate.

Since all virulence factors present in the fish pathogenic isolates were also present in the avirulent isolate, it is not clear by exactly which mechanisms pathogenic *L. garvieae* causes injury to the host. All virulence factors discussed in this chapter, with the exception of haemolysins, are virulence lifestyle factors that indirectly contribute to host damage. These virulence lifestyle factors aid in the infection process by evasion of the host's innate immune system, cofactor homeostasis, systemic invasion and dissemination in the host and adhesion to host tissues. Future studies can focus on investigating differential expression of virulence lifestyle and true virulence genes during growth in the host environment versus laboratory conditions.

3. Heterologous expression of putative *Lactococcus garvieae* virulence factor, glyceraldehyde 3-phosphate dehydrogenase (GAPDH)

3.1. INTRODUCTION

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is a highly conserved enzyme involved in the central energy conversion pathway of glycolysis (Berg *et al.*, 2002). However, advances in the field of protein moonlighting have revealed its involvement in the pathogenic processes of various bacterial pathogens (Pancholi and Fischetti, 1992; Terao *et al.*, 2006; Terrasse *et al.*, 2012). Moonlighting proteins are defined as intact or complete proteins that perform more than one biochemical function, not as a result of the existence of multiple splice variants, gene fusions, proteolytic fragments or promiscuous enzyme activity (Jeffery, 1999; Amblee and Jeffery, 2015).

The first and only account of investigation of a possible moonlighting protein of *Lactococcus garvieae* involved the recombinant expression of GAPDH in *E. coli* with the purpose of assessing its immunogenicity (Tsai *et al.*, 2013). The study indicated that GAPDH is capable of inducing a protective immune response in tilapia. In agreement with the results of Tsai and co-workers (2013), preceding immunoproteomic studies (Shin *et al.*, 2009) have identified *L. garvieae* GAPDH as a common antigen in capsulated and non-capsulated strains. From these observations it can be concluded that GAPDH is present on the extracellular surface of *L. garvieae,* although the function is still unknown.

The aim of this chapter is to clone the GAPDH gene, express and purify the *L. garvieae* GAPDH protein to enable further studies that may provide insight into its function on the cell surface.

3.2. MATERIALS AND METHODS

3.2.1. Directional cloning of *gapC*

The high copy number pGEM®-T Easy bacterial vector (Promega, USA) was used as the parent vector for TA subcloning, while pET-28b(+) (Novagen®) was used as the destination vector. Plasmid vectors used for cloning procedures and their features are summarised in Table 3.1.

Vector maps with reference points and sequences of multiple cloning sites (MCSs) are presented in Fig. 3.1 and Fig. 3.2. The ampicillin resistance gene (*amp*^{R}), filamentous phage f1 sequences and bacterial *ori* origins of replication, the location of the T7 and Sp6 promoters and the *lacZ* coding sequence of pGEM®-T Easy are indicated in Fig. 3.1. The kanamycin resistance gene (*kan*^{R}), the ColE1 pBR322 origin of replication and *lacI* coding sequence of pET-28b(+) are indicated in Fig. 3.2. The sequence of the MCS indicates the binding sites of the T7 promoter and T7 terminator sequencing primers, the restriction enzyme recognition sites, as well as the location of the sequence coding for the His_6-tag (Novagen®).

Table 3.1: Features of plasmids used for recombinant expression of *L. garvieae* GAPDH

Plasmid	Features	Selective marker	Manufacturer
pGEM®-T Easy	T-overhangs for subcloning of *Taq* amplified DNA, *lacZ,* f1 ori, T7 promoter, SP6 promoter	Amp^R	Promega
pET-28b(+)	T7*lac* promoter, T7 terminator, f1 ori, N-terminal His_6-tag and thrombin configuration	Kan^R	Novagen®

3.2.1.1. *In silico* design of expression construct

Primers for the amplification of *gapC* were designed using a publically available *L. garvieae gapC* nucleotide sequence (GenBank accession nr. FJ524849.1) as template. A graphic representation of the expression construct, produced by Geneious version 9 (http://www.geneious.com, Kearse *et al*., 2012), is shown in Fig. 3.3. Oligonucleotides were designed with Geneious version 9 (http://www.geneious.com, Kearse *et al*., 2012) and analysed using an online tool, Integrated DNA Technologies OligoAnalyzer 3.1 (http://eu.idtdna.com/calc/analyzer). The oligonucleotides' melting temperatures were designed to differ by no more than 5°C, but the GC-content of the primers were lower than ideal, due to the low GC-content of the template. Primers were designed with restriction enzyme recognition sites at the 5'-end (as indicated in Table 3.2) to facilitate cloning into the destination vector.

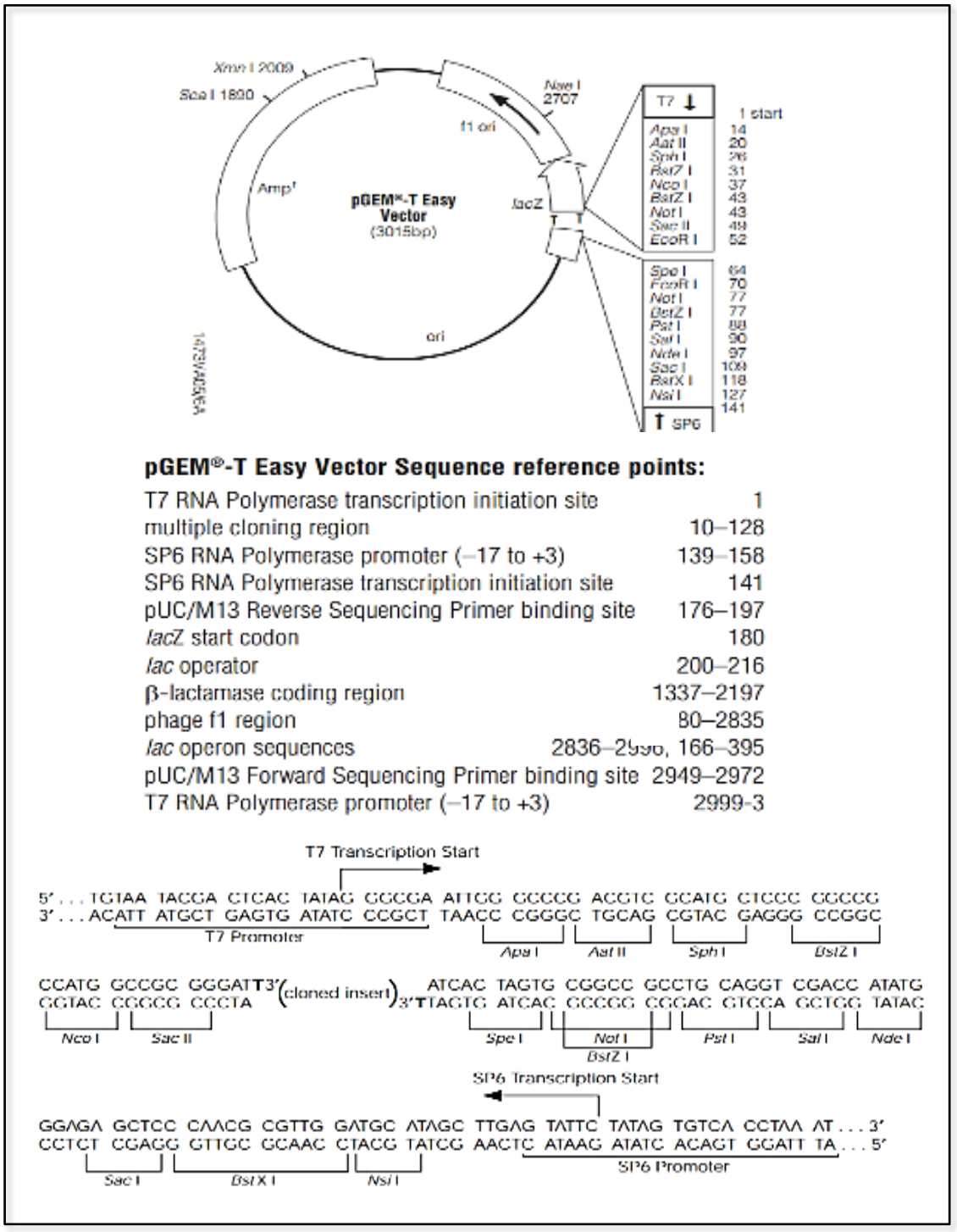


Figure 3.1: Vector map of the parent vector, pGEM®-T Easy. The sequence of the cloning region is provided.

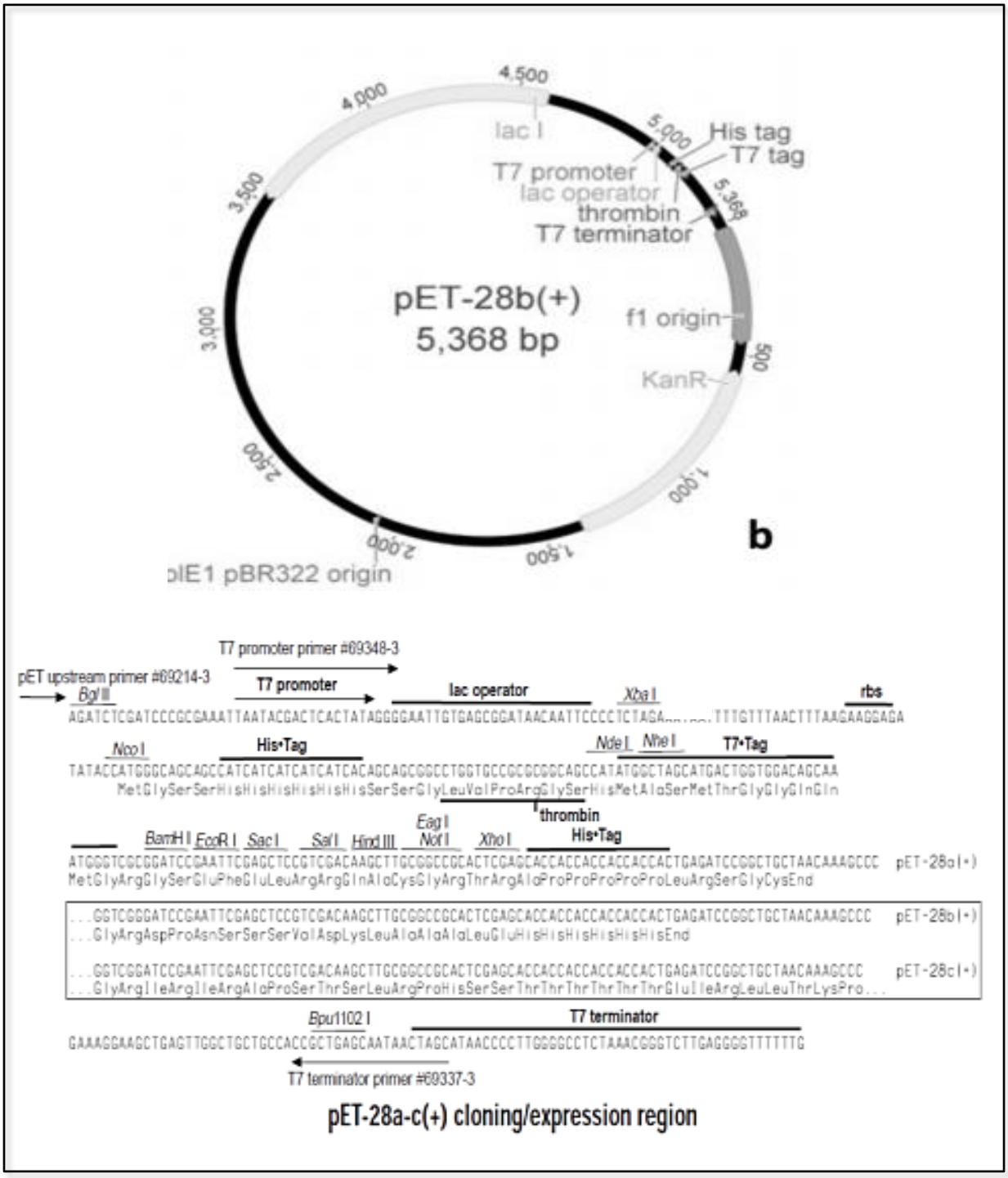


Figure 3.2: Vector map of the destination vector, pET-28b(+). The sequence of the expression region is provided.

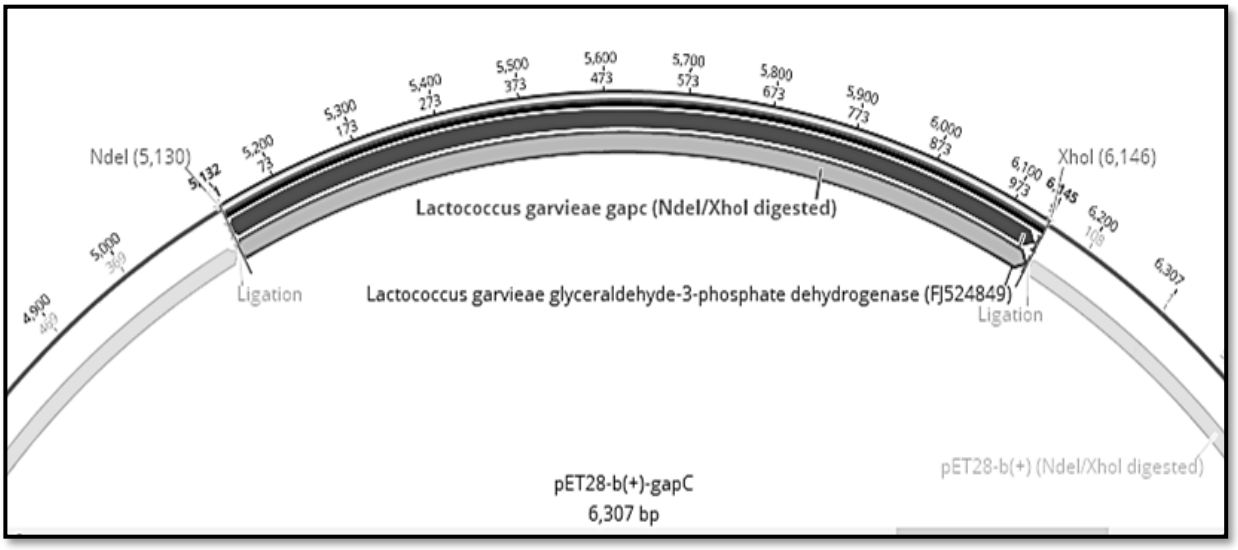


Figure 3.3: *In silico* design of the expression construct was performed using Geneious version 9 (http://www.geneious.com, Kearse *et al.,* 2012). Endonuclease restriction sites at the N-terminus (*Xho*I) and C-terminus (*Nde*I) of GAPDH are indicated.

3.2.1.2. Amplification of *gapC* by PCR

The annealing temperature for PCR amplification of *gapC* was optimised by gradient PCR in a Vacutec® G-Storm™ Thermocycler, with annealing temperatures ranging from 51.7°C to 64.4°C. Final Mg^{2+} concentrations (2-5 mM) were tested at each annealing temperature in increments of 1 mM to determine optimum Mg^{2+} concentration for *gapC* amplification. Decreased elongation temperature and increased elongation time is required for amplification of a template with low GC-content (Su *et al.*, 1996), therefore thermocycling conditions differed slightly from typical PCR cycling conditions, due to the low GC content of the primers and template DNA. Each PCR mixture contained the following reagents at final concentrations parenthesised: dNTPs (0.2 mM), ThermoPol reaction buffer (1x) (New England BioLabs #B9004S), *Taq* DNA polymerase (1.25 U) (New England BioLabs #M0267S), forward and reverse primers (0.2 μM) (Integrated DNA Technologies), $MgCl_2$ (0.5 mM), genomic DNA (1 ng.μL^{-1}) and Milli-Q® H_2O up to the final reaction volume. Reactions were performed in a Vacutec® G-Storm™ Thermocycler under the following conditions: initial denaturation at 95°C for 2 min followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 50-65°C for 30 s, elongation at 64°C for 3 min and a final elongation step at 64°C for 5 min. Gel electrophoresis, excision of DNA bands and gel purification of PCR products were performed as described in Chapter 2, Section 2.2.2.2.

Table 3.2: Oligonucleotide sequences, melting temperatures, GC content and expected product size of primers used for the amplification of *L. garvieae gapC*, with restriction enzyme recognition sites underlined.

Primers	Sequence (5'-3')	T_m(°C)	GC (%)	Restriction site	Product size (bp)
LGgap-F	AAGCCGCATATGATGGTAGTTAAAGTTGGTAT	59.6	37.5	*Nde*I	1011
LGgap-R	CTGCCCTCGAGCTTATTTAGCGATTTTTGC	61.4	46.7	*Xho*I	

3.2.1.3. Subcloning of *gapC* into parent vector (pGEM®-T Easy bacterial vector)

The amplified *gapC* gene was subcloned into pGEM®-T Easy bacterial vector before the transfer thereof to the expression vector, pET-28b(+). This method of cloning relies on the addition of a single deoxyadenine triphosphate (dATP) base to the 3'-end of a PCR product by the terminal transferase activity of *Taq* DNA polymerase. The vector and insert thus pair *via* complementary interaction between the dATP and dTTP-overhang on the ends of the pGEM®-T Easy vector. Ligase then catalyses the formation of a covalent phosphodiester bond between the 3'-hydroxy and 5'-carboxyl ends. The ligation reaction contained 1 μL PCR product [12.63 ng.μL^{-1}], 0.25 μL pGEM®-T Easy [50 ng.μL^{-1}], 1 μL 10 x Ligation buffer (New England BioLabs® Inc.), 1 μL T4 DNA ligase (New England BioLabs® Inc.) and nuclease-free H_2O up to a final volume of 10 μL. A negative control without insert was included. The required amount of PCR product (insert) per ligation reaction was calculated using Equation 3.1. An insert:vector molar ratio of 3:1 was used for calculations. Reaction components were mixed and incubated at room temperature for 1 h.

$$\text{amount of insert (ng)} = \frac{\text{amount of vector (ng)} \times \text{size of insert (kb)}}{\text{size of vector (kb)}} \times \text{insert:vector molar ratio}$$

$$= \frac{12.5 \text{ ng x } 1.01 \text{ kb}}{3 \text{ kb}} \text{ x } \frac{3}{1}$$

$$= 12.63 \text{ ng insert}$$

Equation 3.1: Calculation of the required amount of insert per ligation reaction

Competent *E. coli* cells were prepared by the method of Inoue and co-workers (1990). A pre-inoculum was prepared by inoculating 5 mL LB media (Table A.2, Appendix 1) with 5 μL glycerol stock, and incubating with shaking at 37°C for 10 h. The full pre-inoculum was transferred to 250 mL SOB medium and grown at 18-20°C until an OD_{600} of ~0.55 was reached. The flask containing the culture was placed in ice-water slurry for 10 min, where after the culture was transferred to pre-cooled Falcon tubes and centrifuged at 2 500 x*g* for 10 min at 4°C. Following removal of the supernatant, cells were gently resuspended in 80 mL ice cold transformation buffer (TB) (Table A.1, Appendix I). The suspension was incubated on ice for 10 min, followed again by centrifugation at 2 500 x*g* for 10 min at 4°C. After gentle resuspension in 20 mL ice cold TB; DMSO to a final concentration of 7% was added and the suspension was again incubated on ice for 10 min. Cells were aliquoted in pre-cooled tubes, snap frozen in liquid nitrogen and stored at -80°C.

SIG10 5α competent *E. coli* cells (Sigma-Aldrich, USA) were transformed with ligated pGEM®-T Easy plasmids according to manufacturer's guidelines. Aliquots of frozen competent cells (50 μL) were thawed on ice and 5 μL ligation reaction mixture was added, followed by incubation on ice for 30 min. Cells were heat-shocked at 42°C for 1 min and returned to ice for 2 min. After adding 1 mL LB medium, 20 μL 1 M glucose and 10 μL 2 M Mg^{2+}, cells were incubated at 37°C for 1 h. Cells were centrifuged at 9 000 x*g* for 30 s, 800 μL supernatant was removed and cells were resuspended. Remaining culture was plated onto AIX agar (Table A.2, Appendix I) to enable blue-white selection of transformants. A positive control (pUC19) was included to assess the efficiency of the transformation procedure. A negative control, with no plasmid added, was included to test the potency of the antibiotic. Plates were incubated at 37°C for 18 h.

White colonies were selected and screened for the presence of plasmids containing the insert by performing plasmid isolation followed by *Eco*RI digestion. Randomly selected white colonies were picked with a sterile Eppendorf® yellow pipette tip and inoculated into LB media supplemented with ampicillin (100 μg.mL^{-1}) to maintain selective pressure and incubated at 37 °C for 18 h. Plasmid purification was performed by pelleting 1 mL of culture in 1.5 mL Eppendorf® tubes by centrifugation and resuspension of cells in 350 μL STET buffer (Table A.1, Appendix I), followed by addition of 4 μL lysozyme (50 mg.mL^{-1}). The mixtures were then incubated for 44 s in a boiling water bath in order to lyse cells. After incubating the tubes for 10 min on ice, the mixtures were centrifuged for 15 min at maximum speed to pellet cell debris. The pellets were removed with sterile toothpicks and 420 μL of ice cold isopropanol and 40 μL sodium acetate (pH 5.2) was added to the supernatants to precipitate plasmid DNA. Following centrifugation at 4°C for 5 min at

maximum speed, the supernatants were removed and the pellets washed with ice cold 70% ethanol. Pellets, containing plasmid DNA, were dried in a Concentrator Plus (Eppendorf®) for 15 min at 45°C followed by resuspension in TE buffer at 37°C for 30 min. Purified plasmids were stored at -20°C.

Restriction digest screening of recombinant DNA clones was performed on the purified plasmid DNA with *Eco*RI to confirm the presence of the insert. Digestion reactions contained 4 μL plasmid DNA, 0.5 μL *Eco*RI [10 U.μL^{-1}], 1 μL *Eco*RI Buffer (10x) and nuclease free water up to a final volume of 10 μL. The restriction digestion reaction was incubated at 37°C for 2 h, followed by gel electrophoresis on a 0.8% (w/v) agarose gel to enable visualisation of digested products. Plasmids containing the ± 1 kb insert were submitted for sequencing with SP6 and T7 promoter primers at Inqaba Biotec™.

3.2.1.4. Construction of pET-28b(+)-*gapC*

To excise and prepare the insert for cohesive-end ligation into the destination vector, pGEM®-T Easy-*gapC* was digested with *Nde*I and *Xho*I in a reaction containing 3 μL pGEM®-T Easy-*gapC* DNA, 0.25 μL *Nde*I [10 U.μL^{-1}], 0.5 μL *Xho*I [10 U.μL^{-1}], 2 μL Buffer O (10x) and nuclease-free H_2O up to 10 μL. The parent vector was also digested with *Nde*I and *Not*I in a larger reaction volume to facilitate purification of the digested DNA. The reaction contained 30 μL pGEM®-T Easy-*gapC* DNA, 0.5 μL *Nde*I [10 U.μL^{-1}], 1 μL *Not*I [10 U.μL^{-1}], 8 μL Buffer O (10x) and nuclease-free H_2O up to 40 μL. Similarly, the destination vector was linearised by double digestion with *Nde*I+*Not*I and *Nde*I+*Xho*I, preparing 5' and 3' ends for cohesive-end ligation with the *gapC* DNA fragment. Digestion removed most of the multiple cloning site (MCS) as indicated in Fig. 3.2, though the ATG start codon at the *Nco*I restriction site and the C-terminal His_6-tag remained intact. The linearised vector and excised insert were purified from a 2% low-melt agarose gel as described in Chapter 2, Section 2.2.2.2 Purified *gapC* was ligated into the prepared pET-28b(+) vector in a ligation reaction containing 1 μL T4 DNA ligase (New England BioLabs), 1 μL T4 DNA ligase buffer (10x), 3 μL digested pET-28b(+), 2.23 μL digested *gapC* and nuclease-free H_2O up to 10 μL. The appropriate amount of insert required was calculated as per Equation 3.1, using a molar insert:vector ratio of 5:1. The ligation reaction was incubated at RT for 1 h. Ligated plasmid was transformed into SIG10 5α chemically competent *E. coli* cells (Sigma-Aldrich, USA) as described previously, plated out on LB plates supplemented with kanamycin (100 μg. mL^{-1}) and incubated at 37°C for 18 h.

3.2.2. Heterologous expression of GAPDH in *E. coli* BL21 (DE3)

The *E. coli* strain BL21(DE3) [genotype *fhuA2 [lon] ompT gal* (λ *DE3) [dcm]* Δ*hsdS;* λ *DE3 =* λ *sBamHIo* Δ*EcoRI-B int::(lacI::PlacUV5::T7 gene1) i21* Δ*nin5*] was used as expression host. The BL21 strains, derived from the *E. coli* B strains, are used for high-level expression of recombinant proteins at high levels. The strain BL21 (DE3) contains a phage lambda (*λ*) DE3 lysogen carrying the T7 RNA polymerase gene under control of the *lac*UV5 promoter, a mutant lac promoter that is less sensitive to intracellular cyclic adenosine monophosphate (cAMP) levels. Deficiency in two proteases, Lon and OmpT, reduces enzymatic degradation of heterologously expressed proteins.

After confirming the presence of insert DNA in the pET-28b(+) vector, the purified plasmid was transformed into the expression host, *E. coli* BL21(DE3). For preparation of a pre-inoculum, a colony from a transformed plate was used to inoculate 5 mL LB media ([kana] = 30 μg.mL^{-1}). The culture was incubated at 37°C for 16 h. Auto-induction and IPTG induction were used for heterologous expression. For expression using auto-induction media, 2 mL pre-inoculum was used to inoculate 100 mL ZYP5052 auto-induction media (Table A.2, Appendix I) ([kana] = 50 μg.mL^{-1}) in a 500 mL Erlenmeyer flask. Flasks were incubated at 25°C in an orbital shaker (200 rpm) for 24 h and 36 h, respectively. For IPTG induction, 2 mL pre-inoculum was used to inoculate 100 mL LB media ([kana] = 50 μg.mL^{-1}) in a 500 mL Erlenmeyer flask. The culture was incubated at 37°C in an orbital shaker (200 rpm) until an optical density (600 nm) of 0.8 – 1.00 was reached. Expression was induced by the addition of 1 mM IPTG, and the culture was further incubated at 30°C for 4 h.

3.2.2.1. Expression analysis

The French press cell disruption technique involves passage of a cell suspension through a small orifice under high pressure, causing destruction of cell membranes by exertion of shear stress and decompression upon exiting the orifice (Walker, 2010). Following expression using auto-induction media and IPTG induction, 10 mL of culture was harvested by centrifugation (9000 x*g*; 30 s) and resuspended in Binding Buffer to a final concentration of 0.1 g.mL^{-1} (w/v). Cells were disrupted using a One Shot Cell Disruptor (Constant Systems, Ltd.) at 30 kPsi. The suspension obtained thereafter represented the total protein fraction. Crude cell debris, insoluble proteins and intact cells were removed by centrifugation at maximum speed (20 000 x*g*) for 20 min at 4°C. This constituted the soluble protein fraction. The supernatant was subjected to ultracentrifugation in an Optima L-100 XP Ultracentrifuge (Beckman-Coulter®) using rotor SW 32 Ti at an average RCF of 110 527, at 4°C for 1.5 h. The identity of the recombinantly expressed protein was confirmed by LC-MS/MS as described in Chapter 2, Section 2.2.6.2.

3.2.2.2. Protein purification by immobilised metal affinity chromatography

Recombinant His_6-tagged GAPDH was purified by the gravity flow IMAC method. Following expression, cells were disrupted using the One Shot Cell Disruptor (Constant Systems, Ltd.) binding buffer (Table A.1, Appendix I) containing 8 M urea. The high urea concentration in the cell lysis buffer served to solubilise and denature rGAPDH expressed in cytoplasmic inclusion bodies, allowing the target protein to be obtained in the soluble fraction and enabling exposure of the affinity His_6-tag to the metal ligand. Two affinity matrices containing different metal ions were utilised during IMAC purification, namely TALON® Metal Affinity Resin (Clontech Laboratories, Inc.) and Pierce™ Nickel Chelated Agarose (Thermo Fischer Scientific), a pre-packed column. TALON® Metal Affinity Resins consist of a 6% cross-linked agarose matrix, Sepharose CL-6B (GE Healthcare), containing a tetradentate chelating ligand carboxylmethylaspartate (CMA) complexed with cobalt ions (Co^{2+}). Similarly, Pierce™ Nickel Chelated Agarose also contains a tetradentate chelating ligand, nitrilotriacetic acid (NTA), complexed with nickel ions (Ni^{2+}). Purification was performed according to the manufacturer's protocol for batch/gravity-flow column purification. For preparation of the TALON® Co^{2+}-CMA column, TALON® resin was centrifuged at 700 x*g* for 2 min to pellet the resin and the supernatant was discarded. Approximately 10 bed volumes (0.5 mL) equilibration buffer was added and mixed to pre-equilibrate the resin. The resin was again centrifuged at 700 x*g* for 2 min and the supernatant discarded. The following steps were performed for both the TALON® Co^{2+}-CMA column and the Pierce™ Ni^{2+}-NTA column. A volume of 15 mL supernatant from the ultracentrifuged fractions was loaded onto the column and the flow-through (F) fraction was collected. Binding buffer was then loaded onto the column and the run-off, designated as the wash (W) fraction, was collected. Lastly, elution buffer (Table A.1, Appendix I) was applied to elute binding protein from the column and elution (E1-10) fractions were collected in volumes of 0.5 mL. Collected fractions were visualised on a 12% SDS-PAGE gel as described in Chapter 2 Section 2.2.6.1. Pure elution fractions were pooled and dialysed against TBS buffer (Table A.1, Appendix I) in order to remove urea and allow refolding of the protein. A Pierce™ Slide-A-Lyzer™ Dialysis Cassette (ThermoFischer Scientific™) with a molecular weight cut off value of 10 kDa was used to perform dialysis according to manufacturer's instructions.

3.2.2.3. rGAPDH activity assay

The enzymatic activity of purified rGAPDH was determined by a spectrophotometric assay as described by Krebs (1955), with some modifications. Concentration of purified rGAPDH was determined in a colorimetric assay using the Pierce™ Bicinchoninic Acid (BCA) Protein Assay Kit (ThermoFischer Scientific™). Standard curves were constructed using bovine serum albumin (BSA)

as per manufacturer's instructions. Absorbance values (562 nm) of BSA standards were determined using a Biochrom WPA Biowave II spectrophotometer and plotted against protein concentration to obtain a standard curve that was used to determine the concentration of purified rGAPDH. The spectrophotometric activity assay performed on purified rGAPDH followed the increase in absorbance at 340 nm as the co-factor NAD^+ was reduced to NADH during the conversion of glyceraldehyde 3-phosphate to 1,3-*bis*phosphoglycerate. The continuous enzymatic assay consisted of 2.6 mL 0.15 mM sodium pyrophosphate buffer (pH 8.5) containing 0.03 M sodium arsenate, 0.1 mL 7.5 mM NAD^+, 0.1 mL 0.1 M dithiothreitol and either 0.5 or 0.05 μg purified rGAPDH. The assay mixture was equilibrated to room temperature in a Biochrom WPA Biowave II spectrophotometer and the blank rate was established. A volume of 0.1 mL glyceraldehyde 3-phosphate (0.15 mM) was added to initiate the reaction and A_{340nm} values were recorded from 0-5 min. Initial reaction velocity was measured as an increase in absorption at 340 nm resulting from the reduction of NAD^+ to NADH. The Beer-Lambert Law, shown in Equation 3.2, and Equation 3.3 was used to calculate activity in units per mg, using a molar extinction coefficient of 6.22 x 10^3 $L.mol^{-1}.cm^{-1}$ (Werner *et al.*, 1973) and a path length of 1 cm. One unit is defined as the initial rate of reduction of one micromole of NAD^+ per minute at 25°C and pH 8.5 under the specified conditions.

$$A = \varepsilon bc \therefore c = \frac{A}{\varepsilon b}$$

A = absorbance (340 nm)

ε = molar extinction coefficient of NADH ($L.mol^{-1}.cm^{-1}$)

b = path length (cm)

c = concentration ($mol.L^{-1}$)

Equation 3.2: Beer-Lambert Law

$$Units.mg^{-1} = \frac{\Delta A_{340}.min^{-1}}{6.22 \times mg\ GAPDH.mL\ reaction\ mixture^{-1}}$$

Equation 3.3: Calculation of rGAPDH enzyme activity

3.3. RESULTS

3.3.1. Directional cloning of *gapC*

3.3.1.1. Amplification of *gapC* by PCR

Optimisation of reaction conditions for amplification of *gapC* by PCR indicated that satisfactory amplification of the target gene was observed using annealing temperatures between 51.7-64.4°C, as shown by visualisation of PCR products in Fig. 3.4. A negative control was included for the lowest annealing temperature, and no bands were observed in this reaction. Increased Mg^{2+} concentrations were shown to enhance amplification of the target gene. Based on these observations, an optimal annealing temperature of 59°C and final Mg^{2+} concentration of 4 mM was selected.

3.3.1.2. Subcloning of *gapC* gene into parent vector (pGEM®-T Easy bacterial vector)

Eight randomly selected white colonies obtained by blue-white selection after subcloning *gapC* into pGEM®-T Easy were picked and cultivated under appropriate conditions. Plasmids were extracted, digested with *Eco*RI to screen for the presence of the insert and digest products were visualised on a 0.8% agarose gel. As shown in Fig. 3.5, fragments of ~1 kb (corresponding to the size of the *gapC* CDS) and ~3 kb (corresponding to the size of the vector) were observed in all digested plasmids, indicating successful subcloning of *gapC* gene into the parent vector, pGEM®-T Easy. Digestion of an empty plasmid yielded only the expected ~3 kb fragment that corresponds to the size of the linear vector.

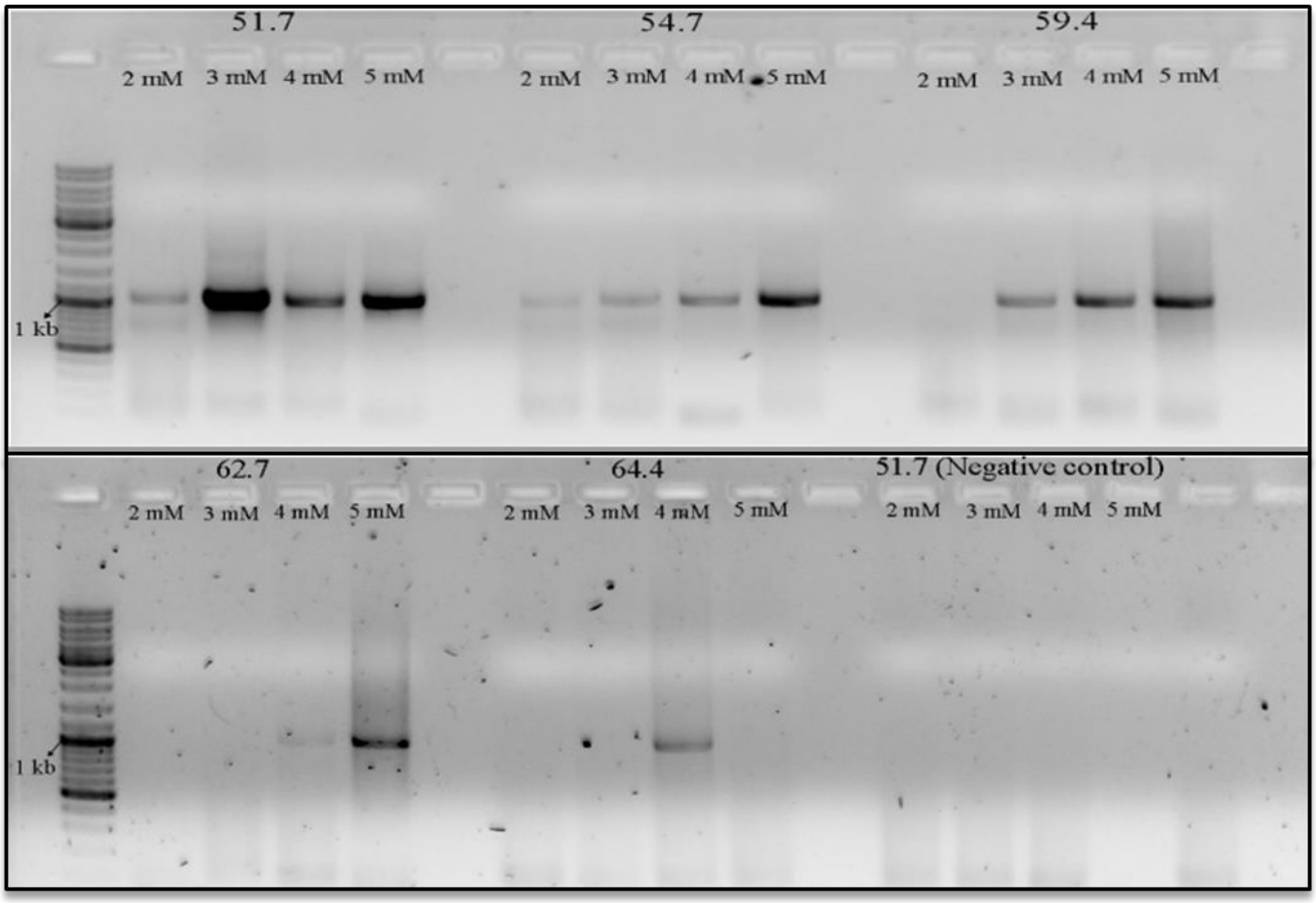


Figure 3.4: A 1% agarose gel showing products of optimisation of reaction conditions for amplification *of L. garvieae gapC*. Annealing temperatures ranging from 51.7-64.4°C and final magnesium concentrations of 2-5mM were tested. No non-specific amplification was observed in the negative control reaction. An optimal annealing temperature of 59°C and final Mg^{2+} concentration of 4 mM was selected.

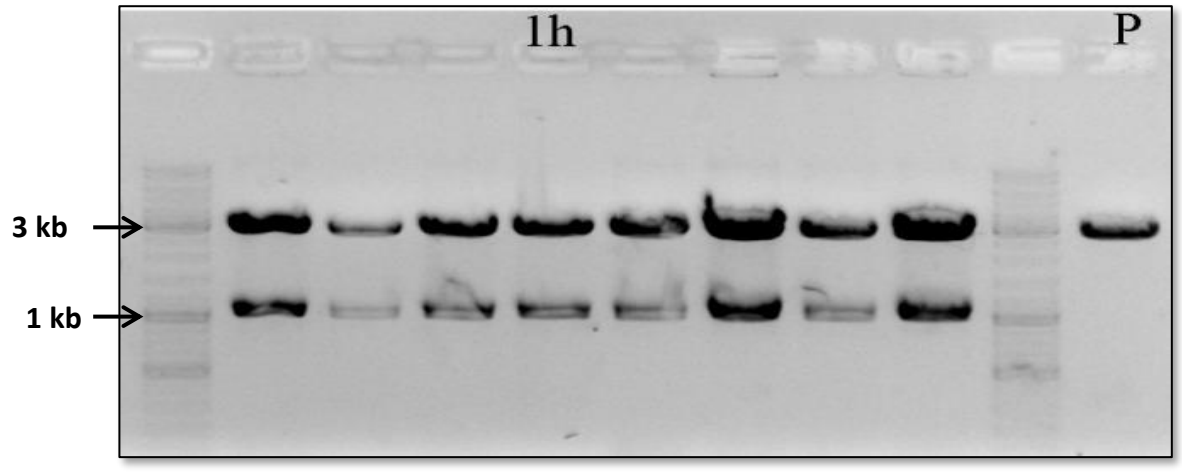


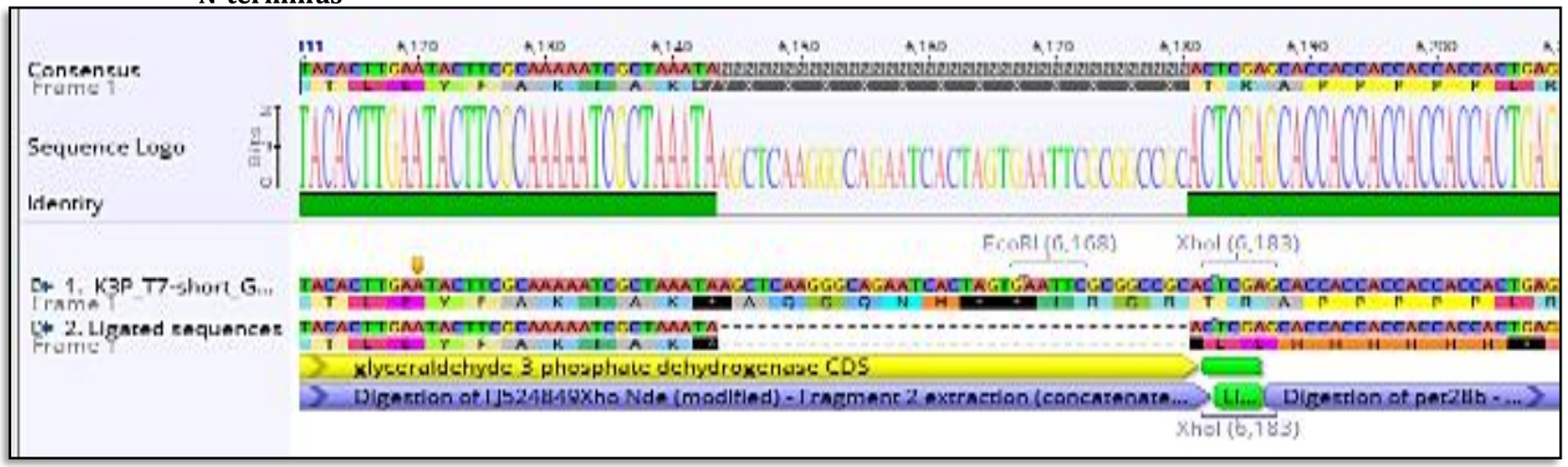
Figure 3.5: Visualisation of *Eco*RI digest products of pGEM®-T Easy plasmids, isolated from colonies obtained by blue-white selection on a 0.8 % agarose gel. Bands of ~1 kb (corresponding to the size of the insert) and ~3 kb kb (corresponding to the size of the plasmid backbone) were observed for all colonies selected. M = marker; P = empty plasmid.

3.3.1.3. Construction of expression construct pET-28b(+)-*gapC*

pGEM®-T Easy-*gapC* was digested in a double restriction digest with *Nde*I and *Not*I in order to extract the *gapC* coding sequence (CDS) from the vector and to prepare the sequence for cohesive end ligation with pET-28b(+) digested with *Nde*I and *Not*I. Restriction digested products corresponding to the ~1 kb size of *gapC* CDS and ~5 kb of the linearised pET-28b(+) vector were first excised and gel purified. The prepared *gapC* CDS was then ligated to the linearised pET-28b(+) vector and successful ligation was confirmed by *Nde*I and *Not*I double digest followed by agarose gel electrophoresis. Plasmids were sequenced using T7 promoter/terminator primers (binding position indicated in Fig. 3.2) to ensure that directional insertion of the CDS had occurred correctly, with no point or frame-shift mutations being introduced during the cloning procedure. The N- and C-termini of the cloned insert are shown in Fig. 3.6.

Initial attempts to ligate the excised insert into the expression vector failed. Sequencing of pGEM®-T Easy-*gapC* DNA with primers T7 and SP6 revealed that a point mutation in the *Xho*I recognition site had occurred, which explained why the attempted ligations had failed. Therefore, *Xho*I had to be replaced by another restriction enzyme in the double digestion of pGEM®-T Easy-*gapC* and pET-28b(+) vectors in order to yield compatible cohesive termini needed for successful ligation. Firstly, the orientation of the insert in pGEM®-T Easy had to be determined in order to select a suitable restriction enzyme. This was achieved by digesting the vector with *Nde*I and visualising the products by agarose gel electrophoresis. Based on the size of the fragments obtained, the orientation of the insert could be deduced. *Not*I was identified as a suitable restriction enzyme to replace *Xho*I. After re-cloning of the insert and vector into BL21 (DE3), alignment of sequence data and the *in silico* expression construct indicated that no mutation occurred during the cloning process, and that the N-terminal His_6-tag and start codon (methionine) were in frame with the CDS. In Fig. 3.6 it can be seen that the C-terminal of the sequence obtained contained a region that did not align with the *in silico*-designed construct. This region is derived from the parent vector pGEM®-T Easy, located between the *Xho*I restriction site of the insert and the *Not*I restriction site on the plasmid backbone.

N-terminus



C-terminus

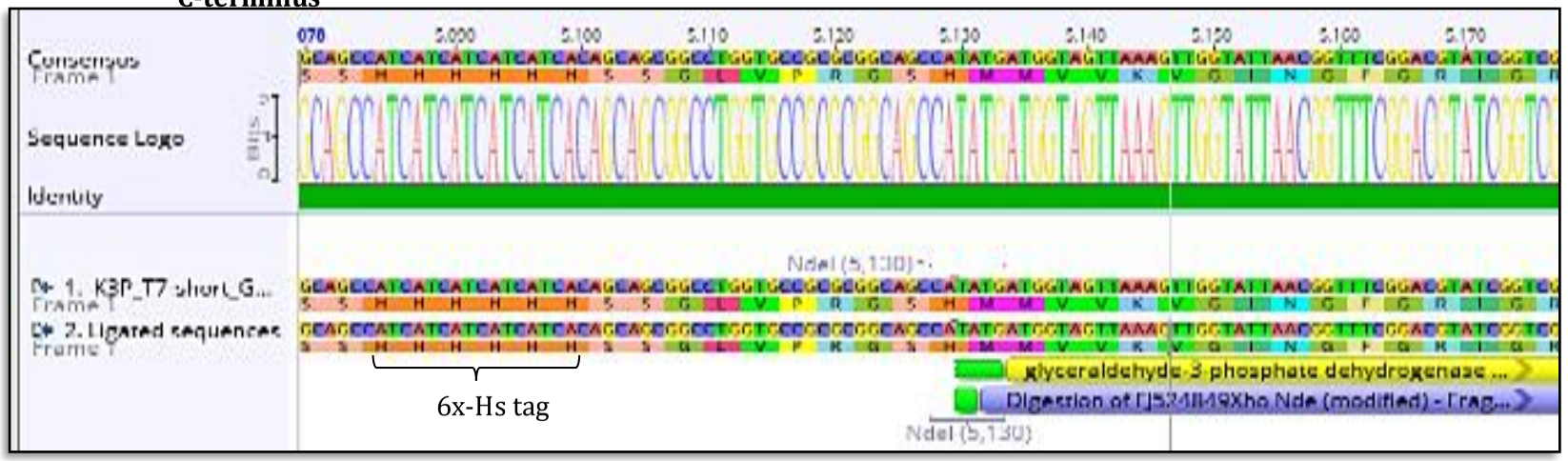


Figure 3.6: Plasmid pET-28b(+)-*gapC* was sequenced using T7 promoter/terminator primers and data was viewed and analysed using Geneious v. 9. Alignment of the sequence obtained and the *in silico* expression construct is presented, showing that the start codon is in frame with the rest of the sequence and no point mutations occurred throughout the coding sequence. The C-terminal His_6-tag is also indicated.

3.3.2. Heterologous expression of GAPDH in *E. coli* BL21 (DE3)

Expression was performed by IPTG induction and cultivation in ZYP5052 auto-induction media. Shown in Fig. 3.7 is the evaluation of rGAPDH expression in total, soluble and insoluble fractions on 12% SDS-PAGE. As a negative control, *E. coli* BL21 (DE3) transformed with the expression vector lacking the *gapC* were included for both IPTG induction and auto-induction experiments, while culture fractions cultivated in the absence of the inducer, IPTG, were also included for the IPTG induction experiment as an additional negative control. The SDS-PAGE analysis revealed expression in total and insoluble fractions, but not soluble fractions. This pattern of expression was observed during both IPTG induction (Fig. 3.7A) and auto-induction (Fig. 3.7B). The lack of expression observed in the soluble fraction suggests that the heterologously expressed protein may be localised to inclusion bodies or the cell membrane. The identity of the expressed protein was confirmed to be GAPDH by LC-MS/MS. Results obtained from the querying of the NCBI database using the Mascot search engine are shown in Table 3.4.

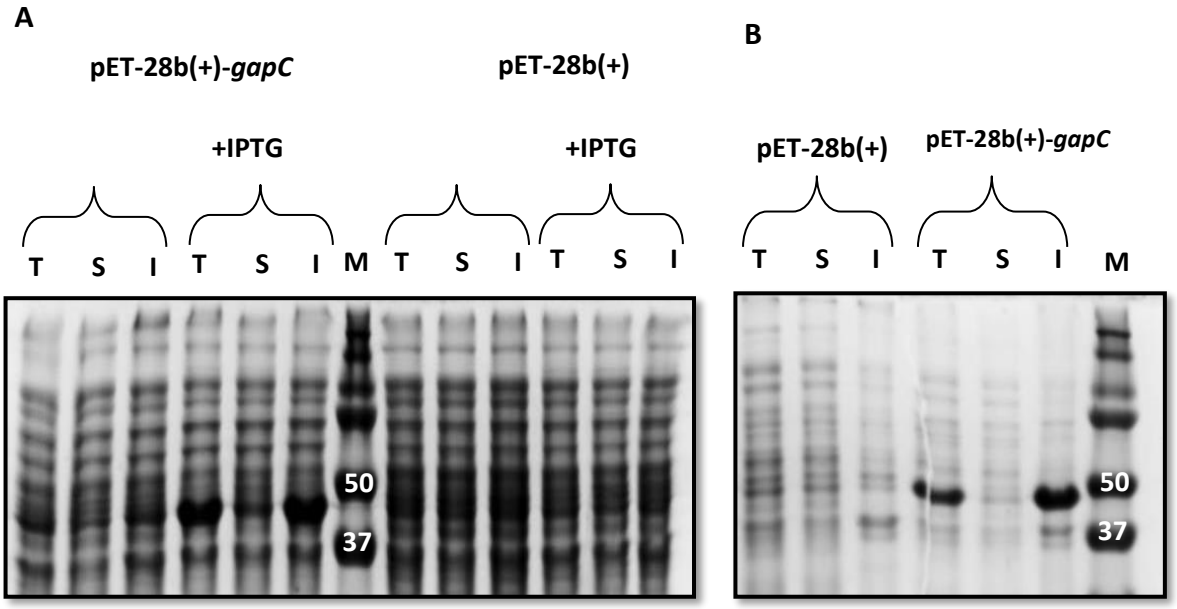


Figure 3.6: Visualisation of total (T), soluble (S) and insoluble (I) protein fractions on 12% SDS-PAGE following expression of rGAPDH in *E. coli* BL21 (DE3). Expression was induced by addition of 1 mM IPTG (A) or culturing in ZYP5052 auto-induction media (B). A distinct band between 37 and 50 kDa is observed in the total and insoluble protein fractions, indicating that rGAPDH was expressed in insoluble inclusion bodies. Molecular weight is indicated in kDa on the marker (M).

Table 3.3: Identification of the band in expected size range using LC-MS/MS and Mascot search engine.

Result	Nominal mass (M_r)	pI	Score
Glyceraldehyde-3-phosphate dehydrogenase [*Lactococcus garvieae*] gi|219880961	35854	5.12	508

3.3.2.1. Protein purification by immobilised metal affinity chromatography

Purification of rGAPDH was executed by affinity chromatography, a liquid chromatographic technique that utilises the selective interaction of an immobilised metal ligand with molecules of interest (Gaberc-Porekar and Menart, 2001). Fractions obtained by IMAC were visualised on 12% SDS-PAGE gels (Fig. 3.8.). Treatment with Triton™ X-100 and urea was applied to denature insoluble inclusion bodies containing rGAPDH. Urea treatment significantly aided in the purification of rGAPDH, as shown by the observed specific enrichment of the target protein in elution fractions of urea-treated samples [Fig. 3.8 (E & F)] compared to untreated [Fig. 3.8 (A&B)] and Triton™ X-100 treated samples [Fig. 3.8(C&D)].

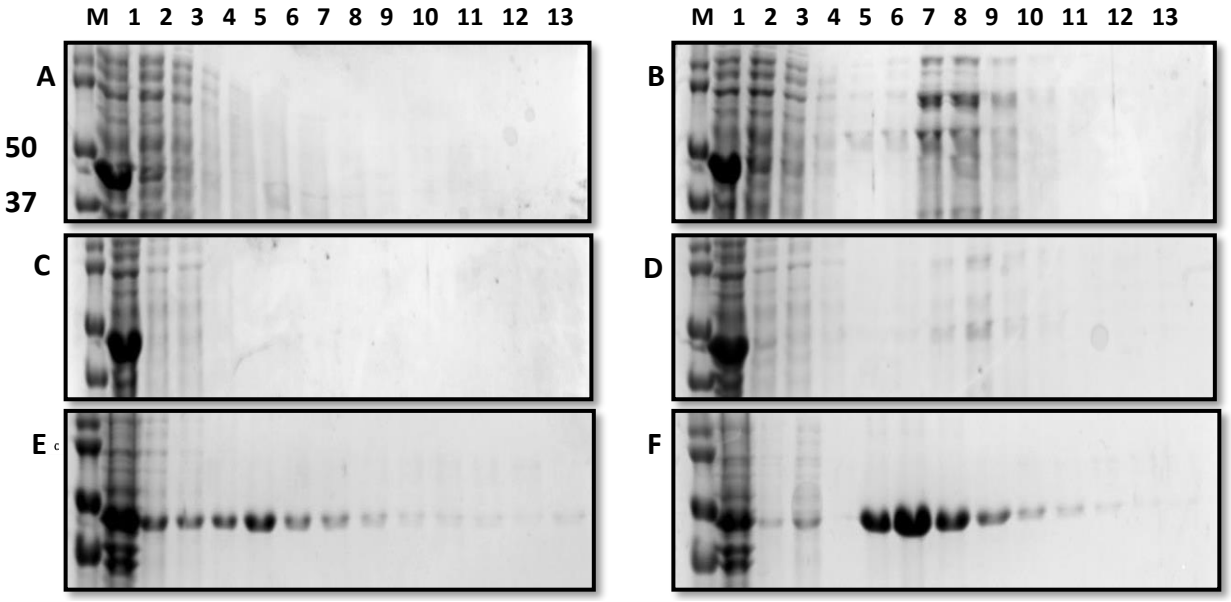


Figure 3.8: Visualisation of protein fractions on 12% SDS-PAGE, obtained by IMAC purification of rGAPDH. Inclusion bodies were solubilised using either 8 M urea or 0.5% Triton™ X-100. Urea treatment proved to be more successful in protein solubilisation than Triton™ X-100. Gels A, C & E - Co^{2+}-CMA; Gels B, D & F - Ni^{2+}-NTA. Gels A & B – untreated; Gels C & D – Triton™ X-100 (0.5%); Gels E & F – Urea (8 M). Lanes: M – marker (kDa); 1 – total protein fraction; 2 – flow through; 3 – wash; 4-13 – elution.

3.3.2.2. rGAPDH activity assay

The enzymatic activity of purified rGAPDH was determined by a spectrophotometric assay. The standard curve obtained by measuring absorbance at 562 nm of protein standards is shown in Fig. 3.9. The continuous enzymatic activity assay on rGAPDH revealed that the enzyme showed no detectable activity. Results of the activity assay using 0.05 and 0.5 μg rGAPDH, both in the presence and the absence of the substrate DL-glyceraldehyde 3-phosphate (G3P), are presented in Fig. 3.10. No increase in absorbance was detected, which can be interpreted as a lack of reduction of the co-factor NAD^+. Therefore, no activity for rGAPDH could be calculated.

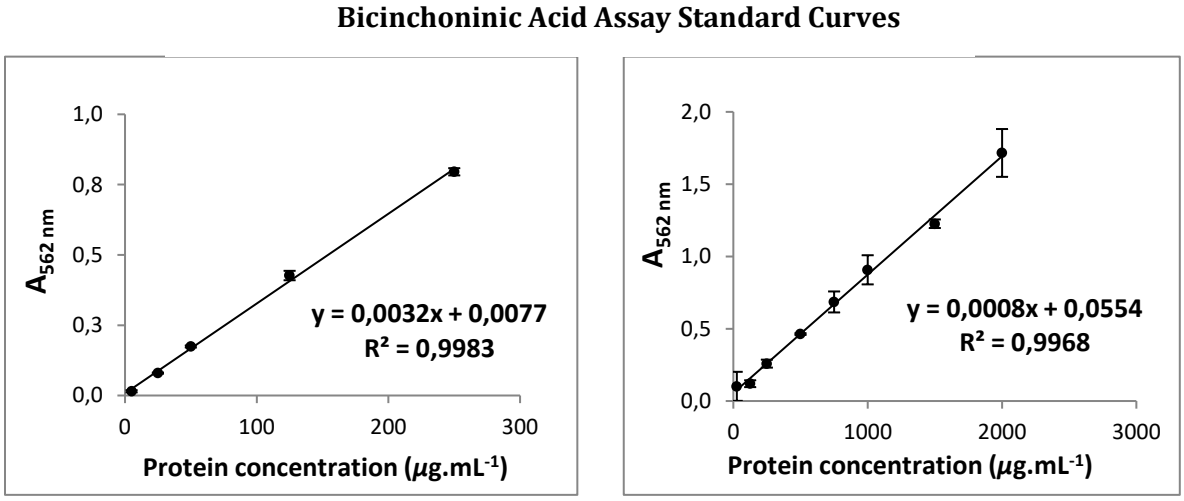


Figure 3.9: Standard curves for two ranges of concentration were constructed using Pierce™ Bicinchoninic Acid (BCA) Protein Assay Kit (ThermoFischer Scientific™) and known concentrations of bovine serum albumin as standards. Error bars represent standard deviation of triplicates.

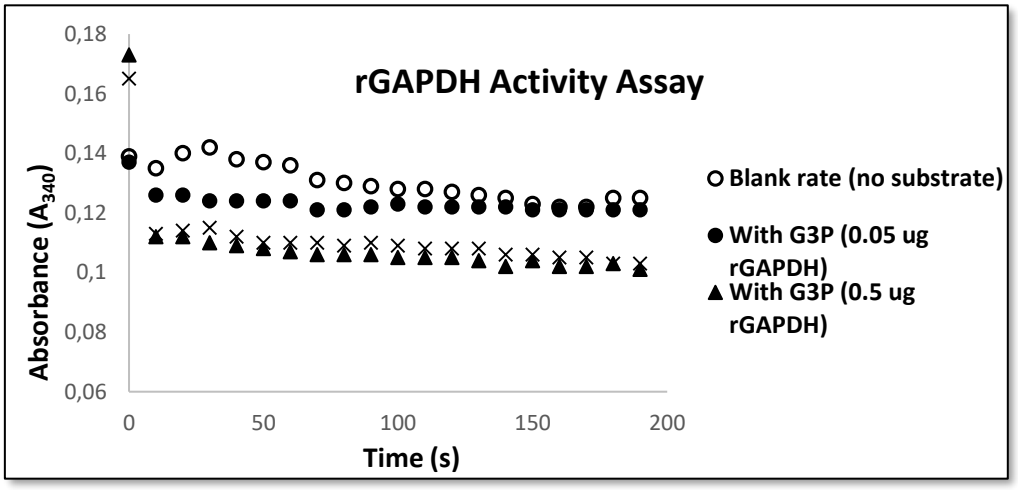


Figure 3.10: Enzymatic activity of purified rGAPDH was assayed using 0.05 μg and 0.5 μg rGAPDH.

3.4. DISCUSSION

A house-keeping enzyme with possible involvement in virulence in *L. garvieae* was cloned and expressed in the current study. The *gapC* gene was amplified by PCR and cloned into the expression vector pET-28b(+). Heterologous gene expression in pET-28b(+) is under the control of the *lac* operator. The vector encodes a lactose repressor and T7 RNA polymerase transcription signal, allowing transcription of the recombinant gene only in the presence of lactose or the non-metabolisable allolactose analogue, IPTG, and absence of glucose. The auto-induction medium ZYP5052 contains glucose and *α*-lactose. Glucose, as the preferred carbon source, initially represses transcription from the T7*lac* promoter during cultivation in order to allow cell proliferation to a sufficient level before the metabolism of the cell is directed towards expression of the recombinant protein. Upon depletion of glucose, recombinant protein expression proceeds by transcription from the T7*lac* promoter induced by α-lactose or the addition of IPTG in non-inducible media (Studier, 2005).

After attempts at ligation had failed, sequencing of the expression construct revealed a point mutation in the *Xho*I restriction site. This was overcome by selecting another restriction enzyme that digested the parent vector on the N-terminal side of the gene. After replacing *Xho*I with *Not*I for digestion of pGEM®-T Easy-*gapC,* the insert could be successfully ligated into the expression vector. Sequencing of the expression construct and alignment with the *in silico* expression construct, shown in Fig. 3.6, revealed that no further mutations had occurred and that recombinant expression could commence. Recombinant expression proceeded in *E. coli* BL21 (DE3), and analysis of expression revealed that rGAPDH could be successfully expressed using IPTG induction and auto-induction medium as indicated by the presence of an intense band in the expected size range of ~37 kDa in Fig. 3.7 that is not present in uninduced or negative control cultures. The use of auto-induction media for recombinant protein production is preferable to IPTG induction in T7 expression systems, as it enables facile simultaneous screening of multiple clones for expression, in comparison with conventional IPTG induction that requires growth monitoring for IPTG addition. Higher culture densities and increased levels of recombinant protein production, in comparison to IPTG induction, are also obtained by auto-induction media (Studier, 2005). For these reasons, auto-induction media was used for expression of rGAPDH.

Following intracellular expression of rGAPDH, cells were lysed using the French press cell disruption technique. This method is convenient for processing of high culture volumes, as it is rapid and inexpensive. However, this method is considered harsh due to the stress placed on cells that may result in less efficient recovery of proteins in the soluble fraction. Gentler methods of cell lysis, including enzymatic and freeze-thaw lysis, are associated with increased protein recovery, but are costlier and more time-consuming than physical lysis techniques (Walker, 2010). High-level recombinant expression in prokaryotic hosts frequently lead to the formation of insoluble inclusion bodies (IBs) within the cytoplasm when performed under conditions conducive to a high rate of protein translation such as high temperature, high inducer concentration and expression under control of a strong promoter (Singh *et al.*, 2015). In the current study, high-level expression resulted in the formation of insoluble, cytoplasmic inclusion bodies, evidenced by the absence of a band in the soluble fraction in the expected size range of ~37 kDa (Fig. 3.7). Solubilisation was attempted by treatment with Triton™ X-100 and urea. It was observed that urea treatment was more successful in the solubilisation of inclusion bodies in comparison to Triton™ X-100, as clearly shown in Fig. 3.8. Formation of insoluble protein aggregates may present both an obstacle and an advantage to the recovery of biologically active protein. Recovery of proteins localised to inclusion bodies during recombinant protein expression requires solubilisation of IBs and refolding of denatured proteins. Classic strategies for IB solubilisation include treatment with high concentrations of denaturants or chaotropic agents, such as urea or guanidine hydrochloride (Singh *et al.*, 2015).

Immobilised metal affinity chromatography (IMAC) is a form of affinity chromatography in which the resin contains an immobilised chelating agent complexed with metal ions. Protein purification and separation by IMAC are based on the interactions of amino acid side chains and metal ions chelated by the proteins with complexed metal ions (Hage, 1999). Purification of proteins by affinity chromatography is most commonly applied to proteins fused to an affinity tag, usually consisting of a series of histidine residues. This affinity tag, termed a "His-tag", is encoded by the expression vector and is fused to either the C- or N-terminus of the recombinant protein by inserting the protein CDS in-frame with repetitive histidine codons, available in several commercial expression vectors. In this study, a N-terminal hexa-histidine tag (His_6-tag) was fused to recombinant GAPDH (rGAPDH) using the vector pET-28b(+). Imidazole side chains of histidine residues display high affinity for metal ions resulting in the preferential binding of proteins with surface-exposed His residues to the resin (Bornhorst and Falke, 2000). Transition metal ions Cu^{2+}, Ni^{2+}, Zn^{2+}, Co^{2+} and Fe^{3+} are most commonly used as ligands for His-tagged proteins during IMAC

purification. The Ni^{2+}–NTA matrix has a high binding affinity (K_d = 10−13 M) for the His_6-tag at pH 8.0. By comparison, the Co^{2+}–CMA matrix has a lower affinity for the His_6-tag than the Ni^{2+}–NTA resin, but allows elution of target proteins under milder conditions. The use of Co^{2+}–CMA resin has also been reported to result in higher specific protein binding compared to Ni^{2+}–NTA resin, resulting in higher elution product purity (Bornhorst and Falke, 2000). Although His_6-tags are most commonly used, affinity tags consisting of varying lengths of His residues have been successfully used in the purification of His-tagged proteins (Gaberc-Porekar and Menart, 2001).

During purification of His_6-tagged rGAPDH by IMAC in this study, both Ni^{2+} - and Co^{2+}-complexed resins were used and compared. Solubilised protein was successfully purified using both Ni^{2+} - and Co^{2+}-complexed resins, although the use of Co^{2+}–CMA resin resulted in the elution of purer protein fractions in comparison to Ni^{2+}–NTA resin, as evidenced by SDS-PAGE visualisation (Fig. 3.8) showing a single band in the vicinity of the target protein's molecular weight (~37 kDa). This observation may be attributed to the lower affinity of Co^{2+}–CMA displayed for the His-tag, as well as less non-specific binding of protein by this resin compared to Ni^{2+}–NTA resin. The rGAPDH band appeared slightly higher than the expected molecular weight of 37 kDa on SDS-PAGE, but the identity of the recombinant protein was confirmed as GAPDH by LC-MS/MS with a molecular weight of 35,8 kDa.

Following solubilisation of rGAPDH using urea, an attempt was made to refold the protein by removing the denaturant by dialysis against TBS. To ascertain whether rGAPDH retained catalytic activity after solubilisation and refolding, a spectrophotometric enzyme activity assay was performed. The enzyme GAPDH follows two substrate reversible Michaelis-Menten kinetics with a random-ordered sequential bi-bi mechanism (Chassagnole *et al.*, 2002). From the results obtained in the activity assay, presented in Fig. 3.10, it is clear that the enzyme's activity was abolished by the denaturation and refolding process. A marked decrease in absorbance immediately after (0-10 s) addition of substrate G3P was observed. This phenomenon appears to have occurred independently of the enzyme, as this behaviour was observed in a negative control assay (with no rGAPDH) as well. However, this effect was more pronounced in the presence of a higher quantity of enzyme (0.5 μg) versus a lower quantity (0.05 μg). The cause of this anomaly is not known. The observed decrease in absorbance is not likely to be caused by the reverse reaction (i.e. oxidation of NADH to NAD^+), because arsenate in the reaction buffer inhibits the reverse reaction by replacing inorganic phosphate during the conversion of D-glyceraldehyde 3-phosphate to 1,3-bisphospho-D-glycerate, yielding 1-arseno-3-phosphoglycerate instead (Hughes, 2002). It needs to emphasised

that the aim of this chapter is not to characterise the enzymatic activity of *L. garvieae* rGAPDH, but to express the protein in order to potentially discover new interaction partners and functions not related to the native dehydrogenase activity of the enzyme. Therefore, the lack of enzyme activity of rGAPDH does not pose an impediment to the continuation of the current study.

3.5. CONCLUSION

The Gram-positive bacterial surface contains an assortment of proteins playing roles relevant to the lifestyle of the bacterium (Desvaux *et al.*, 2006). More than two decades ago, a *Streptococcus pyogenes* surface protein, streptococcal surface dehydrogenase (SDH), was identified as the cytoplasmic glycolytic enzyme GAPDH (Pancholi and Fischetti, 1992), which is considered one of the best studied moonlighting proteins in prokaryotes and eukaryotes (Giménez *et al.*, 2014). The multi-functionality of many so-called "house-keeping" enzymes in both Gram-positive and -negative organisms consequently came under scrutiny. One of the aims of the current study is to determine whether *L. garvieae* GAPDH plays a role in virulence, since GAPDH of a number of streptococcal pathogens is known to moonlight as a virulence factor (Pancholi and Fischetti, 1993; Terao *et al.*, 2006; Terrasse *et al.*, 2012; Seidler and Seidler, 2013; Giménez *et al.*, 2014). The aim of this chapter was fulfilled by the successful recombinant expression of *L. garvieae* GAPDH. An enzymatic activity assay indicated that the native GAPDH activity was lost during solubilisation of the recombinant protein expressed in insoluble inclusion bodies, suggesting that the protein did not refold correctly during the removal of the denaturant by dialysis. However, the loss of dehydrogenase activity is not expected to hinder the continuation of this study.

4. Identification of putative ligands to GAPDH using random peptide phage display

4.1. INTRODUCTION

Glyceraldehyde 3-phosphate dehydrogenase is considered the quintessential moonlighting protein (Tristan *et al.*, 2011; Giménez *et al.*, 2014). The involvement of GAPDH in virulence of streptococcal pathogens has been under investigation for over two decades (Pancholi and Fischetti, 1992; Bergmann, Rohde and Hammerschmidt, 2004; Terao *et al.*, 2006). Previously cited as surface dehydrogenase (Boël, Jin and Pancholi, 2005) or nephritis-associated plasminogen-binding receptor (NAPlr) (Abdelsalam *et al.*, 2015), the range of moonlighting functions of GAPDH extends beyond streptococcal pathogenesis. The functional diversity of GAPDH has been studied in probiotics (Kinoshita *et al.*, 2008; Kainulainen, 2012), plants (Zaffagnini *et al.*, 2013) and in both human normal cellular functions and disease (Sirover, 2005). Each different role requires interaction of GAPDH with novel ligands, and the different functions of GAPDH may also be influenced by post-translational modifications (Pancholi and Fischetti, 1993). Random peptide phage display is one of the oldest and most versatile combinatorial biology tools that has had significant impacts on the study of protein-protein interactions (Sidhu *et al.*, 2003).

The aim of this chapter is to use phage display technology to identify host proteins that potentially interact with *L. garvieae* GAPDH in order to elucidate the role of this moonlighting protein in the pathogenic processes of this bacterium.

4.2. MATERIALS AND METHODS

A commercial random dodecapeptide phage display system, Ph.D.™ Phage Display Library (New England BioLabs® Inc.), was used to identify peptides that interact with recombinant GAPDH. The Ph.D.™ Phage Display Library contains 1.0 x 10^{13} peptides of 12 amino acid residues in length expressed as peptides fused to the minor coat protein (pIII) of filamentous phage M13 (Cwirla *et al.*, 1990; Devlin *et al.*, 1990; Scott and Smith, 1990). The *E. coli* host strain K12 ER2738 [genotype

F´*proA*$^{+}$*B*$^{+}$ *lacI*^{q} Δ*(lacZ)M15 zzf::Tn*10(Tet^{R})*/ fhuA2 glnV* Δ*(lac-proAB) thi-1* Δ*(hsdS-mcrB)5*] (New England BioLabs® Inc.) was used during all phage propagation steps. This is the *fhuA2* (a ferric hydroxamate uptake receptor) encoding version of the strain *E. coli* NM522 in which the episomal fertility factor (F-factor) can be selected for using tetracycline. The fertility plasmid contains a tetracycline-resistance conferring mini-transposon [*Tn10*(Tet^{R})] that enables cultivation under selective conditions. Maintenance of the F-factor is essential for phage M13 propagation in *E. coli*, since M13 is a male-specific phage that infects *via* binding of pIII to the host's F-pilus encoded by the fertility episome (Karlsson, Borrebaeck and Nilsson, 2003). Thus, all cultures prepared for M13 propagation were cultivated in the presence of tetracycline to prevent loss of the plasmid encoding the F-factor. Further characteristics of the host strain include a mutation in the gene encoding the amber (UAG) stop codon tRNA (*glnV*), resulting in the insertion of glutamine instead of the amber stop codon during translation. Amber suppression is required for the propagation of M13 vectors (Oh *et al.*, 2007). A deletion in the gene encoding the *α*-fragment of *β*-galactosidase [Δ*(lacZ)M15*] enables differentiation between plaques formed by M13 library phages and environmental phages *via* blue/white selection.

4.2.1. Affinity selection

A solution of 50 μg.mL^{-1} purified rGAPDH in TBS (pH 7.5) was prepared and 150 μL was added to the wells of a 96-well microtitre plate. The plate was swirled until the surfaces of the wells were covered and incubated overnight at 4°C with mild agitation (30 rpm) in a humidified container. The coating solution was poured off from each plate and residual solution was removed by inverting and firmly tapping the plate on a clean paper towel. Each well was completely filled with blocking buffer [0.1 M $NaHCO_3$ (pH 8.6), 5 mg.mL^{-1} BSA] and incubated at 4°C for 1 h. Blocking buffer was discarded in a similar manner to the coating solution and wells were washed 6 times with TBST (TBS + 0 .1% [v/v] Tween®-20). A 100x representation of the phage library was diluted in 100 μL TBST and pipetted into the coated wells followed by gentle rocking of the plate for 45 min at RT. Non-binding phages were poured off and residue was removed by inverting and firmly tapping the plate on a clean paper towel. Wells were washed 10 times with TBST, followed by elution of binding phages by addition of 100 μL 0.2 M Glycine-HCl (pH 2 .2), 1 mg.mL^{-1} BSA per well and gently rocking the plate for 15 min. Eluate from each well was transferred to separate microcentrifuge tubes and neutralised by addition of 15 μL of 1 M Tris-HCl (pH 9.1). A second and third round of panning was performed as described above, using the amplified eluate of the preceding round as input and increasing Tween®-20 concentration to 0.5 % during the washing steps.

4.2.2. Phage titering

A titering culture was prepared by inoculating 10 mL LB medium supplemented with tetracycline with ER2738 and incubating at 37°C with vigorous shaking until mid-log phase was reached (OD_{600} = ±0.5). Top agar was melted in a conventional microwave and 3 mL was dispensed into sterile test tubes and maintained at 45°C to prevent re-solidifying of the agar. A volume of 1 μL eluate was used for titering. To carry out infection, 10 μL of serially diluted (10^{-1}- 10^{-4}) phage eluates in LB medium was added to 200 μL of ER2738 at mid-log phase of growth in microcentrifuge tubes and incubated at RT for 5 min. Infected cultures were transferred to molten top agar, vortexed briefly and immediately poured onto pre-warmed (37°C) LB/IPTG/X-Gal plates. Plates were tilted to spread the top agar evenly, allowed to cool for 5 min and incubated in an inverted position at 37°C overnight. Plaques on plates containing ± 100 plaques were counted the following day. The number of plaques was multiplied by the dilution factor to obtain the phage titer in pfu.10 μL^{-1}. The phage titre was converted to pfu.mL^{-1} by multiplying with a factor of 10^2.

4.2.2.1. Phage amplification

A culture for amplification of eluted phage was prepared by inoculating 20 mL LB medium and incubating at 37°C until early logarithmic phase (OD_{600} = 0.01–0.05). The remaining eluate was added to the early log phase culture and incubated with vigorous shaking at 37°C for 4.5 h. Cells were pelleted by centrifugation for 10 min at 12 000 x*g* at 4°C and the supernatant transferred to a clean centrifuge tube and centrifuged again. Precipitation of phages was performed by transferring the upper 80% of the supernatant to a clean tube, adding 1/6 volume of 20% poly-ethylene glycol (PEG)/2.5 M NaCl and storing at 4°C overnight. The phage and PEG precipitation mixture was centrifuged at 12 000 x*g* for 15 min at 4°C, the supernatant discarded and the phage pellet suspended in 1 mL TBS. The suspension was centrifuged at 14 000 x *g* for 5 min at 4°C to remove residual cells and the phage supernatant was re-precipitated and suspended in 200 μL TBS. This amplified eluate was titered on LB/IPTG/X-Gal plates as described in Section 4.2.2. Blue plaques were counted to determine phage titer.

To prepare individual phage clones for further characterisation by sequencing, an overnight culture of the host bacterium was diluted 1:100 in LB medium. A sterile pipette tip was used to stab a blue plaque from a titering plate and transferred to a test tube containing 1 mL of the diluted overnight culture and incubated at 37°C for 4.5 h with shaking.

4.2.3. Sequencing of phage DNA and similarity search

Single-stranded DNA templates from individual phage clones were prepared for sequencing (Wilson, 1993). After incubation, cultures were transferred to microcentrifuge tubes and centrifuged at 20 000 x*g* for 30 s and 500 μL of the supernatant was transferred to a new microcentrifuge tube. Phage virions were precipitated by adding 200 µ*L* 20% PEG/2.5 M NaCl and incubated at room temperature for 15 min. The precipitate was centrifuged (20 000 x*g*; 10 min; 4°C) and the supernatant was discarded. To precipitate phage DNA, a volume of 100 μL potassium iodide buffer (pH 8.0) was added to the phage pellet, the tube was vigorously tapped to resuspend the pellet, 250 μL absolute ethanol was added and the mixture was incubated for 15 min at room temperature. The precipitate was centrifuged (20 000 x*g*; 10 min; 4°C) and the supernatant was discarded, followed by washing of the pellet with 500 μL of 70% ethanol (-20°C) and re-centrifugation. The DNA pellet was dried under vacuum at 45°C for 5 min and re-suspended in 30 μL TE buffer (Table A.1, Appendix I). Insert DNA was sequenced using the -96 gIII sequencing primer supplied with the kit. Sequence data was viewed using Geneious version 9 software (http://www.geneious.com, Kearse *et al*., 2012). The 36 bp library insert, located between the *Kpn*I/*Acc*65I and *Eag*I restriction sites indicated in Fig. 4.1, was extracted from the sequence and translated. The peptides were also subjected to a protein-protein BLAST (blastp) search (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins; Altschul *et al.*, 1990) to identify host proteins with similarity to peptides recovered from biopanning against rGAPDH. The NCBI Non-redundant protein sequences database (nr) was queried and results were restricted to the phylum Chordata, to include only proteins from higher eukaryotes in the results. Algorithm parameters were automatically adjusted to search for a short input sequence. The expect value (*E*-value) cut-off was automatically adjusted to 20 000 and PAM30 was selected as a scoring matrix.

4.3. RESULTS

4.3.1. Phage titering

Blue tinted plaques with diameters ranging from 0.5 - 2 mm were observed upon titering of phage eluates. Results of the affinity selection process are summarised in Table 4.1. After the first round of biopanning against rGAPDH, 2.5 x 10^6 pfu.mL^{-1} bound phages were retrieved. A second round of biopanning yielded 4.0 x 10^7 pfu.mL^{-1} phages and after a third round, 5.6 x 10^6 pfu.mL^{-1} bound phages were retrieved. From the observed 4 log-fold increase in recovery rates from the

first to the second round it can be inferred that the library was depleted of the majority of non-binding phages after a single round of biopanning. During round 2 and 3, a five-fold higher concentration of Tween®-20 in the washing buffer was used to increase stringency. However, the higher stringency of selection in the last two rounds did not lower the recovery rate.

Table 4.1: Phage titering results following affinity selection rounds. Recovery percentage represents the ratio of recovered phages to input phages.

Biopanning round	Input (pfu.mL^{-1})	Output (pfu.mL^{-1})	Recovery (%)	Enrichment (fold)
1	1.0×10^{13}	2.5×10^{6}	2.5×10^{-5}	1
2	4.0×10^{10}	4.0×10^{7}	1×10^{-1}	4000
3	4.0×10^{10}	5.6×10^{6}	1.4×10^{-2}	560

4.3.2. Insert sequence analysis and similarity search

DNA sequencing of library inserts recovered after second and third rounds of affinity selection yielded a recurring translated peptide motif DYHDPSLPTLRK after the third round of affinity selection. This motif was present in 5 out of 29 clones (17.2%) sequenced after either two or three rounds of biopanning. The remaining peptides from round 2 and 3 did not yield any clear consensus sequence. Four additional sequences displayed similar but non-identical translated motifs [DYQDPSLPTLRK (*n*=2), DFHDPSLPTLRK, DYHNPSLPTLRK, DYLDQGLPTLRK], with variations occurring mainly between amino acid positions 2–5. All phage clone sequences retrieved with selected blastp results, *E*-values and percentage identity are tabulated in Table B.1 (Appendix II). The similarity search yielded high *E*-values, upward of 10, for numerous host proteins. A total of 132 putative rGAPDH ligands, identified by biopanning and similarity search, were grouped into 40 functional categories. The distribution of functional categories among matched host proteins (*E*-values less than 100) is illustrated in Fig. 4.2.

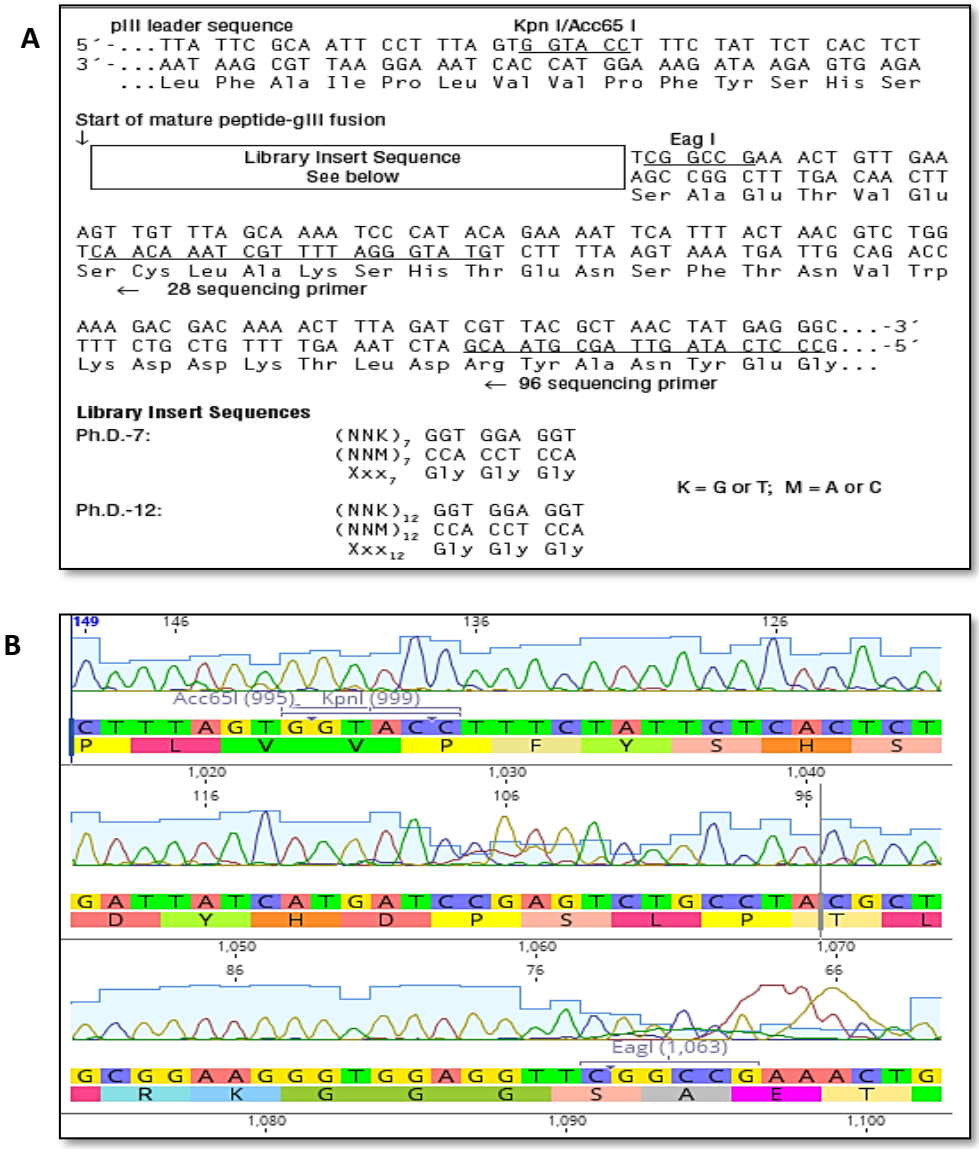


Figure 4.1: (A) Nucleotide sequence of random peptide library insert-gIII fusions. The -28 and -96 sequencing primer binding sites are indicated, as well as restriction endonuclease recognition sites of *Kpn*I, *Acc*65I and *Eag*I. Library insert sequences, consisting of 12 random peptides followed by a triple glycine motif, are also illustrated. (B) Sequencing data obtained from sequencing phage clone G31. The three restriction endonuclease recognition sites of *Kpn*I, *Acc*65I and *Eag*I are indicated as reference points.

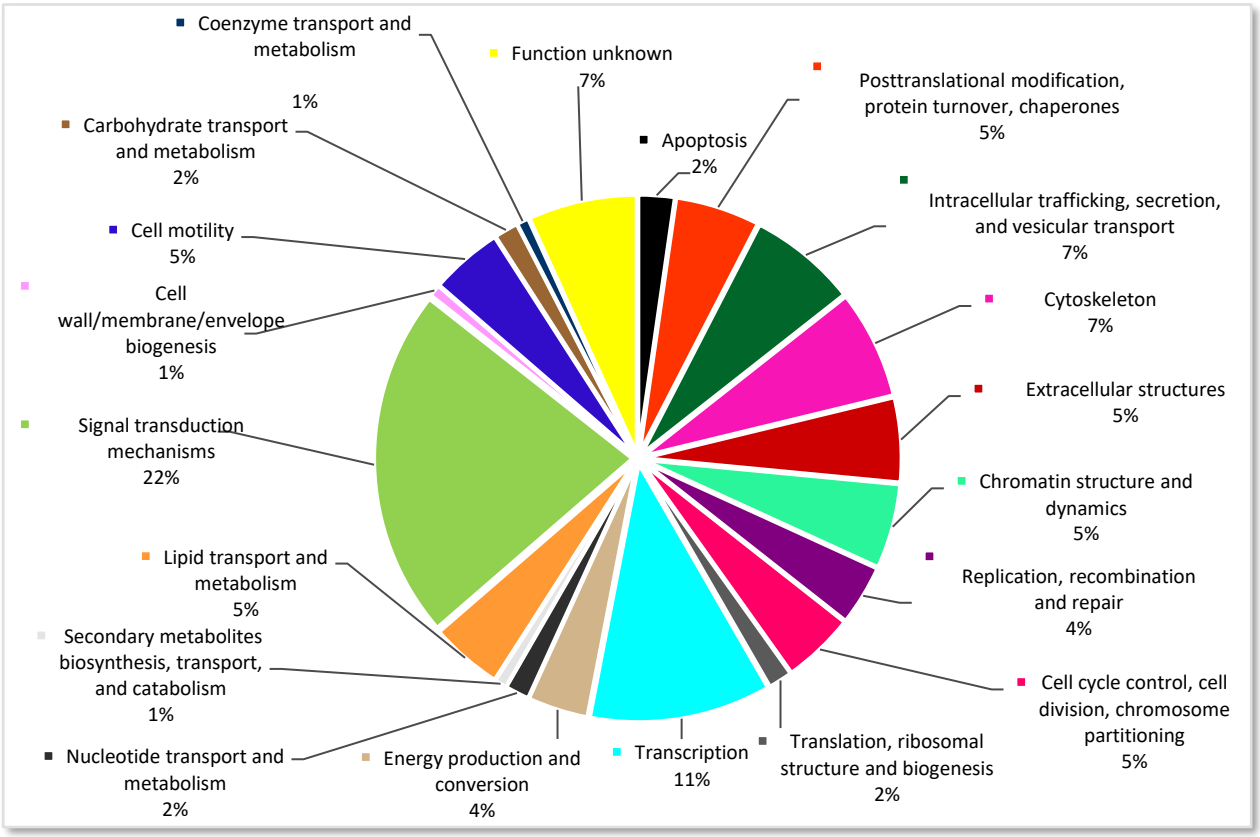


Figure 4.2: Host proteins (E < 100), classified by functional groups, matched to peptides obtained from biopanning against rGAPDH.

4.4. DISCUSSION

Glyceraldehyde 3-phosphate dehydrogenase is a multi-functional enzyme that operates in numerous cellular processes through the phenomenon of protein moonlighting. In this study, a diverse range of host proteins belonging to 40 functional categories were matched to peptides obtained by biopanning against rGAPDH, as illustrated in Fig. 4.2. The most prevalent functional category among host proteins identified was signal transduction (22%). A redox-sensitive cysteine residue that undergoes *S*-thiolation during oxidative stress (Grant *et al.*, 1999) enables GAPDH to regulate signalling pathways in response to oxidative stress. Binding of GAPDH to inositol 1,4,5-trisphosphate receptor, involving cysteine-150 of GAPDH, results in the localised production of NADH eliciting Ca^{2+} release, a finding that provides a link between cellular energetics and Ca^{2+} signalling (Patterson *et al.*, 2005)

With regards to bacterial virulence, GAPDH is most frequently associated with adhesion to host tissues (Pancholi and Fischetti, 1992; Seifert *et al.*, 2003; Bergmann *et al.*, 2004; Jin *et al.*, 2005; Seidler and Seidler, 2013). The functional category of extracellular structures accounts for 5% of host proteins identified, as illustrated in Fig. 4.3. The present study used random peptide phage display to identify several host extracellular matrix (ECM) components as possible ligands of *L. garvieae* rGAPDH. Host ECM proteins are closely associated with eukaryotic cells and are distributed extensively among tissues.

Collagens are a family of glycosylated ECM proteins regarded as the most abundant proteins in metazoans (Exposito *et al.*, 2010). Results identified collagen type VI, VII, XI and XVIII as possible ligands to rGAPDH. The peptide DYQDPSL that occurs in phage clones G34 and G318 matched collagen α-2(XI). Type XI collagen is a minor fibrillar collagen widely distributed in cartilage, skeletal bone, vitreous humour of the eye and neoepithelium of the brain (Luo and Karsdal, 2016). Peptide GTADRALHKE (phage clone G317) was matched to collagen α-6(VI). As a unique beaded filament collagen, Type VI collagen forms a microfibrillar network between the basement membrane and interstitial matrix and is expressed in the ECM of the blood vessels, cornea, cartilage, intervertebral discs and skeletal muscle (Sun and Karsdal, 2016). Two peptide sequences, HLSEATQ and SKGDPHQPG, obtained from phage clones G27 and Gb34 respectively, were matched to collagen α-1(VII). Collagen type VII is a major constituent of anchoring fibrils, the attachment complexes of epithelia in various tissues including oral mucosa, cornea and skin (Chung and Uitto, 2010). Peptide WFWSMDP (phage clone G38) was matched to collagen α-1(XVIII), a basement membrane proteoglycan expressed in vascular and epithelial tissues (Seppinen and Pihlajaniemi, 2011). Laminin is another ECM protein that is found primarily in basement membranes underlying epithelial and endothelial cells. The peptide GDPHQPGG, occurring in phage clone Gb34, was matched to laminin subunit β2. Binding of extracellular GAPDH to laminin has been described in fungal pathogens *Candida albicans* (Gozalbo *et al.*, 1998) and *Paracoccidioides brasiliensis* (Barbosa *et al.*, 2006). One phage clone, G319, was matched to another ubiquitous ECM protein, mucin. Mucin-binding mediated by GAPDH in probiotic bacteria *Lactobacillus plantarum* (Glenting *et al.*, 2013) and *Lactobacillus acidophilus* (Patel *et al.*, 2016) plays an important role in colonisation of the mucin-rich intestine.

Results of this study also identified ligands of GAPDH not relevant to bacterial pathogenesis, reflecting functions performed by eukaryotic GAPDH in eukaryotic cells. The most prevalent peptide, DYHDPSLPTLRK, was matched to the protein sacsin that has been shown to function as a

molecular chaperone (Anderson *et al.*, 2011) responsible for the prevention of protein misfolding. Protein misfolding, leading to aggregration of non-native proteins in the cell, has been implicated in the pathology of various neurodegenerative disorders in humans, including Alzheimer's, Huntington's and Parkinson's disease (Barral *et al.*, 2004; Anderson *et al.*, 2011). Several mutations in the gene that encodes sacsin is responsible for autosomal recessive spastic ataxia of Charlevoix-Saguenay, another neurodegenerative disorder in humans (Kozlov *et al.*, 2011). Although there is no evidence to link GAPDH directly to the pathology of spastic ataxia, a link exists between GAPDH and neurodegenerative disorders in humans. Studies of polyglutamine-repeat neurodegenerative disorders have offered ample evidence to support the role of GAPDH in neurodegeneration. Expanded polyglutamine-repeats occurring in disease proteins selectively bind to various proteins, including GAPDH (Berry, 2004), resulting in the build-up of protein aggregates in the nucleus that may trigger apoptosis (Klement *et al.*, 1998). Knockdown of the molecular chaperone sacsin in tissue culture led to increased toxicity in cells expressing polyglutamine-expanded ataxin, suggesting that sacsin provides protection against mutated ataxin proteins (Parfitt *et al.*, 2009).

The involvement of GAPDH in cell apoptosis is well-documented (Hara *et al.*, 2005; Hara and Snyder, 2006; Colell *et al.*, 2009; Oliveira *et al.*, 2012). In eukaryotic cells, nuclear translocation of GAPDH is associated with apoptotic cell death (House *et al.*, 2003). It has been shown that generation of nitric oxide by nitric oxide synthase stimulates *S*-nitrosylation of GAPDH, prompting binding to Siah-1 (an E3 ubiquitin ligase) that contains a nuclear localisation signal enabling transport into the nucleus. GAPDH functions in the stabilisation of Siah-1, thereby increasing levels of Siah-1 in the nucleus where it degrades various nuclear proteins, leading to cell death (Hara *et al.*, 2005). Peptides from five respective phage clones were matched to E3 ubiquitin-protein ligases.

Several limitations of this study need to be taken into consideration. Affinity selection using a random peptide phage display library can only aid in the identification of protein ligands of the target. Peptides expressed in the Ph.D-12 library are of a random nature, they may not bear resemblance to any ligands expressed *in vivo*, particularly if the site of interaction consists of discontinuous areas of the primary sequence. Natural ligands of the target may be lost during the initial rounds of biopanning, since the affinity selection procedure enriches ligands with high affinity to the peptide. The short length of the peptides obtained from biopanning (12 aa) means that peptides can not be matched to host proteins with absolute certainty. Therefore, it can not be stated with absolute confidence that the host proteins identified in this study are GAPDH ligands. Very high *E*-values were obtained during blastp similarity searches, even though algorithm

parameters were automatically adjusted to search for a short input sequence. Query sequence length and database size greatly influence *E*-values (Altschul, 2006). Thus the extremely short length of the query sequence and the large size of the database queried likely contributed to the high *E*-values obtained.

The recombinantly produced target molecule used in this study, rGAPDH, could not be expressed in the enzymatically active form. While the effect of the activity of the target protein on the validity of the affinity selection process is not known, several ligands with documented *in vivo* GAPDH interaction were identified in the current study. This may be explained by the fact that the numerous moonlighting functions of GAPDH are unrelated to the native dehydrogenase activity of the enzyme, implying that the additional functions of GAPDH are not dependent upon its native conformation and that the active site likely does not partake in these non-native functions.

4.5. CONCLUSION

Since the foundations of phage display technology were laid in 1985 (Smith, 1985), this technique has been successfully applied to various areas in research. The present study exploited the ability of phage display to identify protein ligands to a specific target molecule. In the present study, the multifunctional glycolytic enzyme GAPDH from *L. garvieae* was recombinantly expressed in *E. coli* and used as the target molecule during an affinity selection process. The diversity of possible binding partners identified in this study reflects the multi-functionality of GAPDH in different cellular milieus. The putative ligands of GAPDH identified that are relevant to bacterial pathogenesis, including adhesion to host ECM proteins, are consistent with the reported roles of GAPDH in streptococcal virulence. Other putative ligands, reflecting the roles of eukaryotic GAPDH in normal and diseased cell function, strongly suggest a link between eukaryotic GAPDH in apoptotic processes in neuronal cells, and its subsequent involvement in neurodegenerative disorders in humans. The results obtained in this study indicate that GAPDH may contribute to *L. garvieae* virulence, although only as a virulence lifestyle factor, aiding adhesion to and colonisation of various host tissues but not causing direct damage to the host.

5. General discussion, conclusions and future outlook

The extracellular polysaccharide (EPS) capsule plays a significant role in the virulence and serology of various Gram-positive pathogens and literature cites this as the main virulence factor of *L. garvieae.* This study therefore aimed to characterise the EPS capsules of South African *L. garvieae* isolated from rainbow trout (*Oncorhynchus mykiss*). Unexpectedly, the EPS capsule was not detected in any of the isolates tested in this study. These results indicate that the EPS biosynthesis cluster could have been lost during repeated passaging of these strains under non-selective conditions that is comparable to the case of the KG^+ (non-capsulated) reference strain ATCC® 49156 isolated from diseased yellow tail (Miyauchi *et al.*, 2012). Alternatively, these results could also support the findings of recent research that has shown the capability of non-capsulated isolates to cause disease and mortality in fish, similar to capsulated counterparts (Türe and Altinok, 2016). The current pathogenicity of the strains used in this study is unknown. A set of putative virulence factors, including three haemolysins, were detected in all the isolates from diseased fish as well as the avirulent isolate from raw milk used in this study, *L. garvieae* NCFB 657. From the detection of these genes in piscine and milk isolates it can be concluded that either this set of virulence factors do not contribute to the pathogenicity of *L. garvieae* as true virulence factors (hence the presence thereof in NCFB 657), or that these genes are differentially expressed in piscine and milk isolates. Identification of extracellular proteins of these strains detected lactate dehydrogenase, previously identified as an antigen common to both KG+ and KG- serotypes (Shin *et al.*, 2009), in a piscine strain (A3) but not in NCFB 657. Given its antigenicity (implying extracellular localization) in both strains, coupled with the inability to be detected in the supernatant of NCFB657, these results could again point to differential expression of lactate dehydrogenase in A3 and NCFB 657.

Additional studies regarding differential expression of *L. garvieae* virulence genes under both *in vitro* and *in vivo* conditions could provide answers to the questions raised in this study. The increasing availability of full genome sequences of *L. garvieae* offer extraordinary insights into the virulence factors, epidemiology and serology of this emerging pathogen, and should be employed to

expand the search for previously uncharacterised virulence factors, especially true virulence factors.

The glycolytic enzyme GAPDH is a renowned moonlighting protein implicated in the virulence processes of various microbial pathogens. In order to identify interaction partners and possible moonlighting functions of GAPDH, the protein was expressed recombinantly in *E. coli.* After solubilisation of inclusion bodies formed in the cytoplasm due to high levels of expression, the purified recombinant GAPDH (rGAPDH) did not retain native catalytic activity.

The possible role of GAPDH in *L. garvieae* pathogenesis was investigated by random peptide phage display, in the hope of discovering host ligands to GAPDH that could elucidate an additional function of this moonlighting protein. Among the possible ligands to rGAPDH that were identified were vertebrate extracellular matrix (ECM) proteins collagen (Type VI, VII, XI and XVIII), laminin and mucin. These findings indicate that GAPDH may act as an adhesion, facilitating colonisation and persistence of the bacterium in the host, a notion that is consistent with known functions of extracellular GAPDH in other streptococcal pathogens (Brassard *et al.*, 2004; Seidler and Seidler, 2013). The results can be correlated with symptoms and post-mortem observations of *L. garvieae* infections in fish and humans. The mucins and collagens identified can be found in tissues and organs affected by *L. garvieae* infection: cartilage, skeletal bone, vitreous humour of the eye, brain epithelium, blood vessels, intervertebral discs, muscle and oral mucosa. In both fish and humans, the most probable route of infection is the oral route followed by colonisation and invasion of the gastro-intestinal tract. Indeed, reports of *L. garvieae* infection in humans highlight ingestion of raw fish by patients coupled with the presence of predisposing pathologies of the gastro-intestinal tract as major risk factors (Chan *et al.*, 2011; Gibello *et al.*, 2016). Systemic and localised infection follows when bacteria enter the blood stream *via* compromised sites in the gastro-intestinal tract (Chan *et al.*, 2011; Gibello *et al.*, 2016). Exophthalmia and acute meningitis in fish can be correlated to the ability of the pathogen to bind collagens in vitreous humour and brain epithelium. Clinical manifestations of infections in humans, typically endocarditis (Navas *et al.*, 2013; Heras Cañas *et al.*, 2015), infective spondylodiscitis (Chan *et al.*, 2011) and cerebral infarction (Li *et al.*, 2008), can be correlated to the pathogen's ability to bind collagens in cardiac tissue, cartilage, intervertebral discs and brain epithelium. The high degree of conservation of GAPDH protein sequences can be responsible for the identification of interaction partners relevant to both prokaryotic and eukaryotic cellular processes in this study. The most prominent function of eukaryotic GAPDH identified by phage display in this study is its involvement in signal transduction, with 22% of host

proteins that were matched to peptides obtained by biopanning against rGAPDH belonging to this functional category. Another interesting moonlighting function of eukaryotic GAPDH identified by phage display is its involvement in protein folding and apoptosis. In this study, the most prevalent peptide expressed in phages binding to rGAPDH was matched to sacsin, among other host proteins. Sacsin is a molecular chaperone involved in protein folding and mutated versions of this protein is responsible for a neurodegenerative disease called autosomal recessive spastic ataxia of Charlevoix-Saguenay (Anderson *et al.*, 2011; Kozlov *et al.*, 2011). The association of GAPDH with a molecular chaperone is relevant as the involvement of GAPDH with neurodegenerative disorders caused by the aggregation of misfolded proteins is well-established (Barral *et al.*, 2004; Berry, 2004).

In the current study, GAPDH was not detected in culture supernatants of any of the isolates involved in this study. This finding could indicate that extracellular GAPDH is tightly associated with the cell surface of *L. garvieae,* perhaps in a similar manner to the reported association of GAPDH and peptidoglycan in *Streptococcus pneumoniae.* Alternatively, the absence of GAPDH in the extracellular fraction could simply indicate that GAPDH was not released extracellularly in sufficient quantities to be visualised by SDS-PAGE. It can also be considered that GAPDH is not released extracellularly under *in vitro* conditions, since its main reported moonlighting functions are related to colonisation of and persistence in host tissues during infection. The extracellular locality of GAPDH and its extracellular release during *in vitro* versus *in vivo* conditions may prove to be interesting avenues of future research.

The wide variety of GAPDH interaction partners identified in this study not only serves to highlight the extreme functional diversity of this moonlighting protein, but also points to the versatility of random peptide phage display. However, the functional diversity and highly conserved nature of GAPDH coupled with the power of phage display technology might have contributed to the lack of definitive answers garnered by this study. This study succeeded in identifying GAPDH from *L. garvieae* as a possible microbial surface component recognizing adhesive matrix molecules (MSCRAMM), although it needs to be acknowledged that these results do not conclusively prove the involvement of this protein in the pathogenesis of this specific bacterium. Since GAPDH is highly conserved, even more so in closely related streptococci, the results may simply reflect the already proven ability of other streptococcal GAPDHs to function as adhesins. Studies on GAPDHs of closely related streptococcal species have been able to prove that this protein plays a role in virulence and is even essential to virulence (Terao *et al.*, 2006;

Madureira *et al.*, 2007; Oliveira *et al.*, 2012; Terrasse *et al.*, 2012, 2015). Similar *in vivo* studies using *L. garvieae* strains engineered to inhibit GAPDH export will need to be performed in order to ascertain the involvement of GAPDH in virulence. The high level of interspecies and inter-kingdom conservation of GAPDH may represent an obstacle in the current study, but it certainly can be exploited in further studies. Given the immunogenicity and high level of conservation of GAPDH, the possibility of a multi-purpose vaccine against lactococcosis and other bacterial fish diseases can be investigated in future.

Summary

Bacterial infections cause severe economic loss in the rapidly growing aquaculture industry. *Lactococcus garvieae,* a Gram-positive bacterium, causes lethal, hyperacute septicaemia in numerous fish species of importance to aquaculture worldwide. The virulence factors of this pathogen is not well understood, although the exopolysaccharide (EPS) capsule is considered the most important virulence determinant. A conserved metabolic enzyme, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), may function as a virulence factor of *L. garvieae via* the phenomenon of "moonlighting", a term that refers to the ability of a protein to perform more than one function. Previous investigations have also identified *L. garvieae* GAPDH as an antigen, suggesting that GAPDH is localised to the extracellular environment. The general aim of this study was to improve the current understanding of factors contributing to *L. garvieae* virulence by means of the following objectives:

- Identify putative virulence factor genes of South African *L. garvieae* isolates by PCR and sequencing.
- Identify extracellular proteins from South African *L. garvieae* isolates.
- Clone, express and purify *L. garvieae* GAPDH.
- Identify putative ligands to recombinant GAPDH using a commercially available random peptide phage display library.

Isolates obtained from diseased rainbow trout (*Oncorynchus mykiss*) from locations in present day Gauteng and Mpumalanga, South Africa, were screened for the presence of a set of putative virulence factor genes by PCR. The presence of EPS capsules were tested by negative staining and amplification of EPS biosynthesis genes by PCR. All virulence factor genes were found in the isolates tested, including an avirulent strain NCFB 657. However, negative staining indicated the absence of EPS capsules in the virulent isolates and NCFB 657 and EPS biosynthesis genes could not be amplified. Extracellular proteins detected and identified by LC/MS-MS included L-lactate dehydrogenase, enolase, 60 kDa chaperonin and phosphoenolpyruvate-protein phosphotransferase. The metabolic enzyme enolase is known to moonlight as a virulence factor in several streptococcal pathogens, similar to GAPDH.

The GAPDH of *L. garvieae* was successfully cloned and expressed *via* growth in auto-induction media as insoluble inclusion bodies using *Escherichia coli* BL21 (DE3) as expression host, although native dehydrogenase activity was abolished following denaturation, refolding and purification. A random peptide library was panned against inactive recombinant GAPDH in order to identify possible ligands. After three rounds of biopanning, a recurring peptide motif (DYHDPSLPTLRK) was detected among the sequences binding to rGAPDH. Host proteins matching retrieved peptide sequences exhibited diverse functions related to bacterial pathogenesis as well as normal eukaryotic cell function. The possible ligands to rGAPDH that may be relevant to *L. garvieae* pathogenesis, collagen, mucin and laminin, are consistent with the role of GAPDH as adhesin in streptococcal pathogens. Functional categories related to eukaryotic cell functions were, however, more prevalent among peptides obtained from biopanning against rGAPDH. The two most prevalent categories included signal transduction mechanisms and transcription.

Various disadvantages of using random peptide phage display for the discovery of protein ligands need to be considered when interpreting results obtained by this study. Short query sequences searched against an expansive protein database does not yield conclusive results; therefore, all the host proteins identified by similarity search in this study should not be viewed as undisputed ligands of GAPDH. Further studies will need to be conducted in order to prove the *in vitro* and *in vivo* interaction between *L. garvieae* GAPDH and host proteins identified in this study, but these results may suggest preliminarily that GAPDH is involved in *L. garvieae* pathogenesis as an adhesin. Bacterial virulence is a multifactorial process dependent upon host and environmental influences. This study has shown that the capacity of *L. garvieae* to cause disease should not be linked to a sole lifestyle virulence factor, such as an EPS capsule.

A. Appendix I

Table A.1: Composition of buffers used in this study

Buffer	Components	Concentration
Elution buffer	Tris-HCl (pH 7.4)	50 mM
	NaCl	0.5 M
	Imidazole	0.5 M
Binding buffer	Tris-HCl (pH 7.4)	50 mM
	NaCl	0.5 M
	Imidazole	20 mM
Blocking buffer	$NaHCO_3$ (pH 8 .6)	0.1 M
	BSA	5 $mg.mL^{-1}$
Buffer *Eco*RI (1x) (Thermo Scientific™)	Tris-HCl (pH7.5)	50 mM
	$MgCl_2$	10 mM
	NaCl	100 mM
	Triton X-100	0.02% (v/v)
	BSA	0.1 $mg.mL^{-1}$
Buffer Orange (O) (1x) (Thermo Scientific™)	Tris-HCl (pH7.5)	50 mM
	$MgCl_2$	10 mM
	NaCl	100 mM
	BSA	0.1 $mg.mL^{-1}$
Saline sodium citrate buffer (SSC) (20x)	NaCl	175.3 $g.L^{-1}$
	$Na_3C_6O_7.2H_2O$	88.2 $g.L^{-1}$
	SDS	1 $g.L^{-1}$
STET Buffer	Sucrose	8% w/v
	Triton X-100	5% v/v
	EDTA	50 mM
	Tris-HCl (pH 8.0)	50 mM
Transformation buffer *	HEPES (pH 6.7)	10 mM
	$CaCl_2$	15 mM
	KCl	250 mM
	$MnCl_2$	55 mM
Tris-acetate-EDTA (TE) Buffer	Tris	0.1 M
	EDTA	0.05 M
Tris-buffered saline	Tris-HCl (pH 7 .5)	50 mM
	NaCl	150 mM

All buffers were autoclaved for 15 min at 120 °C prior to use, unless stated otherwise.

***filter sterilised**

Table A.2: Composition of media used in this study

Media	Components	Concentration
LB	Tryptone	10 $g.L^{-1}$
	NaCl	10 $g.L^{-1}$
	Yeast Extract	5 $g.L^{-1}$
AIX agar	Tryptone	10 $g.L^{-1}$
	NaCl	10 $g.L^{-1}$
	Yeast Extract	5 $g.L^{-1}$
	Bacteriological agar	15 $g.L^{-1}$
	Ampicillin*	50 $\mu g.mL^{-1}$
	X-Gal*	20 $\mu g.mL^{-1}$
	IPTG*	4.8 $\mu g.mL^{-1}$
ZYP5052 Auto-induction Medium	Tryptone	10 $g.L^{-1}$
	Yeast extract	5 $g.L^{-1}$
	$(NH_4)_2SO_4$*	25 mM
	KH_2PO_4*	50 mM
	Na_2HPO_4*	50 mM
	Glycerol*	5 $g.L^{-1}$
	Glucose*	0.5 $g.L^{-1}$
	α-lactose*	2 $g.L^{-1}$
	$MgSO_4$*	2 mM

All media were autoclaved for 15 min at 120 °C prior to use.

***added after autoclaving.**

Table A.3: Composition of solutions used in this study

Solution	Components	Concentration
Fairbanks A staining solution	Isopropanol	25% (v/v)
	Acetic acid	10% (v/v)
	Coomassie Brilliant Blue R-250	0.05% (w/v)
Fairbanks D destaining solution	Acetic acid	10% (v/v)
Dithiothreitol (DTT)	NH_4HCO_3	0.1 M
	DTT	10 mM
Trypsin		500 $ng.\mu L^{-1}$
Digestion Solution	NH_4HCO_3	100 mM
	Trypsin	10 $\mu g.mL^{-1}$

B. Appendix II

Table B.1: Similarity search (blastp) results of peptides obtained from biopanning against rGAPDH

Phage Clone	Amino acid sequence	blastp result	Query Subject	*E* value	Identity (%)
G31, G33, G313, G315 & G316	DYHDPSLPTLRK	PREDICTED: testis, prostate and placenta-expressed protein isoform X2 [*Ornithorhynchus anatinus*]	^{2}YHDPSLPTLR^{11} ^{50}YH-PSLPTLR^{58}	8.9	90
		Sacsin [*Lonchura striata domestica*]	^{4}DPSLPTLR^{11} ^{3726}DPSLPTLR^{3733}	12	100
		PREDICTED: TBC1 domain family member 8B isoform X1 [*Dasypus novemcinctus*]	^{2}YHDPSLPTL^{10} ^{825}YHDPSLPYL^{833}	25	89
		NUAK family SNF1-like kinase 2, partial [*Picoides pubescens*]	^{3}HDP-SLPTLRK^{12} ^{428}QDPQSLPTLRK^{438}	25	82
		PREDICTED: DNA repair protein XRCC4 isoform X1 [*Loxodonta africana*]	^{3}HDPSLP-TLRK^{12} ^{317}HDPSLPQTLKK^{327}	25	82
G34 & G318	DYQDPSLPTLRK	PREDICTED: NUAK family SNF1-like kinase 2 [*Picoides pubescens*]	^{3}QDP-SLPTLRK^{12} ^{435}QDPQSLPTLRK^{445}	2.2	91
		PREDICTED: sacsin-like [*Taeniopygia guttata*]	^{4}DPSLPTLR^{11} ^{3287}DPSLPTLR^{3294}	12	100
		serine/threonine-protein phosphatase 4 regulatory subunit 3 [*Xenopus laevis*]	^{1}DYQDPSLPTLRK^{12} ^{225}EY-DPSLPTQRK ^{235}	36	75
		PREDICTED: crossover junction endonuclease MUS81 isoform X1 [*Condylura cristata*]	^{3}QDPSLPTLRK^{12} ^{112}QDPPLPVLRK^{121}	36	80
		PREDICTED: collagen alpha-2(XI) chain isoform X1 [*Sarcophilus harrisii*]	^{1}DYQDPSL^{7} ^{290}DYQDPSL^{296}	51	100
		PREDICTED: WASH complex subunit strumpellin [*Salmo salar*]	^{2}YQDPSLP^{8} ^{995}YQDPSLP^{1001}	51	100
		PREDICTED: calcineurin-binding protein cabin-1 [*Ictidomys tridecemlineatus*]	^{1}DYQDPSL^{7} ^{302}DYQDPSL^{308}	51	100
Gb37	DFHDPSLPTLRK	mediator of RNA polymerase II transcription subunit 14 (predicted)	^{2}FHDPSLPTL^{10} ^{373}FHDPSLPAL^{381}	6.2	89

		[*Otolemur garnettii*]			
		PREDICTED: sacsin-like [*Taeniopygia guttata*]	[4]DPSLPTLR[11] [3287]DPSLPTLR[3294]	12	100
		PREDICTED: obscurin [*Esox lucius*]	[1]DFHDPS-LPTLRK[12] [3212]DFHDRSILPGLRK[3224]	25	77
		NUAK family SNF1-like kinase 2, partial [*Picoides pubescens*]	[3]HDP-SLPTLRK[12] [428]QDPQSLPTLRK[438]	25	82
		PREDICTED: DNA repair protein XRCC4 isoform X1 [*Loxodonta africana*]	[3]HDPSLP-TLRK[12] [298]HDPSLPQTLKK[308]	25	82
		PREDICTED: claudin-2 [*Haliaeetus leucocephalus*]	[1]DFHDPSLP[8] [146]DFHNPSLP[153]	25	88
		PREDICTED: phospholipid-transporting ATPase IA isoform X1 [*Xenopus laevis*]	[1]DFHDPSL[7] [457]DFHDPSL[463]	51	100
G320	DYHNPSLPTLRK	PREDICTED: testis, prostate and placenta-expressed protein isoform X1 [*Ornithorhynchus anatinus*]	[2]YHNPSLPTLR[11] [50]YH-PSLPTLR[58]	8.9	90
		PREDICTED: claudin-2 [*Haliaeetus leucocephalus*]	[1]DYHNPSLP[8] [146]DFHNPSLP[153]	36	88
G36	DYLDQGLPTLRK	chromodomain-helicase-DNA-binding protein 6 [*Sorex araneus*]	[3]LDQGLPT--LR[11] [212]LDQGLPTPSLR[222]	51	82
		titin-like [*Callorhinchus milii*]	[1]DYLDQGL[7] [3322]DYLDQGL[3328]	72	100
		NUT family member 2G-like [*Pteropus vampyrus*]	[1]DYLDQGL[7] [407]DYLDQGL[413]	72	100
G37	RNHFASLPTVRK	SLIT and NTRK-like protein 5 [*Oryctolagus cuniculus*]	[2]NHFASLP[8] [538]NHFASLP[544]	102	100
		chondroadherin-like protein isoform X2 [*Condylura cristata*]	[2]NHFASLP[8] [428]NHFASLP[434]	102	100
		copine-8 isoform X3 [*Homo sapiens*]	[4]FA-----SLPTVRK[12] [379]FALKLPISLPTVRK[92]	206	64
		E3 ubiquitin-protein ligase RNF19A [*Oreochromis niloticus*]	[6]SLPTVRK[12] [26]SLPTVRK[32]	293	100
Gb312	NNMWTKYVAMAT	cilia- and flagella-associated protein 54 [*Ceratotherium simum simum*]	[2]NMWTKY[7] [116]NMWTKY[121]	36	100
		PREDICTED: HEAT repeat-containing protein 4 isoform X1 [*Xenopus tropicalis*]	[1]NNMWTK[6] [153]NNMWTK[158]	72	100
		Dynein heavy chain 3, axonemal, partial [*Tauraco erythrolophus*]	[2]NMWTKYV[8] [1941]NMWTEYV[1947]	145	86

G32	SDTSYSRAAVRP	DENN domain-containing protein 4C-like isoform X1 [*Scleropages formosus*]	^{1}SDTSYSRAAVR^{11} ^{1186}SNRSYSRAAVR^{1196}	12	82
		Tricarboxylate transport protein, mitochondrial [*Tupaia chinensis]*	^{5}YSRAAVRP^{12} ^{70}YSRAAVRP^{77}	13	100
		PREDICTED: equatorin [*Microtus ochrogaster*]	^{1}SDTSYSRAA^{9} ^{263}SDTSYSRSA^{271}	25	89
		protein Shroom3 isoform B [*Patagioenas fasciata monilis*]	^{2}DTSYSRAAVRP^{12} ^{1446}DSSYSRAVVSP^{1456}	72	73
		PREDICTED: nesprin-2 [*Callorhinchus milii*]	^{1}SDTSYSRAAVR^{11} ^{2053}SDTSYNRGYVR^{2063}	292	73
		ubiquitin carboxyl-terminal hydrolase BAP1 isoform X2 [*Mesocricetus auratus*]	^{6}SRAAVRP^{12} ^{301}SRAAVRP^{307}	416	100
G317	GTADRALHKELK	Neuron navigator 3, partial [*Pelecanus crispus*]	^{4}DRALHKE^{10} ^{1685}DRALHKE^{1691}	102	100
		Myosin-M heavy chain [*Chelonia mydas*]	^{2}TADRALH----KELK^{12} ^{125}TEERALHGDAKKELK^{139}	102	60
		PREDICTED: ras-related protein Rab-11A [*Callorhinchus milii]*	^{1}GTADRALHKE^{10} ^{18}GTEDRALRKE^{27}	102	80
		PREDICTED: collagen alpha-6(VI) chain [*Latimeria chalumnae]*	^{1}GTADRALHKE^{10} ^{704}GTQDRAFHKE^{713}	145	80
		cullin-associated NEDD8-dissociated protein 2 [*Gavia stellata*]	^{5}RALHKELK^{12} ^{407}RALHKQLK^{414}	145	88
		PREDICTED: ADP-ribosylation factor-binding protein GGA1 isoform X2 [*Homo sapiens*]	^{1}GTADRA---LHKEL^{11} ^{4}GTANRATNPLNKEL^{17}	145	64
		actin-related protein 10 isoform X1 [*Vicugna pacos*]	^{5}RALHKEL^{11} ^{169}RALHKEL^{175}	145	100
		PREDICTED: torsin-1A [*Felis catus*]	^{2}TADRALHKEL^{11} ^{21}TTDRALQKDL^{30}	145	70
		E3 ubiquitin-protein ligase Topors-like [*Pogona vitticeps*]	^{5}RALHKELK^{12} ^{17}RPLHKELK^{24}	206	88
		PREDICTED: tumor necrosis factor receptor superfamily member 3-like [*Latimeria chalumnae*]	^{1}GTADRALH^{8} ^{452}GTADRSLH^{459}	206	88
		PREDICTED: glutamate receptor-interacting protein 2 [*Dipodomys ordii*]	^{2}TADRALHKE^{10} ^{142}TADVALHKE^{150}	293	89
		FERM, RhoGEF and pleckstrin domain-containing protein 2 isoform X2 [*Phascolarctos cinereus*]	^{2}TADRALHKEL^{11} ^{382}TA-RALHKDL^{390}	416	80

G27	HLSEATQTNRMS	PREDICTED: iporin [*Nanorana parkeri*]	[2]LSEATQTNRM[11] [1354]LSEAKQTNRL[1363]	0.022	80
		PREDICTED: cell cycle checkpoint protein RAD1 isoform X1 [*Esox lucius*]	[6]TQTNRMS[12] [217]TQTNRMS[223]	0.031	100
		collagen alpha-1(VII) chain-like [*Cyprinus carpio*]	[1]HLSEATQ[7] [941]HLSEATQ[947]	0.12	100
		PREDICTED: protocadherin-17-like [*Cyprinodon variegatus*]	[2]LSEATQTNRMS[12] [810]LSTDTQANRMS[820]	0.12	73
		PREDICTED: E3 SUMO-protein ligase RanBP2-like isoform X1 [*Calidris pugnax*]	[3]SEATQTNRM[11] [2685]SESTQENRM[2693]	0.18	78
Gb33	VTLYPSGSSELT	importin-4 [*Cricetulus griseus*]	[1]VTLYPSGS-SEL[11] [813]VTLYPSGRLSEL[824]	12	83
		teneurin-4 isoform X1 [*Xenopus laevis*]	[1]VTLYPSGSS--EL[11] [351]VTLYPSGGTGLEL[363]	102	69
		nectin-4-like [*Xiphophorus maculatus*]	[1]VTLYPSGS[8] [122]ITLYPSGS[129]	145	80
		cytohesin-4 [*Phaethon lepturus*]	[4]YPSGSSEL[11] [4]YPSGSSDL[11]	206	88
		otogelin [*Microcebus murinus*]	[2]TLYPSGS[8] [441]TLYPSGS[447]	292	100
Gb34	CSKGDPHQPGGW	bridging integrator 2 [*Haliaeetus leucocephalus*]	[4]GDPHQPGG[11] [429]GDPHQPGG[436]	12	100
		laminin subunit beta-2-like isoform X2 [*Sinocyclocheilus grahami*]	[4]GDPHQPGG—W[12] [1010]GDPQQPGGTCW[1020]	102	73
		ADAMTS-like protein 2 isoform X5 [*Phascolarctos cinereus*]	[4]GDPHQPGG[11] [547]GDPHHPGG[554]	145	80
		myosin-6-like [*Corvus brachyrhynchos*]	[5]DPHQPGGW[12] [115]DPSQPGGW[122]	206	88
		tetraspanin 17, isoform CRA_a, partial [*Homo sapiens*]	[7]HQPGGW[12] [115]HQPGGW[120]	293	100
		collagen alpha-1(VII) chain [*Sarcophilus harrisii*]	[2]SKGDPHQPG[10] [1441]SKGDPGQPG[1449]	591	89
		collagen alpha-4(IV) chain-like [*Austrofundulus limnaeus*]	[3] KGDPHQPGG [11] [67]KGDPGQPGG [675]	591	89
		adhesion G protein-coupled receptor L1-like [*Ficedula albicollis*]	[6]PHQPGGW[12] [188]PHQAGGW[194]	591	86
G312	HAMSPVFLSKYA	protocadherin Fat 1 isoform X1 [*Haplochromis burtoni*]	[4] SPVFLSK--YA[12] [2275]SPVFLSKSYYA[2285]	51	82
		scavenger receptor cysteine-rich type 1 protein M160-like isoform X1 [*Poecilia formosa*]	[3]MSPVFLS[9] [1]MSPVFLS[7]	72	100

G310	HSSAMFGQLMPT	ATP-binding cassette sub-family G member 4-like [*Cynoglossus semilaevis*]	^{5}MFGQLMPT^{12} ^{448}MFGALMPT^{455}	51	88
G39	GTLGPLFHWTVA	rhophilin-1 [*Ursus maritimus*]	^{1}GTLGPLFHW^{9} ^{177}GNLGPLFHW^{185}	3.1	89
		heat shock protein 30-like [*Cynoglossus semilaevis*]	^{3}LGPLFHWTVA^{12} ^{1324}LAPLGHWTVA^{133}	72	73
		integrin alpha-IIb isoform X1 [*Oreochromis niloticus*]	^{5}PLFHWTV^{11} ^{138}PLFHWNV^{144}	206	86
Gb310	FALRTPHVGGNF	von Willebrand factor A domain-containing protein 8 [*Pogona vitticeps*]	^{5}TPHVGGN^{11} ^{1573}TPHVGGN^{1579}	206	100
		PREDICTED: DNA-directed RNA polymerase III subunit RPC4-like [*Cyprinus carpio*]	^{4}RTPHVGG^{10} ^{12}RTPHVGG^{18}	206	100
		myosin-14 isoform X2 [*Mus pahari*]	^{1}FALRTPHVGG^{10} ^{29}FAPRTPNVGG^{38}	292	80
Gb311	SPEYLPMRPGPA	PREDICTED: E3 ubiquitin-protein ligase TRIM39-like isoform X1 [*Pygocentrus nattereri*]	^{3}EYLPMRPG^{10} ^{127}EYLPMRPG^{13}	1.1	100
		PREDICTED: tyrosine-protein phosphatase non-receptor type 14 [*Anolis carolinensis*]	^{6}PMRPGPA^{12} ^{782}PMRPGPA^{788}	51	100
		nuclear factor of activated T-cells, cytoplasmic 3-like isoform X1 [*Oncorhynchus kisutch*]	^{6}PMRPGPA^{12} ^{850}PMRPGPA^{856}	51	100
Gb36	AETVESCLAKSH	PREDICTED: TRAF2 and NCK-interacting protein kinase isoform X1 [*Esox lucius*]	VESCLAKSH^{12} ^{266}IESCLVKSH^{274}	102	78
Gb39	VTVLPLQVVSGP	Dynein heavy chain domain 1-like protein [*Pteropus alecto*]	^{1}VTVLPLQVV^{9} ^{87}VSVLPLQVV^{95}	18	89
		Soluble scavenger receptor cysteine-rich domain-containing protein SSC5D, partial [*Ornithorhynchus anatinus*]	^{1}VTVLPLQVVSGP^{12} ^{172}VTVLPL--VTGP^{181}	102	75
		tumor suppressor p53-binding protein 1 isoform X4 [*Gavialis gangeticus*]	^{5}PLQVVSGP ^{12} ^{1642} PLQVVAGP ^{1649}	206	88
G25	SLTHVSVNIAAS	inter-alpha-trypsin inhibitor heavy chain H3-like isoform X1 [*Boleophthalmus pectinirostris*]	^{5}VSVNIAAS^{12} ^{141}VSVNIAAS^{148}	72	100
		PREDICTED: adhesion G-protein coupled receptor F1-like [*Oreochromis niloticus*]	^{2}LTHVS-VNIAAS^{12} ^{94}LRHVSIVNIAAS^{105}	72	83
		Alpha-actinin-3 [*Larimichthys crocea*]	^{4}HVSVNIAAS^{12} ^{690}HVSVDIAGS^{698}	416	78
G38	SALPWFWSMDPS	PREDICTED: ATP-binding cassette sub-family B member 9 isoform X1 [*Pelodiscus sinensis*]	^{4}PWFWSM^{9} ^{116}PWFWSM^{121}	8.8	100

		Putative caspase-14-like protein [*Myotis brandtii*]	[2]ALPWFW[7] [373]ALPWFW[378]	36	100
		PREDICTED: protein O-mannose kinase-like isoform X1 [*Branchiostoma belcheri*]	[3]LPWFWS[8] [23]LPWFWS[28]	36	100
		PREDICTED: kinesin-like protein KIF13A [*Ciona intestinalis*]	[6]FWSMDP[11] [62]FWSMDP[67]	51	100
		PREDICTED: collagen alpha-1(XVIII) chain-like isoform X2 [*Notothenia coriiceps*]	[5]WFWSMDP[11] [21]WFWSKDP[27]	51	86
G24	TIVGGRSAFWWS	growth hormone-releasing hormone receptor [*Microcebus murinus*]	[6]RSAFWW[11] [105]RSAFWW[110]	13	88
		FYVE, RhoGEF and PH domain-containing protein 6 [*Chelonia mydas*]	[6]RSAFWWS[12] [1168]RSSFWWS[1174]	51	86
		PREDICTED: teneurin-4-like [*Panthera tigris altaica*]	[4]GGRSAFWW[11] [30]GGRPGFWW[37]	207	75
		protocadherin gamma-C5-like isoform X1 [*Oryzias latipes*]	[5]GRSAFWW[11] [29]GRQAFWW[35]	293	86
		PREDICTED: mannan-binding lectin serine protease 1 isoform X2 [*Ictalurus punctatus*]	[2]IVGGRSAF[9] [475]IVGRTAF[482]	293	88
G26	MGSAPFWDSVRD	Neutral ceramidase, partial [*Struthio camelus australis*]	[5]PFWDSVRD[12] [412]PFWDSIRD[41]	4.4	88
		PREDICTED: serine/threonine-protein kinase DCLK1-like isoform X1 [*Kryptolebias marmoratus*]	[4]APFWDSVRD[12] [607]APFWDSVSD[615]	4.4	89
		PREDICTED: SUN domain-containing protein 1 isoform X1 [*Kryptolebias marmoratus*]	[2]GS-APFWDSVR[11] [311]GSLASFWDSVR[322]	25	82
G319	FTTPMLNNSRVH	PREDICTED: complement C1s subcomponent-like [*Takifugu rubripes*]	[1]FTTPMLNN[8] [591]FTTPMLGN[598]	72	88
		PREDICTED: kelch-like ECH-associated protein 1 [*Nanorana parkeri*]	[1]FTTPMLNNSRV[11] [424]FVTPM-NNSRI[433]	72	73
		PREDICTED: RNA polymerase II elongation factor ELL2, partial [*Egretta garzetta*]	[1]FTTPMLN--------NSRVH[12] [261]FTTPLMNKKQRISPLNSRVQ[280]	72	45
		PREDICTED: histone-lysine N-methyltransferase SUV420H1 isoform X1 [*Balaenoptera acutorostrata scammoni*]	[3]TPMLNNSRV[11] [430]TPM-NNSRV[437]	102	89
		PREDICTED: raftlin-like [*Sinocyclocheilus anshuiensis*]	[2]TTPMLNNSRVH[12] [332]TTPMLN--RVQ[340]	102	73
		PREDICTED: tyrosyl-DNA phosphodiesterase 2 [*Tinamus guttatus*]	[1]FTTPMLNNSRV[11] [112]FTAIMLKNSRV[122]	102	73

	PREDICTED: mucin-3A-like [*Sinocyclocheilus anshuiensis*]	[1]FTTPMLNNSRV[11] [1966]YTTQLLNDSRV[1976]	145	64
	plexin-C1 isoform X1 [*Pogona vitticeps*]	[2]TTPMLNNSR[10] [162]TTHMLNDSR[170]	145	78

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