Heterologous expression of a putative *lodAB* operon derived from

Thalassomonas viridans

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Declaration

I, Dugald Joseph Rowland, hereby declare that "Heterologous expression of a putative *lodAB* operon derived from *Thalassomonas viridans*" is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used have been indicated and acknowledged**.**

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Signed: …… **……**

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Abstract

The growing incidence of antibiotic resistance in bacteria to current antimicrobial compounds is a major concern worldwide. Identifying novel antimicrobial compounds is vital to mitigate the increasing prevalence of antibiotic-resistant bacteria and other pathogens. One of the methods used to find novel antimicrobial compounds is genome mining. The genome mining of marine organisms, which have been poorly represented in research, may reveal a potential source of new antimicrobial compounds which can be used as antibiotics. This study focused on the characterisation of an antibacterial protein identified by the genome mining of a marine microorganism.

Thalassomonas viridans is a microorganism that is known to produce antibiotic secondary metabolites (Adams, 2019). The genomic annotation of the *T. viridans* genome indicates that there are two sequences with similarity to the *lodA* gene that codes for l-lysine ε-oxidase (LodA) - first described as an antimicrobial protein produced by the marine bacteria *Marinomonas mediterranea*. LodA is an extracellular enzyme that has antibacterial properties associated with the production of hydrogen peroxide (H_2O_2) as a product of lysine oxidation (Gómez D *et al.*;2006). The *lodA* sequences submitted to a wider BLAST analysis showed that similar sequences are present in the genomes of a number of other microorganisms.

In this study the heterologous expression of the *lodA* genes from *T. viridans* was conducted in *Escherichia coli* to produce, purify, and characterise the proteins. Multiple methods of *E. coli* expression to produce the protein were explored, and multiple activity assays were employed to characterise the proteins produced. The LodA protein was produced at low concentrations and no activity was detected on a wide range of amino acid substrates.

Key words: *antibiotic resistance, marine natural products, non-ribosomal peptides, Thalassomonas viridans, marine invertebrate larvae, antimicrobial compounds, genome mining, transcriptional profiling, heterologous expression, synthetic DNA, liquid chromatography mass spectrometry, bacteriophages, antimicrobial proteins, activity assay.*

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Finally, I'd like to thank my wife Jamie for her constant encouragement, love, and support. Without her I would not be in the position I am now.

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Dedication

This work is dedicated to my family, especially my wife (Jamie Rowland), for her love, patience, kindness and sacrifices.

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Abbreviations

°C	Degree Celsius
µg/ml	Microgram per millilitre
µl	microlitres
µm	micrometre
ACN	Acetonitrile
AMPs	Antimicrobial proteins
antiSMASH	Antibiotics and secondary metabolites analysis shell
APS	Ammonium persulfate
AT	Acyltransferase
ATCC	American type culture collection
ATP	Adenosine triphosphate
BCWH's	Bacterial cell wall hydrolases
BGC	Biosynthetic gene cluster
BLAST	Basic Local Alignment Search Tool
bp	base pair
C domain	Condensation domain
cDNA	Complementary DNA
CLB's	Colicin-like bacteriocins
CLF	Chain length factor
cm	centimeter
CoA	Co-Enzyme A
contig	Contiguous
DAS	Distributed Annotation System
dH_2O	Distilled water
DNA	Deoxyribose nucleic acid

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EDTA	Ethylenediaminetetracitic acid
ESKAPE	*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, and Enterobacter*
EtBr	Ethidium Bromide
FPLC	Fast performance liquid chromatography
xg	Gravitational force
g	grams
g/L	Gram per liter
GPC	Gel Permeation chromatography
HPLC	High performance liquid chromatography
ID	Identity
IMBM	Institute for Microbial Biotechnology and Metagenomics
kDa	Kilo Dalton
L	litre
LB	Lysogeny broth
LC	Liquid chromatography
MA	Marine agar
MB	Marine broth
MMM	Marine Minimal Media
MWCO	Molecular weight cut-off
mg/ml	Milligram per millilitre
min	minutes
ml	Millilitres
mM	millimolar
mm	millimetre
NaAc	Sodium Acetate
NCBI	National Centre for Biotechnology Information

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ng	Nanogram
NRPs	Non-ribosomal peptides
OD	Optical density
PCR	Polymerase chain reaction
PKs	Polyketides
PRISM	Prediction Informatics for Secondary metabolomes
RAST	Rapid Annotation using Subsystem Technology
RNA	Ribonucleic acid
rpm	Revolutions per minute
rRNA	Ribosomal RNA
SDS	Sodium dodecyl Sulphate
SDS-PAGE	Sodium-dodecyl sulphate-polyacrylamide gel electrophoresis
sec	seconds
SMs	Secondary metabolytes
TAE	Tris-Acetate-EDTA buffer
TE	Tris-EDTA buffer
TEMED	Tetramethylethylenediamine
UV	Ultraviolet
w/v	Weight per volume
WGS	Whole genome sequencing
WHO	World Health Organization

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Chapter 1 Literature Review

1.1. Introduction

For decades we have depended on antibiotics to treat and prevent various forms of infection or disease.

The use of microorganisms as a source of antibiotics dates back to the discovery of penicillin in 1932 (Fleming A., 1929) and streptomycin in 1943 (Schatz, *et al.*, 1944). Since then, although the principal characteristics of the drug have remained the same, progress in the development of new antibiotic drugs has depended on the modification of molecules to change the mode of interaction (Kohanski, *et al.*, 2010a). The mode of action of an antibiotic exerts selective pressure on bacteria, which can cause them to develop resistance due to rapid evolution, such as the acquisition of resistance genes like β-lactamase or the modification of target proteins (Kolář, *et al*, 2001; Auckloo and Wu, 2016). There has also been a decrease in the number of novel effective antibacterial compounds discovered due to practical (such as rediscovery), and regulatory barriers (Auckloo and Wu, 2016).

The World Health Organisation (WHO) has noted that the increase in antibiotic resistance may result in a "post-antibiotic" period where the once minor infections can be deadly, and lifesaving procedures will no longer be effective (WHO, 2018). As the incidence of antibiotic resistance increases, there is an urgent requirement for new antibacterial agents that have a novel mode of action (WHO, 2018). Currently, a large proportion of the exploited antibiotics are secondary metabolites (SMs) that are plant-derived (Bérdy, 2005), and most of the remainder are derived from terrestrial Actinobacteria (e.g., vancomycin & streptomycin) (Bérdy, 2005; Thirumurugan *et al.*, 2012; Jackson *et al.*, 2018). There is thus a new focus on the marine environment for antibiotic compound discovery because of the reduced efficacy of terrestrially derived compounds due to antimicrobial resistance (Haefner, 2003; Wiese and Imhoff, 2019).

Derivatives from marine organisms offer a large pool of novel compounds that may have antibacterial activity (Carroll *et al.*, 2020). Some of these may be useful in mitigating the advancement of antibacterial resistance and should be characterised to determine whether they may be effective novel antimicrobial compounds.

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Genomic investigation of marine organisms that have little representation in research may be a potential source of new antibiotics. An example is *Thalassomonas viridans*, where the entire genomic sequence has been made available, but little research has been carried out on characterising the SMs it produces (as at October 2022). Some of the SMs, notably proteins, produced by *T. viridans* may be isolated and characterised through the utilisation of DNA synthesis and homologous expression. This study focused on the antibacterial potential of these proteins.

1.2. Alternatives to antibiotics.

A summary of some of the alternatives to the antibiotics currently used was compiled by Ghosh *et al* (Ghosh *et al.*, 2019) (Table 1.1) where they list the advantages and disadvantages for each of the alternatives. Some of these are secondary metabolites and others (CRISPR/Cas9 & Synthetic mimics) are novel laboratory modifications of known genomic and metabolic components that are involved in the development of antimicrobial resistance. While lysins are hydrolytic enzymes produced by bacteriophages that are specific to the bacteria that each one targets, they are often grouped with antimicrobial peptides as opposed to being included in the use of the entire source bacteriophage in phage therapy (Vázquez, García and García, 2018; Ghosh *et al.*, 2019). These alternatives need to be broadly applied in conjunction with the reduction in the use of current antibiotics. This will help circumvent the growing incidence of antimicrobial resistance.

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Table 1.1 - Major Alternatives to Antibiotics and Their Advantages and Disadvantages.

Strategy	**The advantage over Conventional antibiotics**	**Possible disadvantages**
Phage therapy	- Self-replicating pharmaceuticals - Selective towards specific strains of bacteria - Amenable to genetic engineering	- Immunogenicity - Pharmacokinetics Release of bacterial endotoxins - Inadequate preparations – failure to remove endotoxins and pyrogenic substances. - Resistance development
Lysins	- Amenable to genetic engineering - Selective towards specific strains of bacteria - Not prone to resistance development	- Production - Lack of sufficient knowledge
CRISPR/Cas9	- Can be tuned for a variety of antimicrobial applications. - Reversal of antibiotic usage - Specificity towards pathogenic strains	- Expensive large-scale production - Toxicity
Antimicrobial peptides	- Not prone to resistance development - Broad-spectrum activity is an advantage, depending upon application	- Expensive large-scale production - Susceptible to proteolysis - Toxicity
Bacteriocins	- Specificity towards pathogenic strains of bacteria - Resistance to heat and UV	- Expensive large-scale production - Susceptible to proteolysis
Synthetic mimics of antimicrobial peptides.	- Ease of synthesis - Not prone to resistance development - Broad-spectrum activity is an advantage, depending upon application	- Toxicity - Route of administration
Innate defence regulatory peptides.	- Work by modulating the immune system. - No resistance development as no direct antimicrobial activity	- Expensive large-scale production - Susceptible to proteolysis
Probiotics	- Easy availability	- Used mostly for intestinal infections
Antibodies	- Selective towards specific strains of bacteria - Do not damage the microflora	- High cost of production - Poor shelf life

Adapted from, "Alternatives to Conventional Antibiotics in the Era of Antimicrobial Resistance" - (Ghosh *et al.*, 2019)

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The following section provides an overview of the modes of action of some of the strategies mentioned in Table 1.1. It briefly describes how they work and why they are advantageous in combatting antibiotic resistance.

Bacteriophages destroy sensitive bacteria by binding to the cell surface and injecting their own genetic material into the cell (Ghosh *et al.*, 2019). This "hi-jacks" the cell biosynthetic machinery to replicate the phage and it's components resulting in cell destruction (Ghosh *et al.*, 2019). Cell destruction, and the cell surface binding properties of phages result in a reduced possibility for resistance to develop (Auckloo and Wu, 2016). In addition, the specificity of phages leaves beneficial bacterial communities intact (Ghosh *et al.*, 2019). Lysins are an enzymatic component of bacteriophages that hydrolyse bacterial cell walls and are normally formed at the end of the phage cycle (Vázquez, *et al.*, 2018). These lysins hydrolyse either the glycosidic bonds or the amide bonds in the peptidoglycan polymer resulting in osmotic lysis and cell death and can be used in a purified form (Vázquez, *et al.*, 2018). Lysins have the same benefits as bacteriophages in that there is a reduced risk of resistance development and specificity ensures beneficial bacterial communities are not degraded. In addition, lysins do not depend on the cycle of the target cells to be effective (Vázquez, *et al.*, 2018).

Secondary metabolites (SMs) are small molecules that have widely diverse functions in the original organism and can be classed into several groups: ribosomal and non-ribosomal peptides (RP / NRPs), polyketides, terpenes, and alkaloids (Biswas, et al, 2016). Larger antimicrobial proteins are often grouped with ribosomal peptides. Ribosomally synthesized and post-translationally modified peptides (RiPPs) are made up of amino acids that undergo further modification in many ways, such as *N*- and *C*-terminal sequences being removed from a core sequence (Letzel, *et al.*, 2014; Arnisona, *et al.*, 2014). The core sequence can be further modified (e.g., cyclisation of cyanobactin) - these are generally less than 10kDa in size (Letzel, *et al.*, 2014; Arnisona, *et al.*, 2014). The NRPs are made up of several building blocks, other than the primary amino acids, that are assembled and 'edited' by complex architectures of multi-subunit enzymes (Jensen, 2016). Polyketides are also a large group of diverse compounds that are derived from the modification of acyl-CoA thioesters by specific synthases (Korman, *et al.*, 2010). The other two groups, terpenes and alkaloids, cover a large number of SMs. Terpenes are often aromatic compounds consisting of isoprene subunits that

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have many side modifications (Louie *et al.*, 2020). Alkaloids are also cyclic compounds that contain nitrogen and are often toxic and are more commonly derived from plants (Verpoorte, 2005).

Many SMs generated by microorganisms surviving in environments that are extremely variable and biologically competitive, such as the sea, are often effective against Gram-positive and Gram-negative bacteria that are pathogenic to humans (Auckloo and Wu, 2016; Biswas, *et al.*, 2016). This may be due to lack of exposure, where the microorganisms that are pathogenic to humans have not encountered any competition like that found in marine environments. The SMs may be classified by the cellular system or component they interact with and whether they are bacteriostatic or bactericidal (retard cell growth or kill cells) (Kohanski, *et al.*, 2010).

The SMs that are of particular interest are those that function in two ways; interruption of the cell wall/membrane or interfering with the metabolism of the cell (Kohanski, *et al.*, 2010; Auckloo and Wu, 2016). Both methods reduce the potential for the emergence of drug resistance due to complete cell death that results from the action of these SMs (Kohanski, *et al.*, 2010). The interference with the metabolism of the cell includes disrupting protein expression by creating double-stranded DNA breaks by inhibiting topoisomerase activity by quinolone bonding (Kohanski, *et al.*, 2010). This bonding prevents the detachment of the topoisomerases from the DNA and because of this, a break forms at that point preventing the rest of the DNA repair/replication/transcription machinery from progressing (Kohanski, *et al.*, 2010). If a cell is no longer able to repair or read the DNA, most metabolism will stop, and the cell will eventually die. Interruption of the cell wall results in more immediate cell death by either causing the cell to rupture or preventing transport through it (Auckloo and Wu, 2016). In both cases, there is less opportunity for the microorganism to develop resistance. In some cases, the damage to the cell wall or membrane is caused by a product (such as H_2O_2) from the activity of antimicrobial peptides or proteins.

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1.3. Antimicrobial Proteins

Antimicrobial proteins and peptides (AMPs) are a diverse group of antimicrobial agents (Auckloo and Wu, 2016; Zhang and Gallo, 2016). Some of these are small peptides that attach to the cell wall and degrade the cell membrane structure through ionic interaction, resulting in cell destruction (Ghosh *et al.*, 2019). A few AMP-type antibiotics have been developed, namely polymyxin B, gramicidin and daptomycin (Greber and Dawgul, 2017; Ghosh *et al.*, 2019). These target Gram-negative bacteria but clinical trials have shown that there may be a high rate of unacceptable side effects such as systemic toxicity (Greber and Dawgul, 2017; Ghosh *et al.*, 2019). Bacteriocins are considered AMPs, but they have additional modes of action, such as inhibiting the biosynthesis of peptidoglycans (e.g., interaction with lipid II – a precursor for the bacterial cell wall) (Ghosh *et al.*, 2019). An additional benefit of bacteriocins is that they are far more target specific than other AMPs and HDPs (Ghosh *et al.*, 2019).

1.3.1. Amino acid oxidases

L-amino acid oxidases (LAAOs; EC 1.4.3.2) facilitate the oxidative deamination of an amino acid with the release of ammonium, hydrogen peroxide (H_2O_2) and a specific organic acid (e.g. an α-keto acid) which is dependent on the substrate and the location of the ammonium group on the molecule (Lucas-Elío *et al.*, 2006; Yu and Qiao, 2012; Kasai *et al.*, 2015). LAAOs are found widely in nature; the first being described in snake venom and subsequently described in various microorganisms, insects, and algae (Yu and Qiao, 2012; Kasai et al., 2015). The snake venom LAAOs are the best characterized in terms of biochemistry, toxicology, physiological interaction, and medical applications (Yu and Qiao, 2012). While the non-snake venom LAAOs that have been characterised exhibit a wide range of stabilities in terms of pH, temperature, and substrate specificity - less work has been published on this group (Yu and Qiao, 2012).

LAAOs are stereospecific for L-isomers of the amino acid that they act on (Yu and Qiao, 2012). This means that they interact only with one of the two possible enantiomers for a given free amino acid or its derivative. The L- and D- enantiomers are mirror images of each other and cannot be overlayed on each other (i.e. left and right hands) and each of these has a different biological activity (Aliouche, 2023). This conformational difference determines how a molecule can fit or bind into an active site of the enzyme so that the active domains are able to deaminate the amino acid (Yu and Qiao, 2012).

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Many of the LAAOs form dimers, and each subunit requires a non-covalently bonded Flavin adenine dinucleotide (FAD) cofactor (Dym and Eisenberg, 2001; Yu and Qiao, 2012) or another cofactor / activating compound. Structurally, LAAOs consist of at least three domains, cofactor-binding, substrate binding and helical region (Yu and Qiao, 2012). These conserved domains allow for structural and domain similarity searches to be conducted with tools such as UniProt and tblastn as described later. Using a calculated structural search on an annotated genome can highlight potential new LAAOs that can be employed for the development of antibiotics.

In general, LAAOs are synthesized with a signal peptide and may undergo post-translational modification (such as the generation of a cofactor) in addition to secretion due to toxicity of the active components in the cell, such as the generation of H_2O_2 and interaction with other enzymes (Yu and Qiao, 2012; Sehanobish *et al.* 2014). Furthermore, it is assumed that the free ammonium that is released in the enzymatic activity is absorbed as an important contribution to other amine-based metabolism processes for the cell (Yu and Qiao, 2012). The combination of the toxicity of the active compound as well as the presence of a secretion signal result in proteins that are not always simple to produce by heterologous expression.

The antibacterial effect of LAAOs is due to the local production of H_2O_2 and is evidenced by the loss of this effect when catalases are included in the reaction mix (Mai-Prochnow *et al.*, 2008; Kasai *et al.*, 2015). H_2O_2 induces DNA damage and cell death through cytotoxicity as well as the reactive oxygen species released in its interactions (Mai-Prochnow *et al.*, 2008; Kasai *et al.*, 2015). This antibacterial effect is used in the generation and maintenance of biofilms by some marine bacteria (Mai-Prochnow *et al.*, 2008). Biofilms are three-dimensional organic polymer structures that encase suspended micro-colonies of bacteria (Mai-Prochnow *et al.*, 2008). Studies have shown that subpopulations within the biofilm die off, including both primary bacterium as well as competing microorganisms, this is often associated with the activity of prophages as well as autolytic proteins (AlpP) (Mai-Prochnow *et al.*, 2008). The die-off events trigger a dispersion of phenotypically diverse lines with higher metabolic activity (Mai-Prochnow *et al.*, 2008).

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Two well-characterised LAAO enzymes that are involved in the catabolism of L-lysine, are Lysine α-oxidase (LαO) and lysyl oxidase (LOX) (Lukasheva and Berezov, 2002; Lukasheva *et al.*, 2021). The former produces α-keto-ε-aminocaproate and H_2O_2 the latter does not act on free L-lysine (Gómez *et al.*, 2006). Most of the research on LαO has focused on its anti-cancer properties, associated with the local production of H_2O_2 and depletion of free amino acids (Lukasheva *et al.*, 2021). LOX on the other hand is not associated with antibacterial/antitumor activity and is instead associated with connective tissue formation in mammalian models (Smith-Mungo and Kagan, 1998)

1.3.2. L-lysine ε-oxidase

L-lysine ε-oxidase (LodA) is a LAAO flavoprotein that is part of a family of enzymes that catalyse the oxidative deamination of L-amino acids as described above (Ratner, 1955). LodA was first described as a novel enzyme with antimicrobial activity and was isolated from *Marinomonas mediterranea* (Gómez *et al.*, 2006). The initial purification and partial characterisation of LodA, found that it was effective against several Gram-positive and Gram-negative microorganisms including nosocomial *staphylococcus aureus* and *Pseudomonas sp.* (Gómez *et al.*, 2006). This effect against such a wide range of microorganisms, including those that are resistant to traditional antibiotics is due to the H_2O_2 formed in the deamination of the substrate (Ehara *et al.*, 2002; Lucas-Elío *et al.*, 2006; Chen, Lin and Sheu, 2010).

The activity of LodA is highly specific for the oxidative deamination of L-lysine to 2-aminoadipate 6-semialdehyde in the below reaction (Gómez *et al.*, 2010), and follows the same basic reaction process as other LAAOs where the organic product is 2-aminoadipate 6-semialdehyde. The 6-semialdehyde 2-aminoadipic acid spontaneously cyclizes to d-1-piperidine 6-carboxylate (Matsuda and Asano, 2010) and can be further converted to indolizidine alkaloids, cephalosporins, some of which may be further modified as other antibiotics or can be used in the biosynthesis of lysine ((KEGG), 2019). Interestingly 6-semialdehyde 2-aminoadipic acid is the same as mammalian allysine which is a precursor for collagen and connective tissue synthesis.

$$\textbf{L-lysine} + O_2 + H_2O \rightarrow \textbf{2-aminoadipate 6-semialdehyde} + NH_3 + H_2O_2$$

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Further sequencing and annotation of the *M. mediterranea* genome revealed an additional sequence highly similar to that of LodA, and the *lodA/B* operon, this has been confirmed to produce a glycine ε-oxidase (GoxA) and has been confirmed to have similar antimicrobial activity (Campillo-Brocal, Lucas-Elio and Sanchez-Amat, 2013). The sequence similarity and conserved domains between both LodA and GoxA suggests that this is a distinct family of enzymes.

In the characterisation of LodA, a UV-Vis spectral analysis does not show the peaks at 388nm and 466nm associated with FAD as with other LAAOs (Gómez *et al.*, 2006; Lukasheva *et al.*, 2021). In addition, experiments on the dependency of LodA for FAD did not show that this was the case (Gómez *et al.*, 2006).

The active form of LodA can only be generated by posttranslational modifications through the action of co-expressed LodB (Gómez *et al.*, 2006). This protein forms a cysteine tryptophylquinone (CTQ) cofactor, where a di-oxygenated tryptophan (Trp581) residue is cross-linked to a cysteine (Cys516) residue in the active site (Chacón-Verdú *et al.*, 2015; Sehanobish *et al.* 2015). This PTM of LodA is uncommon among the LAAOs and the CTQ formed acts as an oxidase as opposed to a dehydrogenase (Sehanobish *et al.* 2014). In studies conducted by Chacón-Verdú *et al.* (2015), mutation of these residues prevented the generation of CTQ. In addition, when either of the proteins is not present, the stability of the partner protein is compromised leading to a marked decrease in its cellular levels (Chacón-Verdú *et al.*, 2015). In *M. mediterranea* LodA is secreted to the external medium when the culture reaches the stationary phase but LodB on the other hand, has only been detected intracellularly and is not secreted (Gómez *et al.*, 2006).

The studies characterizing LodA have used H_2O_2 detection to confirm activity. The Amplex Red reaction was developed for the detection of H_2O_2 produced by the activity of lysyl oxidase (Palamakumbura and Trackman, 2002). When the Amplex Red reagent is combined with H_2O_2 in the presence of horseradish peroxidase, resorufin, a fluorescent compound is produced that can be detected using a spectrophotometer with excitation wavelength set at 550nm and an emission detection wavelength set at 590nm (Lucas-Elio *et al.*, 2005; Sehanobish *et al.*, 2015).

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LodA derived from *M. mediterrania* is resistant to hydrolytic enzymes (Lucas-Elio *et al.*, 2005), this was determined by exposing LodA to neuraminidase, O-glycosidase, proteinase K, a-amylase, lysozyme, trypsin, chymotrypsin and lipase in a ratio of 0.2µg/Unit of LodA in a buffer suitable for each challenge enzyme (Lucas-Elio *et al.*, 2005). The effect of each of the enzymes was determined by assessing the effect on the inhibition diameter as well as a non-denaturing SDS-PAGE assay to confirm fragmentation (Lucas-Elio *et al.*, 2005). In addition, it has also been noted that it is particularly resistant to heat, up to 70 °C where antimicrobial activity was still detected after an hour (1 h) incubation at this temperature -this may be due to dimer formation as suggested by Elio *et al.* (2005). This was confirmed for the LodA from *M. mediterrania* which exists as a dimer due to the appearance of two bands when the 140kDa band from the non-denaturing SDS-PAGE is re-assayed in denaturing conditions (Sehanobish *et al.*, 2015). These bands match those from denaturing SDS-PAGE conducted on concentrated LodA samples (Sehanobish *et al.*, 2015).

As an aside, H_2O_2 is currently used in the design of enzymatic biosensors (Vanegas and Gomes, 2016). Indeed, there is at least one paper detailing the employment of lysine oxidases for the clinical detection of lysine in serum samples based on the release of H_2O_2 (Chauhan *et al.*, 2012). The high specificity of LodA and GoxA for L-lysine and Glysine respectively makes them ideal candidates for this application.

1.4. The Marine Environment and New Antibiotics.

The marine environment covers two-thirds of the earth's surface and represents an extensive resource of novel bioactive agents, including antibiotic/antimicrobial drugs (Haefner, 2003; Auckloo and Wu, 2016). The complexity of the marine environment, including that it is liquid means that there are many selective pressures on the organisms found there, resulting in a wide range of secondary metabolite activities (and possible antibiotics) (Demain and Fang, 2000; Vijayalakshmi *et al.*, 2008; Thirumurugan *et al.*, 2012; Jensen, 2016). Different environmental pressures result in organisms producing different chemistries, such as the secretion of variations compounds that may be more effective under marine conditions. In addition, variability in the marine environment influences the competition for resources, forcing organisms to need to have multiple "tools" such as secondary metabolites to survive. The SMs that are generated by these marine organisms due to the competitive complexity of the marine environment are often very different from those found in terrestrial

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environments (Biswas, *et al.*, 2016; Jensen, 2016). These SMs give the organism a competitive advantage in their environment and may be produced as a stress response or to some other signalling event (Demain and Fang, 2000; Thirumurugan *et al.*, 2012; Jensen, 2016).

Until modern isolation and characterisation techniques were developed, little research had been done to isolate the useful SMs (Baam *et al.*, 1963) mainly due to the difficulty in cultivation, isolation, and characterisation in a laboratory (Auckloo and Wu, 2016). More recently, not only multi-celled marine organisms are being investigated for the production of antibiotic SMs activity, but also symbiotic microorganisms associated with them as well as those isolated from bacterial communities (sediments *etc.*) (Auckloo and Wu, 2016). A summary of current antimicrobial compounds derived from marine organisms (Table 1.2) has been compiled.

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Table 1.2 - Summary of Antibiotics/Antibacterial Agents Derived from Marine microorganisms.

Chemical Class	Isolated Compound	Source	Potential Target	Mode of Action
Protein/ polypeptide	Hormaomycin B and C	*Actinomycete* strain (SNM55)	*Kocuria rhizophila* NBRC 12708	-
	Marthiapeptide A	*Marinactinospora thermotolerans (SCSIO 00652)*	*M. luteus; S. aureus; B. subtilis; B. thuringiensis*	-
	Desotamide B	*Streptomyces scopuliridis* (SCSIO ZJ46)	*S. aureus; S. pneumoniae;* MRSE shhs-E1	-
	Marfomycins A, B, E	*Streptomyces drozdowiczii (SCSIO 10141)*	*S. aureus; E. faecalis*	-
	Bacteriocin PSY2	*Lactococcuslactis* strain PSY2	*Arthrobacter sp., Acinetobacter sp., B. subtilis, E. coli, Listeria monocytogenes, Pseudomonas aeruginosa, S. aureus*	-
	Etamycin	*Actinomycete* strain (CNS-575)	Hospital- and community- accociated methicillin-resistant *S. aureus, Streptococcus pysogenes, Streptococcus agalactiae, Coccobacilli, Moraxella cararrhalis*	Obstruction of protein synthesis in bacterial cells
	Salinamide A	*Streptomyces* sp. CNB-091	*Enterobacter cloacae* ATCC 13047 and *Haemophius influenza* ATCC 49247	Inhibition of bacterial RNA polymerase
	Antibiotic PM181104	*Kocuria (MTCC 5269)*	Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococci* (VRE)	
	Salinamide F	*Streptomyces* sp. CNB-091	*S. aureus, E. coli, Enterococcus feacalis, H. influenza, Neisseria gonorrhoeae, E. cloacae*	Inhibition of bacterial RNA polymerase
Polyketide/ macrolactones	Abyssomicin C	Marine *Verrucosispora*	Methicillin-resistant *S. aureus*	Restrict the biosynthetic precursor of folic acid

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	Lobophorin E & F	Actinobacterial strain SCSIO 01127	*S. aureus* ATCC 29213, *E. faecalis* ATCC 29212, *Bacillus thuringensis* SCSIO BT01	-
	Talaromycesone A & B	*Talaromyces* sp. strain LF458	*Staphylococcus epidermis*, methicillin-resistant *S. aureus*	-
	7-O-methyl-50 -hydroxy-3 heptenoate-macrolactin	*Bacillus subtilis* MTCC 10403	*Aeromonas hydrophila*, *V. parahemolyticus* ATCC 17802	Affect the lipophilic membrane
	Aromatic polyketide compound 4	*Streptomyces* sp.JRG-04	*S. aureus* MTCC 3160	Disrupt the cell membrane
	Macrolactins X, Y, Z	*Bacillus* sp. 09ID194	*B. subtilis* (KCTC 1021), *E. coli* (KCTC 1923), *Saccharomyces cerevisiae* (KCTC 7913)	-
	Macrolactin S	*Bacillus* sp.	*E. coli, S. aureus, B. subtilis*	-
	Lindgomycin and ascosetin	*Lindgomycetaceae* strains KF970 & LF327	*B. subtilis, S. epidermidis*, methicillin-resistant *S. aureus*	-
	8-Chloro-9-hydroxy-8,9-deoxyasperlactone, 9-chloro-8-hydroxy-8,9-deoxyasperlactone, 9-chloro-8-hydroxy-8,9-deoxyaspyrone	*Aspergillus ostianus* st. TUF 01F313	*Ruegeria atlantica*	-
	Gageomacrolactin 1-3	*Bacillus subtilis*	*Grampositive and Gramnegative Pathogenic bacteria*	
Anthroquinone class	Cosmomycin B	*Streptomyces cyaneofuscatus* M-27	-	-
	Aranciamycin I & J	*Streptomyces* sp. (CMB-M0150)	*Mycobacterium bovis BCG, B. subtilis*	-
	Zafrin	*Pseudomonas stutzeri*	*S. aureus, Salmonella typhi*	Destructive effect on the cytoplasm
Polybrominated biphenyl class	MC21-A 1	*Pseudoalteromonas phenolica* sp. O BC30T	-	Render the cell membrane of bacteria permeable

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	MC21-B 2	*Pseudoalteromonas phenolica* O-BC30T	Methicillin-resistant *S. aureus, B. subtilis, Enterococcus serolicida*	Disruption of the cell wall of the membrane of sensitive pathogens
Terpenoid class	Terretonin G	*Aspergillus* sp. OPMF00272	*S. aureus* FDA209P, *B. subtilis* PCI219, *Micrococus luteus* ATCC9341	-
	Chlorine-containing terpenoid dihydroquinones	*Streptomyces* sp. Strain CNQ-525	Methicillin-resistant *S. aureus*, vancomycin-resistant *E. faecium*	-
Alkaloid class	Diazepinomicin (ECO-4601)	*Micromonospora* strain	Certain Gram-positive bacteria	-
	Stachyin B	*Stachybotrys* sp. MF347	*B. subtilis, S. epidermidis,* methicillin-resistant *S. aureus*	-
	Trichodin A	*Trichoderma* sp. strain MF106	*B. subtilis, S. epidermidis*	-
Chlorinated benzophenone	Pestalone	*Pestalotia* sp. CNL-365	Methicillin-resistant *S. aureus,* vancomycin-resistant *E. faecium*	-
Phenazine antibiotic	LL-14I352a	Halophilic marine bacterium	*E. coli*	Disruption of cytoplasmic membrane and ability to restrict DNA synthesis
	a-pyrone I	Novel *Pseudomonas* sp.	*B. subtilis, S. aureus, M. catarrhalis, E. faecium*	Interruption of the cellular uptake of amino acids and acetates leads to disruption of the membrane

Modified from "Antibiotics Derived from Marine Organisms: Their Chemistry and Biological Mode of Action" - (Auckloo and Wu, 2016), "Antibiotics from Deep-Sea Microorganisms: Current Discoveries and Perspectives" - (Tortorella *et al.*, 2018) and "Antibiotics from Marine Bacteria" - (Stonik, *et al.*, 2020). Discovery period, 2013 – 2019, accessed 01 September 2022.
(- = not published)

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Indeed, there have been studies on many novel compounds produced by marine bacteria, such as abyssomicin C, diazepinomicin and homaomysin B & C (Auckloo and Wu, 2016). Marine fungi produce an equally wide range of antibacterial SMs, such as terretonin G, chlorine-containing antibiotics and oxaphenalone dimers (Auckloo and Wu, 2016). Unfortunately, many of the SMs derived from marine organisms cannot currently be chemically synthesised due to their complexity and/or unusual structure *(Thirumurugan et al.*, 2012). Synthetic bioproduction would be required to produce these SMs in significant quantities.

1.4.1. Marine soft-bodied organisms as sources of useful secondary metabolites.

The marine environment has the highest diversity of soft-bodied organisms, and Mollusca as a phylum is extremely diverse in species and thus morphology (Benkendorff, 2010; Blunt *et al.*, 2016). The soft-bodied nature of Mollusca, despite many having a hard exoskeleton, means that they have minimal physical barriers to microbial infection (Blunt *et al.*, 2016). To counteract this, many produce active compounds as defensive mechanisms (Vijayalakshmi *et al.*, 2008; Benkendorff, 2010). One of the known active compounds is the antimicrobial peptide myticin found in mussels (Cheng-Hua, *et al.*, 2009; Benkendorff, 2010). However, some of the SMs that act as antibiotics, are not produced by the mollusc itself but are rather produced by symbiotic microorganisms such as *Pseudoalteromonas tunicata* (Marine Natural Products 2021). *P. tunicata* was first isolated from the tunicate *Ciona intestinalis* and produces the SM violacein (Marine Natural Products 2021). The search for novel SMs has thus been expanded to incorporate commensal and symbiotic microorganisms associated with molluscs and other soft-body organisms (Blunt *et al.*, 2016).

Oysters, marine bivalves belonging to the Mollusca phylum, are known to host symbiotic microorganisms in their gills and other tissues (Zhukova *et al.*, 2022). These microorganisms may provide SMs for defence against other microorganisms (Zhukova *et al.*, 2022). *T. viridans* is a halophilic, Gram-negative, rod-shaped, strictly aerobic bacteria that has a single flagellum (Macián *et al.*, 2001). It was first isolated (labelled as XOM25^T) from oysters cultivated off the coast of Valencia, Spain in 1994 as distinct cultures that produce a blue-green diffusible substance (Ortigosa, *et al.*, 1995). This short-lived green compound is produced in both solid and liquid media (MB, Difco). However, extensive characterisation of the secondary metabolites produced by *T. viridans* has not yet been conducted (as of October 2022)

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although a draft genome was published in 2015 (Olonade *et al*., 2015). Genome mining of *T. viridans* (Section 2.1) has been employed to investigate the potential for SM production with reference to databases of known SMs. Several bioactivity-guided investigations have confirmed the production of antibacterial activity by this strain, and fractionation has confirmed that there is likely more than 1 bioactive compound contributing to this activity (Adams, 2019; Pheiffer *et al.*, 2022). To date, lanthipeptides are the only SMs that have been characterised from this strain, but these did not confer antimicrobial activity (Vermeulen *et al.*, 2022).

In molluscs alone, it is suggested that the chemical diversity available (including structural similarities) exceeds the species diversity due to the nature of the environments in which they are found (Benkendorff, 2010). However, in 2017 only 60% (17 in total) of the previous decade's annual average number of new metabolites was reported from molluscs - none of which are specifically antibiotic in nature (Carroll *et al.*, 2020). Over the same period, 240 novel or new-to-environment compounds were described. This highlights the potential for discovering novel products from the marine environment, especially from bacteria - commensal, symbiotic or otherwise.

1.5. Approaches to novel compound/antibiotic discovery from microbes.

The signal or mode of induction for an antimicrobial peptide (AMP) of interest may not be present in a standard laboratory culture media, making it impossible to induce the production of uncharacterised AMPs in the origin microorganism (Demain and Fang, 2000). Trial and error are often used to isolate AMPs from an unknown pathway, but this is a time-consuming process that relies on variations in the growth conditions to attempt to trigger AMP production (Adams, 2019; Pheiffer *et al.*, 2022; Vermeulen *et al.*, 2022). To overcome this, genome mining can be employed to interrogate the potential for AMP production. This method has been employed in the research associated with *T. viridans* (Adams, 2019; Pheiffer *et al.*, 2022; Vermeulen *et al.*, 2022), but a comprehensive study of AMPs has not been conducted.

Genome mining considers the genome first in the search for new compounds and the chemical nature of the products can be predicted by biosynthetic logic and similarity to known characterised compounds and pathways (Medema and Fischbach, 2015;

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Navarro-Muñoz *et al.*, 2020). The use of bioinformatics tools on large genomic data sets circumvents the limitations of traditional isolation and identification, but this increase in information and the need to annotate it, introduces its own limitations such as being dependent on extrapolation on existing data of known AMPs. This means that novel AMPs will have to be characterised in some way (using traditional techniques) to build the database and the resulting hypothetical AMPs revealed by genome mining would be expected to share some characteristics.

There are a number of bioinformatics and *in silico* tools that can be used for the assessment and management of genomic data to determine the presence of potentially useful AMPs and the capabilities of these tools have increased rapidly in the past decade (Shaker *et al.*, 2021). *In silico* tools cover those that can computationally determine the potential presence and/or mechanisms of an AMP or potential SMs (Shaker *et al.*, 2021). These include homology structural prediction and ligand-binding prediction. The complexity of bioinformatics tools and systems has been simplified by the development of web-based platforms such as KBASE (www.kbase.us) where a large portion of the genomic and *in silico* tools have been curated. This platform combines a wide range of tools in a unified graphical user interface that allows for assembly, annotation, sequence analysis, metabolic modelling, RNA sequence and expression analysis, and comparative genomics.

These tools include the Antibiotics and Secondary Metabolite Analysis Shell (antiSMASH) (Medema *et al.*, 2011; Blin *et al.*, 2019), The SEED/Rapid Annotation using Subsystem Technology (RAST) (Overbeek, *et al.*, 2005; Aziz, *et al.*, 2008; Overbeek, *et al.*, 2014), PROKKA (Seemann, 2014), Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1997, 2005; Johnson *et al.*, 2008) and Biosynthetic Gene Similarity Clustering and Prospecting Engine (BiG-SCAPE)/CORe Analysis of Synthetic Orthologues to prioritize Natural product biosynthetic gene clusters (CORASON). In addition to these tools for genomic analysis, the Universal Protein Resource (UniProt) platform combines some of these tools in conjunction with structural prediction analysis referencing multiple curated databases to infer the similarity of gene sequences to those of well-characterised proteins (Bateman, 2019).

AntiSMASH, a software pipeline that can identify and annotate possible secondary metabolite gene clusters in the target genome, is specifically designed for NRPS/PKS identification by

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inferred function based on homology (Medema *et al.*, 2011; Weber *et al.*, 2015). The RAST/SEED server is based on subsystems that generally reflect a pathway, protein family, or collection of functional roles that are associated with structural complex or biological process (i.e., glycolysis comprising of a set of functional roles - glucokinase, glucose-6-phosphate isomerase and phosphofructokinase) (Overbeek, *et al.*, 2005; Aziz *et al.*, 2008; Overbeek *et al.*, 2014). In addition to the subsystems mapping by the RAST tool, the SEED database is incorporated to integrate annotations that improve genome mapping (Overbeek *et al.*, 2014). PROKKA is a tool for the annotation of large genomes and is primarily a Unix-based system that coordinates other gene annotation tools aimed at improving the accuracy of the annotation of bacterial sequences (Seemann, 2014). The BLAST alignment web tool enables the search for similar SMs based on either protein or gene sequence similarity. This tool references NCBI-linked databases and is split into different methodologies. One such methodology is Tblastn which identifies gene sequences in the database that encode proteins with similar sequences to the reference protein sequence (Altschul *et al.*, 1997, 2005; Johnson *et al.*, 2008). This also enables the assessment and identification of conserved domain and similarity to other protein/gene sequences based on functional families (Johnson, *et al.*, 2008). BiG-SCAPE/CORASON is a combination of tools that are aimed at building phylogenetic links between BGC and incorporates the Minimum Information about a Biosynthetic Gene cluster (MIBiG) database (Navarro-Muñoz *et al.*, 2020).

All alignment, annotation and analysis tools depend on similarities to existing known genomic structures and thus sequence extrapolation needs to be used to find conserved domains that represent Biosynthetic Gene Clusters (BGCs) or gene sequences for AMP families (Floudas *et al.*, 2006). The success of these tools depends on the reference library used (Marchler-Bauer *et al.*, 2009). However, the increase in the size of the available databases, collaborations and curation of data means that these are becoming invaluable tools for the discovery of BGCs and the associated SMs. Evaluating an organism's entire genome for SMs almost immediately after isolation of a bacterium vastly reduces the amount of time needed to determine if any useful, or potentially useful, SMs are produced by it. This approach may not reduce initial costs for SM discovery, as genomic sequencing is currently expensive, but it allows for a targeted approach to isolate and characterise SMs and AMPs. In terms of proteins, the similarity in the conserved domains in the DNA sequences could imply that there is some

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structural similarity in the active protein as well and, by extension, functional similarity (Floudas *et al.*, 2006b). By searching for the conserved domains of known SMs in gene sequences, in conjunction with heterologous expression systems and artificial DNA synthesis, multiple potential SMs can be investigated without the need for extensive gene editing and isolation experiments. All these tools also allow for large-scale investigation and discovery of potentially useful AMPs and will be vital in the development of a functional hypothesis for a specific protein family, especially with reference to antibiotics and their role in the natural environment.

The ability to artificially synthesise a DNA sequence has assisted the search for bioactive secondary metabolites without the need to culture/isolate individual organisms (Sanchez-Amat, Solano and Lucas-Elío, 2010). In addition, variations can be made to specific sections of the DNA sequence for comparative assessment and development of isolation and purification protocols. It also provides a background for the *De novo* design of proteins of interest based on the relationship between gene sequence and protein functional structure (Floudas *et al.*, 2006). Furthermore, with the combination of heterologous expression of proteins from synthetic DNA and "knock-out" gene modification in the natural host, the SM pathways can be investigated to further characterise AMP and assess the differences in function between them.

Examples of compounds that have successfully been identified and characterised using the process outlined above are lanthipeptides and polytheonamide-type peptides (Bhushan *et al.*, 2019). Both are important novel compounds in expanding the diversity of available antibiotics (Bhushan *et al.*, 2019). Lanthipeptides as novel antibiotic peptides were heterologously expressed in *E. coli* from gene sequences identified in *Corynebacterium lipophiloflavum* DSM 44291 and *Streptococcus agalactiae* ATCC 13813 (van Heel *et al.*, 2016). Owing to polytheonamide-type peptides' novelty and cytotoxicity, they have been heterologously expressed in *Rhizobium leguminosarum* from sequences identified in *Microvirgula aerodenitrificans* (Bhushan *et al.*, 2019).

In some cases, as above, the AMP may even be toxic or have byproducts that are toxic to the expression host, further frustrating the production of the AMP for characterisation and antibiotic screening (Glick, 1995; Gabor, Alkema and Janssen, 2004). In addition, there are a

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number of other factors to be considered for heterologous expression, such as: the size of the total construct required for the synthesis of the SM of interest, the metabolic load placed on the host in producing the product, whether the AMP is fully synthesised, or if the produced proteins are incorporated into insoluble inclusion bodies (Gabor, Alkema and Janssen, 2004). The minimal requirements for successful heterologous gene expression are a transcription promoter site, a ribosome binding site (-20 to -1 upstream of the start codon), and a bacterial start codon, all of which must be compatible with the host's expression pathways (Gabor, *et al.*, 2004). The selection of the appropriate host organism is also directed by the requirement for cofactors, chaperones, and specific post-translation modification enzymes (Gabor, *et al.*, 2004). An example of this is where the expression of polytheonamide-type peptides was shifted to *R. leguminosarum* to achieve the correct formation of the peptides (Bhushan *et al.*, 2019). In this instance *E. coli* was not able to produce the complete protein with modifications because it does not have a complete cobalamin biosynthetic pathway, whereas *R. leguminosarum* has the required machinery (Bhushan *et al.*, 2019). An additional complication in the use of synthetic DNA for heterologous expression is that cofactors may need to be expressed in a specific sequence or quantity for the target product to be active.

The specific requirements for cofactors, and the specific post-translation modification enzymes required for active heterologous enzyme production add to the time needed to isolate, identify, and characterise a possible novel antibiotic compound. However, in some cases, even though these heterologous expression systems may take longer, they may make the process of isolating, identifying and characterising the SMs from single or tandem gene sequences much simpler. In these instances, the biosynthetic process may be relatively simple and/or the host carries the required cellular machinery to produce the SM. In the case of biosynthetic gene clusters, where a larger number of genes encode the multi-enzyme machinery required to synthesise the compounds, the use of direct DNA synthesis and heterologous expression may not be as effective.

Using genome mining to search for novel antibiotics, in conjunction with heterologous expression systems, as a method of exploiting the diversity of marine bacteria may help to turn the tide against the increasing prevalence of antibiotic-resistant bacteria and other pathogens. In addition, this method may relieve the pressure of producing large quantities of an AMP for further downstream applications (such as clinical testing) by not relying on the

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cultivation of the source organism, and leveraging the potential production scalability of *E. coli* and other host organisms.

The assessment of AMPs for antimicrobial activity from any source is time-consuming and new methods must be employed to characterise these compounds. With the use of genomics and heterologous expression systems, novel AMP or analogues of existing ones may be assessed faster than isolating them from the origin organism or plant. Although this can improve the rate of new antimicrobial discovery, it does not reduce the rate at which pathogens develop antibiotic resistance. It may, however, contribute to keeping ahead of progress of antibiotic resistance.

1.6. Project Background and Research Objectives.

The annotation of the *T. viridans* genome indicated that there are two sequences with similarity to the *lodA* gene that codes for l-lysine ε-oxidase (LodA) first isolated and characterised from *M. mediterranea* (Gómez D *et al.*, 2006). A large number of other microorganisms carry similar sequences, or at least sequences that have the same conserved domains when using the BLAST web tool (Johnson *et al.*, 2008). LodA is an extracellular enzyme that appears to have antibacterial properties associated with the production of H_2O_2 as a product of lysine oxidation (Gómez D *et al.*, 2006). Expression of both the *lodA* and *lodB* gene sequences is required for the production of functional LodA in *M. mediterranea* (Matsui and Asano, 2015; Sehanobish *et al.*, 2015).

T. viridans harbours two *lodAB* operons and a third lone *lodB* gene. This study aims to assess the expression of these genes in *E. coli,* determine whether they have any antimicrobial activity, and characterize the expressed proteins.

Objectives:

- Genomic annotation of *T. viridans* using various tools to identify possible antimicrobial proteins / peptides similar to LεO from *M. mediterranea.*
- Assess the genomic relationships of the *lodAB* gene sequences.
- Heterologously express proteins in pairs and purify proteins for characterisation.
- Characterise proteins and assess for antimicrobial activity.

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Chapter 2 Materials & Methods

Unless otherwise noted H_2O refers to reverse osmosis (RO), de-ionized and filtered (0.2µm) water from the laboratory supply. Room temperature (RT) was measured to range from 20°C - 22°C.

2.1. Genome Mining of *Thalassomonas viridans* – Secondary Metabolite Identification.

The draft genome of *Thalassomonas viridans* was used for the initial detection and annotation of secondary metabolites (SMs) and antimicrobial peptides / proteins (AMPs). This genome was publicly released and available on NCBI (accession number: JYNJ00000000) (Olonade, *et al.*, 2016), and was submitted to the RAST (https://rast.nmpdr.org/rast.cgi) (Overbeek *et al.*, 2014) and antiSMASH (Blin *et al.*, 2019) servers for analysis in April 2019. Additional long-read technology sequencing was performed to complete the genome (Pheiffer *et al.*, 2022). The final genome of *T. viridans* was resubmitted to RAST, antiSMASH, and PROKKA (Seemann, 2014) servers for reconfirmation of potential SMs and AMPs and to identify their relative position on the genome in March 2020.

Various tools were used for the annotation and alignment of gene sequences from *T. viridans*. The first of which is antiSMASH (Medema *et al.*, 2011; Weber *et al.*, 2015). In conjunction, the RAST server incorporating the SEED database (Overbeek, *et al.*, 2005; Aziz *et al.*, 2008; Overbeek *et al.*, 2014) was also used to annotate the genome. The long-read gene sequence data was then uploaded and stored on the PROKKA server (Seemann, 2014).

Once annotation of the genome was complete, the BLAST alignment tool was employed to search for similar sequences, either protein or gene, in NCBI-linked databases (Johnson *et al.*, 2008).

2.2. General chemicals, antibiotics & enzymes.

The chemicals used in this study were supplied by Fluka, Thermo Fisher Scientific (Massachusetts, USA), Merck Chemicals and Laboratory Supplies (Darmstadt, Germany), and Sigma Aldrich Chemical Company (Deissenhofen, Germany). The protein and DNA size markers were purchased from New England Biolabs (Massachusetts, USA). The restriction endonucleases were purchased from Fermentas Life Sciences Ltd. (Vilnius, Lithuania). Antibiotics were purchased from Sigma Aldrich Chemical Company (Deissenhofen, Germany) and Thermo Fisher Scientific (Massachusetts, USA) and used as described in Table 2.1.

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Table 2.1 - Antibiotics used in this study.

Antibiotics	**Preparation**
Ampicillin (Amp)	200mg/ml in H_2O 200µg/ml final concentration
Chloramphenicol (CHL)	34mg/ml in 100% ethanol stock 34µg/ml or 12.5µg final concentration
Kanamycin (KAN)	200mg/ml in H_2O 50µg/ml final concentration
Streptomycin (STR)	50mg/ml in H_2O 50µg/ml final concentration

2.3. Media

Marine Broth (MB2216), purchased from BD (Becton Dickinson, Berkshire, U.K.) for the culturing of *T. viridans*. This media contains a distribution of minerals and salts designed to replicate the major components of seawater as well as peptone and yeast extract for bacterial growth. Marine Minimal Media (MMM) is a defined media that was used for the culturing of *T. viridans* in order to isolate any AMPs.

Media used for both growth and protein expression in *E. coli* are the following: Lysogeny Broth (LB), Lysogeny Agar (LB Agar), Terrific Broth (TB) and ZYM5052 auto-inducing complex media. The composition of each media is listed in Table 4.1 in the Appendix. All media were autoclaved at 121°C for 20 minutes, except where components are temperature sensitive, in which case they were filter sterilized and added to the media once cooled.

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2.4. Bacterial strains used in this study.

The bacterial strains used in this study are listed in Table 2.2.

Table 2.2 - Bacterial strains used in this study.

Strain	Genotype	Reference*
Thalassomonas viridans (XOM25)		(Pheiffer *et al.*, 2022)
Escherichia coli BL21(DE3)	F^- ompT hsdS(rB– mB–) gal dcm λ(DE3)	Invitrogen, USA
E. coli BL21(DE3) pLysS	F^- *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm* (DE3) pLysSRARE (Cam^R)	(Novagen) Merck KGaA, Darmstadt, Germany
E. coli BL21(DE3) pLysS pNH223	strain carrying amidase gene of *Geobacillus pallidus* RAPc8	(Cameron, 2002)
E. coli MC1061 (K12)	F^- Δ(*ara-leu*)7697 [*araD139*]$_{B/r}$ Δ(*codB-lacI*)3 galK16 galE15 (GalS) λ^- e14$^-$ *mcrA*0 *relA*1 *rpsL*150 *spoT*1 *mcrB*1 *hsdR*2 (Str^R) ($r_K^-m_K^+$) Zeo^R	(Casadaban and Cohen, 1980)
E. coli JM109 (K12)	F´ *traD36 proA+B+ lacIq Δ(lacZ)M15/ Δ(lac-proAB) glnV44 e14- gyrA96 recA1 relA1 endA1 thi hsdR17*	(Hanahan, 1985)
E. coli ArcticExpress (DE3)RIL	B F^- *ompT hsdS*($r_B^-m_B^-$) *cdm*$^+$ Tet´ *gal* λ(DE3) *endA* Hte [cpn10 cpn60 Gent'] [*argU ileY leuW* str']	Agilent, USA
Klebsiella pneumoniae	Drug resistant test organisms	Laboratory Stocks
Staphylococcus aureus ss. 33591		Laboratory Stocks
Escherichia coli ss.25922		Laboratory Stocks
Escherichia coli ss.35218		Laboratory Stocks
Pseudomonas putida		Laboratory Stocks
Enterobacter cloacae		Laboratory Stocks

* The initial supplier or publication is detailed under the reference.

E. coli BL21(DE3) pLysS is a lactose permease (*lacY*) mutant, is protease deficient and contains a plasmid encoding for the tRNAs: *argU, argW, glyT, ileX, leuW, metT, proL, thrT, thrU & tyrU* (Zhang and Studier, 2004). This high-stringency expression host allows the expression of genes encoding tRNAs for rare codons (Zhang and Studier, 2004). In addition to T7 inhibition of transcription, the T7 lysozyme also acts on the peptidoglycan layer of the cell wall (Zhang and Studier, 2004). This improves the recovery of heterologously expressed proteins by enabling the complete rupturing of cells with reduced mechanical force (Zhang and Studier, 2004). This strain is selected for using chloramphenicol resistance (Zhang and Studier, 2004).

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E. coli Arctic Express (DE3)RIL is a T7 expression system where incorrect protein folding and codon bias are reduced by enabling low-temperature heterologous expression of proteins (Agilent, USA). Low-temperature expression is achieved through the inclusion of chaperonins Cpn10 and Cpn60 from *Oleispira antarctica* (Agilent, USA). These chaperonins are highly similar to those native to *E. coli*, but they are active at temperatures between 4°C and 12°C (Agilent, USA). At these temperatures, protein folding is slowed and should increase the abundance of active and soluble heterologously expressed protein (Agilent, USA). In addition, the RIL strain addressed codon bias by the inclusion of additional argU, ileY, and leuW tRNA genes to increase arginine, leucine, and isoleucine recognition (Agilent, 2010).

2.4.1. Freezer stocks

Freezer stocks were made of all the bacterial strains and associated clones from single colonies of the respective bacterial strains with antibiotics as required, that were inoculated in separate 5ml LB in sterile glass tubes. These were then incubated overnight at 37°C on a shaking table (125 rpm). The following day, 250µl was withdrawn from each tube and transferred to 2ml Eppendorf tubes where 250µl 30% glycerol solution was added. These were mixed by inversion and stored at -80°C.

2.5. Construct Synthesis

The Duet-1 plasmid system (Novagen, Germany) was used in this study. This system of plasmids carries the *T7lac* promotor site, which enables induction of expression by the addition of IPTG or using autoinduction with lactose (Novagen, 2011). Synthetic constructs of each of the *lodA* and *lodB* sequences were ordered from Biomatik (USA) as inserts already incorporated into Duet-1 plasmids as per Table 2.3. Plasmid maps and sequence inserts for all plasmids are available in figures 4.1 to 4.6 in the Appendix.

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Table 2.3 - Plasmids used in this study.

Plasmid	Sequence inserts	Description	Reference
pRSFDuet-1	vector	*T7/lac* promotor, Kan^R, N-terminal His·Tag MCS1	Novagen
RSF-lodA1	*lodA #1* – 2130 bp gene sequence for LodA1	*T7/lac* promotor, Kan^R, N-terminal His·Tag	This study
pCOLADuet-1	vector	*T7/lac* promotor, Kan^R, N-terminal His·Tag MCS1	Novagen
COLA-lodB1	*lodB #1* – 1176 bp gene sequence for LodB1	*T7/lac* promotor, Kan^R, N-terminal His·Tag	This study
pETDuet-1	vector	*T7/lac* promotor, Amp^R, N-terminal His·Tag MCS1	Novagen
pET-lodA2	*lodA #2* – 2247 bp gene sequence for LodA2	*T7/lac* promotor, Amp^R, N-terminal His·Tag	This study
pET-lodA1.2	*lodA #1* – gene sequence sub-cloned from RSF-lodA1	*T7/lac* promotor, Amp^R, N-terminal His·Tag	This study
PCDFDuet-1	vector	*T7/lac* promotor, Sm^R, N-terminal His·Tag MCS1	Novagen
CDF-lodB2	*lodB #2* – 1158 bp gene sequence for LodB2	*T7/lac* promotor, Sm^R, N-terminal His·Tag	This study
PCDFDuet-1	vector	*T7/lac* promotor, Sm^R, N-terminal His·Tag MCS1	Novagen
CDF-lodBL	*lodB Lone* – 1155 bp gene sequence for lone LodB	*T7/lac* promotor, Sm^R, N-terminal His·Tag	This study
pNH223	Plasmid carrying amidase gene of *Geobacillus pallidus* RAPc8	*T7* promotor, Amp^R, N-terminal T7·Tag, C-terminal His·Tag, $RAPc8^+$	(Cameron, 2002)

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2.6. Preparation of competent cells.

Electro-competent and chemical competent cells were prepared from a single colony of each of the *E. coli* strains according to existing protocols (Chung and Miller, 1993; Sambrook and Russel, 2001)

2.7. Transformation of Electro- & Chemical Competent cells.

Chemically competent *E. coli* BL21(DE3) cells (50µl) were transformed by the addition of approximately 5µg plasmid DNA in sterile 2 ml Eppendorf tubes. Cells were incubated on ice for 30 minutes prior to the addition of the plasmid solution. Immediately after the addition of the plasmid solution, the cells were heat shocked at 37°C for 5 minutes and then returned to the ice for a further 2 minutes. After the time on ice 1 ml of LB was then added to the tubes and the cells were incubated at 37°C for 60 minutes for recovery. Following incubation 100µl of the transformed cells were then plated onto LB-agar with the appropriate antibiotic/s and further incubated at 28°C overnight.

Electro-competent *E. coli* BL21(DE3) pLysS (80µl) cells were transformed and/or co-transformed by the addition of 8µg plasmid DNA. Prior to electroporation, the plasmid stocks were dialyzed on a 0.025µl nitrocellulose filter floated on H_2O. The competent cells were defrosted on ice prior to the addition of the dialyzed plasmid solution. Treated cells were then transferred to a precooled 0.8 cm cuvette and placed in a Bio-Rad MicroPulser™ (USA). The cells were then subjected to a single pulse of 2.5 kV. Immediately thereafter 1ml room temperature LB was added to the cells and then transferred to a 1.5ml Eppendorf tube and incubated at 37°C for 1 hour. After incubation, 100µl of the transformed cells were then plated onto LB-agar with the appropriate antibiotic/s and further incubated at 28°C overnight.

For co-expression, where 2 plasmids are transformed to *E. coli* BL21(DE3) pLysS, the cells were first transformed with either COLA-lodB1 or CDF-lodB2 by electroporation as above. After successful transformation, the cells were then made electro-competent as for the untransformed cells. Aliquots of each of the transformed LodB *E. coli* were then co-transformed with either RSF-lodA1 or CDF-lodB2 plasmids using electroporation as described above.

E. coli ArcticExpress (DE3)RIL transformations were treated according to the Agilent manual for these cells.

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2.8. Nucleic acid extraction

2.8.1. Crude gDNA extraction from *Thalassomonas viridans*.

A single colony of *T. viridans* was isolated from a marine agar plate. This was inoculated to 50 ml Marine Broth and cultured at room temperature (20°C - 22°C) on an orbital platform at 150 rpm. After 4 days 200µl was then withdrawn and centrifuged at 15 000 *xg* for one (1) minute. The pellet was reserved and re-suspended in 50µl nuclease-free H_2O. This was then heated to 95°C for 10 minutes and centrifuged again at 15 000 *xg* for one (1) minute. The supernatant was reserved as the fraction containing genomic DNA. A colony from the same plate was streaked out onto another marine agar plate and kept at room temperature to observe the production of a green/blue compound.

2.8.2. Plasmid extraction

Where plasmids needed to be extracted for stock storage and cloning/sub-cloning, cultures of each clone were incubated overnight at 37°C with shaking. The relevant antibiotic for each plasmid and/or strain was included for selective control (see Tables 2.2 and 2.3). Plasmid DNA was extracted with either the Plasmid Mini-Prep Kit or the Plasmid Midi-Prep Kit (Qiagen) as per the manufacturer's instructions. In addition, large-volume extractions were also conducted using the alkaline lysis method (Sambrook and Green, 2016), see Appendix Table 4.2. Briefly, 1.5ml of the overnight culture was centrifuged at 16 000 x*g* for 30 seconds at 4°C, the supernatant was discarded. The pellet was then resuspended in 110µl of Solution 1 by vortexing, followed by 200µl of Solution 2. This was gently mixed by inversion, five times and kept at room temperature for 5 minutes. To the sample solution, 150µl of ice-cold Solution 3 was added and mixed gently by inversion, five times and stored on ice for a further 5 minutes. Tubes were centrifuged at 16 000 *xg* for 10 minutes at 4°C and the supernatant was reserved. Two volumes of 100% ethanol were added to the reserved supernatant and mixed by vortex and then stored on ice for 5 minutes or at 4°C overnight. Tubes were then centrifuged at 16 000 *xg* for 10 minutes and the resulting pellet was gently resuspended in 1ml 70% ethanol by inverting five times. The ethanol wash was repeated twice then the supernatant was carefully removed and the pellet containing DNA was air-dried at room temperature. The recovered plasmid DNA was then re-suspended in 50µl TE (pH 8.0) [Tris, 0.12 g; EDTA, 0.03 g; H_2O to 80ml, pH adjusted using HCl; final volume 100ml].

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2.9. Nucleic acid analysis

DNA purity and concentration were determined by using the Nanodrop® ND-1000 (Nanodrop Technologies, Inc., USA) and the Qubit™ Fluorometer v1.0 (Life Technologies, USA).

2.9.1. DNA agarose gel electrophoresis

Agarose gel electrophoresis was used to assess 16S rRNA and plasmid DNA digests. Concentrations from 0.8 – 2% [W/V] were used in 1x TAE buffer (0.48% [w/v] Tris-base, 0.04% [w/v] EDTA and 1.1% [v/v] glacial acetic acid, pH 8.3). Ethidium Bromide (EtBr) at 0.5µg/ml, was included in the solution prior to pouring into a gel tray to solidify. A 10X concentrated loading dye used for loading the DNA samples consisted of 20% [v/v] glycerol, 0.1 M EDTA, 1% [w/v] SDS and 0.25% [w/v] bromophenol blue. Electrophoresis was conducted in 1x TAE buffer at 80 volts for 60 – 90 minutes. DNA size markers were included, either 1kbp (New-England Biolabs) or phage Lambda DNA completely digested with PstI endonuclease (Thermo Scientific) (λ-Pst) for the determination of the fragment sizes. An Alpha Imager®HP 2000 (Alpha Innotech, USA) with UV and relevant filters were used to visualise the DNA in the gel after the completion of the run.

2.9.2. Polymerase Chain Reaction (PCR) for Amplified Ribosomal DNA Restriction Analysis of *Thalassomonas viridans.*

Polymerase Chain Reaction (PCR) was used in this study for Amplified Ribosomal DNA Restriction Analysis (ARDRA) to confirm that the bacterium is indeed *T. viridans* before proceeding with culture isolate experiments. ARDRA is based on the amplification of the 16S rRNA gene by PCR, followed by restriction enzyme digestion and then agar gel electrophoresis. The resulting restriction pattern was then compared to the expected pattern for *T. viridans*.

PCR was set up with the following parameters: 50µl reactions in triplicate consisting of 1x reaction buffer (Dream Taq 10x), 0.25 µM of each of the primers (forward and reverse), 2mM dNTP mix (dATP, dTTP, dCTP and dGTP), 1.25 U Dream Taq® DNA polymerase, 2µl of *T. viridans* crude extract from the culture as described previously (Section 2.8.1), and made-up to the final volume with nuclease-free water. The PCR conditions and primers used are detailed in Table 2.4 below. The amplifications were carried out in a BIO-Rad T100 automated thermal cycler.

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Table 2.4 - Primers and PCR cycling conditions.

Primer	Target sequence	Sequence (5'-3')	Product size (bp)	Thermal cycling parameters	Reference
E9F U1510R	16S rRNA gene	GAGTTTGATC CTGGCTCAG GGTTACCTTG TTGTTACACTT	1500	Initial denaturation at 95°C, for 3 minutes, 30 cycles of 95°C for 30 seconds, 52°C for 30 seconds, 72°C for 2 minutes and final extension at 72°C for 5 minutes.	(Klindworth *et al.*, 2013)

2.10. Restriction enzyme digestion conditions for ARDRA, plasmid confirmation and cloning.

2.10.1. Restriction digest conditions for ARDRA analysis.

The 16S rRNA PCR amplicon was digested for diagnostic purposes using SmaI, HindIII and Eco1471. In all cases 22µl total volume consisted of 2µl of the restriction enzyme stock, 2µl Tango 10x buffer, 10µl PCR product and made up to volume with H_2O. The reaction mixes were incubated for 2 hours at 30°C for SmaI and 37°C for Eco1471 and HindIII as per the manufacturer's instructions. The reactions were then stopped by heating to 60°C for 10 minutes. Agarose (1%) gels were used for visualisation of the restriction digest products.

2.10.2. Restriction conditions for plasmid confirmation and sub-cloning.

Plasmid identity was confirmed using restriction enzymes analysis and were carried out in either 50µl PCR or 1.5ml Eppendorf tubes. Each 20µl reaction volume included approximately 1 U of the restriction enzyme (of both in the case of double digests) per µg of plasmid and was incubated at 37°C for at least 2 hours. Restriction enzymes SacI and NotI were used for restriction analysis and double digests to retrieve the pETDuet-1, pCDFDuet-1 and pCOLADuet-1 plasmid backbones as well as the *LodA1* DNA insert from their respective plasmids. The enzyme reaction for these two was inactivated by heating to 80°C for 20 minutes. EcoRI, BglI, XbaI were used for diagnostic digest. BglI was inactivated using 0.5M EDTA, EcoRI and XbaI were inactivated by heating to 65°C for 20 minutes. In each case, the relevant manufacturer's buffer system was used per instructions. Agarose (1%) gels were

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used for visualisation of the restriction digest products. “Empty” vectors (i.e., without a DNA insert) from all plasmids received were created by double digestion using SacI and NotI to liberate the inserts. The relevant fragments were excised from agarose following electrophoresis and visualisation, followed by T4 DNA Polymerase blunting of the DNA ends and blunt-end T4 ligation.

2.10.3. Plasmid DNA extraction from agarose gels.

A 0.8% (w/v) agarose gel was prepared and run. The DNA was then visualised using long wavelength UV (365nm) and the relevant bands were excised from the gel. The Nucleospin® Gel and PCR Clean-up kit (MACHEREY-NAGEL, USA) or the QIAEX II gel Extraction Kit (QIAGEN, Germany) was then used as per the manufacturer’s instructions to isolate the DNA.

2.11. Sub-cloning of *lodA#1* DNA inserts into pETDuet-1 to form pET-lodA1.2.

The sequence *lodA#1* was sub-cloned from pRSFDuet to pETDuet to avoid antibiotic kanamycin marker conflicts when conducting co-expression of LodA1 and LodB1. Each plasmid requires a different marker to ensure that they are both maintained in the host.

The pETDuet backbone was derived by restriction digestion from the pET-lodA2 plasmid received from Biomatik (USA). Restriction enzymes SacI and NotI were used to isolate both the backbone and the *lodA#1* insert from their respective plasmids. A schematic of the process is shown in annexure figure 4.7.

2.11.1. T4 Polymerase blunting

T4 Polymerase (ThermoFisher, USA) was used to remove the incompatible overhang and recessed ends to create the vectors without inserts for each plasmid. Each 30µl reaction contained 500µg linear plasmid DNA (post gel extraction), 1x reaction buffer (NEB Buffer 2.1), 100 µM each dNTP and 1 U T4 polymerase and incubated at 12°C for 15 minutes. To avoid incompatibility with downstream processes, the reaction was inactivated at 75°C for 10 minutes. The linear plasmid DNA was then ligated with T4 DNA Ligase.

2.12. T4 DNA Ligase ligation

Ligation reactions were performed using T4 DNA Ligase (NEB, USA). The 20µl reaction contained the vector backbone DNA and insert DNA (or only the blunted vector backbone), 1x T4 DNA Ligase buffer, nuclease-free H_2O and 1µl (400 U) T4 Ligase solution. The reaction

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mix was incubated at 16°C overnight. T4 Ligase was inactivated at 65°C for 10 minutes. After cooling on ice, plasmids were transformed to chemical-competent *E. coli* JM109 by heat shock.

2.13. Heterologous expression & co-expression.

Heterologous expression in *E. coli* BL21(DE3) or *E. coli* BL21(DE3) pLysS, harbouring either one or two plasmids, each carrying a *lod* sequence (A, B or A & B) was conducted in 50ml LB, with single or multiple antibiotics as required at room temperature (20°C - 22°C) on a shaking platform (175 rpm) for various time periods before harvesting. Expression was induced by the addition of 0.4 mM to 1 mM IPTG at OD600 approximately 0.6.

Expression of RSF-lodA1 and COLA-lodB1 in *E. coli* BL21(DE3) pLysS was also performed in 200ml to 400ml ZYM5052 auto-inducing complex media or TB. These cultures were incubated on a shaking table (175 rpm) at room temperature or at 15°C. The cells were cultured for 72 hours before harvesting. Expression was induced with IPTG as described for cultures grown in TB.

Expression in *E. coli* ArcticExpress (DE3)RIL was done as per the Agilent Manual (2010) where TB was used as the media and incubated at 12°C for up to 72 hours.

Expression of the amidase gene of *Geobacillus pallidus* RAPc8 in *E. coli* BL21(DE3) pLysS for a positive control for the phenol-nitroprusside assay was done according to (Cameron, 2002).

2.14. Sample preparation of Heterologously Expressed Proteins.

All cells post expression were harvested by centrifugation at 4400 *xg* for 20 minutes at 4°C. The supernatant was discarded, and the pellet was suspended in a suitable volume of potassium phosphate buffer (50 mM pH 7.0). The pellets from expressions carried out in *E. coli* BL21(DE3) pLysS cells were frozen at -80°C for 2 hours or overnight and then defrosted at 37°C. The pellets from expressions carried out in *E. coli* BL21(DE3) were not subject to the freeze-thaw process. Cells were sonicated on ice for five (5) 30 second intervals at 60% power (Bandelin Sonopuls sonicator with MS 73 Titanium microtip horn). The lysate was centrifuged at 4400 *xg* for 20 minutes at 4°C and the supernatant was reserved for further purification and/or analysis. The pellet was reserved for SDS-PAGE analysis to determine if any of the target proteins were being expressed in an insoluble form.

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Additional sample preparation steps were conducted on the extracted amidase according to Cameron (2002). Briefly, the cell free extract post sonication was heat treated at 75°C and then centrifuged at 4000 *xg* for 20 minutes, the supernatant was then reserved as the fraction containing amidase and stored at -80°C.

2.15. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

Total protein and purified proteins were analysed using SDS-PAGE according to the Laemmli (1970) method. 7% to 12% acrylamide gels (See Appendix Table 4.4) were loaded with samples combined with an equal volume of 2:1 loading buffer [63 mM Tris-HCl (pH 6.8), 0.3 M β-mercaptoethanol, 20% glycerol, 0.002% Bromophenol Blue, 4% SDS) and denatured at 95°C for 10 minutes. The samples were initially run at 80 V until the greatest MW protein band reached the top of the resolving gel, then run at 100 V until the dye front reached the end of the gel. On completion of the run, the gel was removed from the cassette and rinsed with H_2O and then placed in a staining solution [0.10% Coomassie Blue R-250, 50% Methanol, 10% Glacial Acetic Acid]. The gels in the staining solution were then agitated for at least 2 hours, but generally overnight. Destaining solution [50% Methanol, 10% Acetic acid] was then used to destain the gels for at least 3 hours, but generally overnight. Colour Prestained Protein standard, Broad Range (11-245kDa) (product #7719) from NEB (USA) was used for the determination of protein size. The equipment used was a PowerPac Basic power supply (Bio-Rad) and Mini-PROTEAN tank and casting system (Bio-Rad).

2.16. His-tag Affinity Chromatography

To purify the recombinant LodA and LodB proteins after expression, His-Bind resin (Novagen) and the corresponding buffers were used in accordance with the manufacturer's instructions (for buffer components see Appendix Table 4.4). A column containing 2ml of His-Bind resin solution was prepared, which settled to a 1ml bed volume. The column was first washed with 3ml of sterile H_2O, followed by 5ml of 1X charge buffer, and then with 4ml of 1X binding buffer. In all cases, the buffers and samples were allowed to drain by gravity. Post-expression, cell-free extracts were generated and applied to the column. The column was washed with 10ml. of 1X binding buffer, followed by 6ml of 1X wash buffer. The proteins bound to the His-tag were then eluted using 6ml of 1X elute buffer and collected. The eluted proteins were subjected to buffer exchange and SDS-PAGE as described in sections 2.17 and 2.19.

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2.17. Sample Concentration and Buffer exchange.

2.17.1. Buffer Exchange

When necessary (such as after His-Tag purification and culture supernatant assays), samples were concentrated and subjected to buffer exchange using Amicon Ultra-15 centrifugal filter devices with a molecular weight cut-off of 50,000 Da (Millipore, Germany). To prevent filter clogging, the samples were pre-filtered using 0.2 µm syringe filters before loading them into the centrifugal filter devices. The tubes were then centrifuged at 5,000 *xg* for 30 minutes, and the resulting filtrate was discarded (except for the initial His-Tag purification samples). Fresh buffer (potassium phosphate buffer pH 7.0) was added to the remaining sample (approximately 200µl), and the tube was centrifuged again under the same conditions. This process was repeated 3 times, without adding buffer after the final centrifugation. His-Tag elution samples were further processed on Amicon Ultra-15 3,000 Da molecular weight cut-off centrifugal filter devices.

As an alternative, buffer exchange and dialysis were performed using SnakeSkin (Thermo Scientific, USA). A segment of SnakeSkin, containing 3.5ml of the sample per 1 cm, was loaded, and closed according to the manufacturer's instructions. The sample was then dialyzed overnight in 2 L of Potassium Phosphate Buffer (50 mM pH 7.0), followed by an additional 2 L of Potassium Phosphate Buffer (50 mM pH 7.0) for 3 hours.

2.17.2. Acetone precipitation

Acetone precipitation was used as an alternative to concentrate His-Tag elution samples.

Four times the volume of the sample of ice-cold acetone was added to each sample. These samples were kept at -20°C overnight. The following day, the samples were then centrifuged at 14 000 *xg* for 10 minutes. The supernatant was discarded, and the pellet was air-dried at room temperature for 30 minutes. The pellet was then re-suspended in a suitable volume of potassium phosphate buffer (50 mM pH 7.0).

2.18. Bicinchoninic Acid (BCA) Assay Protein Quantification.

Protein concentrations were determined using the Peirce BCA Protein Assay (Thermo Scientific) according to the manufacturer’s procedure. Colourimetric readings were then measured using a Spectrostar Nano spectrophotometer (BMG Labtech, Germany) at 562nm

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and plotted against a standard curve constructed on the same day using bovine serum albumin with concentrations 0 to 2000 µg/ml, see Appendix (Figure 4.9).

Standard curves were plotted using MS Excel with absorbance on the dependant (X) axis and the standard protein concentration was plotted on the variable (Y) axis. A polynomial trend line was then added to the data set. The concentration of the unknown samples was then solved for Y, where $Y = ax^2+bx+c$ (Thermo Scientific, 2012).

2.19. Activity assays

As described in section 1.3.2, the oxidation of L-lysine by LodA produces two compounds (H_2O_2 and NH_4^+) that can be detected in several different assays. In this study assays to detect each of the products were employed to improve the identification of active samples. The liquid assays each have their limitations as described below. Lysine-α-oxidase (LαO) from *Trichoderma viride* was used as a positive control for the H_2O_2 product detection assay and was purchased from Sigma. The substrates used are listed in Table 2.5.

Table 2.5 - Amino acid substrates used for activity assays.

Lysine*	Glycine*	Glutamine	Threonine	Glutamate	Aspartic Acid
Histidine	Arginine	Serine	Asparagine	Cysteine	Proline
Alanine	Valine	Isoleucine	Leucine	Methionine	Tyrosine
Phenylalanine	Tryptophan	Acetamide*			

*Lysine and Glycine are the confirmed substrates for LodA from *M. mediterranea*, and acetamide has been included due to its simple structure that has an accessible amide group.

2.19.1. Phenol-hypochlorite – Ammonia Detection Assay

One of the methods used to confirm lysine oxidase activity in this study was a modified version of the phenol-hypochlorite ammonia assay developed by Fawcett and Scott (1960). This assay detects the release of NH_4^+ resulting from the enzymatic deamination of L-lysine. This modification of the assay involved miniaturizing it for use in a 96-well plate and plate reader, this improved the number of samples that could be assayed as well as reduced the sample volume required for each assay.

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The phenol-hypochlorite assay is a colourimetric assay that measures the intensity of colour change, which is directly proportional to the NH_4^+ concentration. Two reagents were prepared: reagent "A," consisting of 0.59M phenol and 1mM sodium nitroprusside ($Na_2[Fe(CN)_5]NO$), and reagent "B," consisting of 0.11 M sodium hypochlorite (NaClO) and 2 M sodium hydroxide (NaOH). The alkaline medium results in the generated ammonia reacting with the sodium nitroprusside to form indophenol blue (Helder and de Vries, 1979).

Reaction mixtures of 150µl were set up in 500µl PCR tubes in triplicate. Each reaction mixture contained a known concentration of substrate (0.1 to 0.5mM lysine), based on the work by Goméz *et al*. (2006), and a known concentration of cell-free extract or semi-pure enzyme in potassium phosphate buffer (50mM pH 7). The reaction mixtures were incubated at room temperature and 25µl test aliquots were removed at timed intervals, and then added to 87.5µl reagent "A" in the corresponding well of a 96-well PCR plate. The solution was mixed thoroughly to stop the enzymatic reaction. After taking all the time aliquots, 87.5µl of reagent "B" was added to each test mixture and incubated for 5 minutes at 50°C in a PCR machine. The samples were then transferred to a 96-well flat-bottom microplate (Greiner, Germany). Absorbance at 600nm was measured using a Spectrostar Nano spectrophotometer (BMG Labtech, Germany). The results were analysed against a standard curve to determine ammonia concentration. The standard curve was developed using 0.005mM to 1.0mM ammonium chloride in potassium phosphate buffer (pH 7.0) (see Appendix Figure 4.10). The blanks consisted of a known concentration of substrate and potassium phosphate buffer (50mM pH 7).

The positive control for this assay was carried out as above (Scott and Fawcett, 1960) using the heat-treated cell free extract of the amidase expression (see Section 2.16). The rection mix contained acetamide 0.1 mM as the substrate and 20 µl heat-treated cell free extract. The reaction was caried out for 8 minutes at 50°C and test aliquots were removed at 2 minute intervals. One unit (U) of activity is defined as the amount of enzyme that catalyses the production of 1µmol of NH_4^+ per minute under standard assay conditions.

2.19.2. Phenol red/Horseradish Peroxidase – H_2O_2 detection assay

One of the methods used to confirm the activity of lysine oxidase in this study used a modified version of the phenol red/horseradish peroxidase (HPR) colourimetric assay to detect the

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hydrogen peroxide (H_2O_2) generated during the oxidation of lysine (Pick and Keisari, 1980). The modification involved miniaturizing the assay for use in a 96-well plate and plate reader to reduce the amount of purified enzyme needed for a reaction. In addition, the exclusion of the pH adjustment step enabled the plotting of kinetic activity at 557nm absorbance. The absorbance at 557nm was chosen for assays as it exhibited the most significant colour change with an increase in H_2O_2 concentration for the pH of the assays (pH 7.2), while the absorbance at 610nm used in the original format did not offer a significant colour shift without pH adjustment. The decomposition of H_2O_2 (3.851mM) at high pH was shown to not be significant under 3 hours (Yazıcı and Deveci, 2010).

For each assay, each 200µL reaction mixture contained a known concentration (0mM to 33mM) of substrate (lysine or one of the essential amino acids), a known concentration of (20µg/ml to 90µg/ml) His-Tag purified enzyme (or positive control enzyme), 0.8mM phenol red sodium salt, and 90µg/mL horseradish peroxidase in potassium phosphate buffer (10mM pH 7.2) with 1.25mM NaCl. All assays were conducted at room temperature. Absorbance was read at 557nm on a Spectrostar Nano spectrophotometer (BMG Labtech, Germany) and interpolated against a standard curve developed using 0.3mM to 1.6mM H_2O_2 in potassium phosphate buffer (10mM pH 7.2) and excess HRP (90µg/ml) (see Appendix Figure 4.11).

For kinetic assessments, each reaction mix was set up in triplicate and started by the addition of the enzyme, and absorbance was read on a Spectrostar Nano at room temperature for 3 or 10 minutes at 10 second intervals. Lysine α-oxidase (LαO) (1.69 µg/ml) from *T. viride* was used as a positive control in the reaction and the Spectrostar Nano was set to 37°C (Georgiou and Sideri, 2000). Each reaction triplicate was started and measured independently. Reaction rates were determined by calculating the concentration of generated H_2O_2 from the absorbance reading and plotting the data over the course of the reaction. Furthermore, the reaction data was normalized by subtracting the background activity data of the reaction mixture without any enzyme. The initial rate was calculated as the change in H_2O_2 concentration per minute. The enzyme units and enzyme activity are defined by the below equations based on the initial 20 seconds of each reaction.

Enzyme units = (Average ΔmM / min) x (1/Vol.) x (1/protein conc.)

Enzyme activity = enzyme units / (vol. x protein conc.) [vol. = ml & protein conc. = mg/µl)

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Two blanks were included in the assay: one did not contain substrate (lysine), and the other did not contain any amino acid oxidase (either LαO or LodA) enzyme. Data was corrected against the blank values. The H_2O_2 standards were produced and assayed immediately prior to the activity assays.

2.19.3. High-Performance Liquid Chromatography – HPLC

High-Performance Liquid Chromatography (HPLC) was explored as a method to confirm chromatographically if there was any degradation of amino acid substrates due to the oxidase activity of amino acid oxidase. LαO was used as a positive control to determine if a retention shift or intensity shift in the chromatogram was evident for the enzymatic oxidation of lysine. HPLC is usually conducted in conjunction with derivatisation of the amino acids to improve detection, but it has been used in conjunction with mass spectrometry without derivatisation to quantify the presence of lysine metabolites, including pipecolic acid, a spontaneous product from 2-Aminoadipate 6-semialdehyde (Matsuda and Asano, 2010b; Pena *et al.*, 2016).

Reaction mixtures of individual lysine and separate reaction mixtures of lysine with LαO in potassium phosphate buffer (10mM pH 7.2) were used for comparison. Various lysine concentrations (4.15mM, 8.3mM, 16.6mM) were used to assess for retention shift and intensity change comparisons. The reaction mixes (1ml) for the positive control (LαO) were treated at 37 °C for 2 hours. For LodA1 His-tag purified protein (1ml) the reactions were incubated at room temperature overnight. All reactions were repeated in triplicate.

A Dionex Ultimate 3000 HPLC system was used for analysis. Sample volumes for injection ranged from 20µl to 200µl for the samples (depending on the intensity of the observed chromatograms) and were injected onto the column, either a Hypersil Gold C18 column (Thermo Scientific) (at 30°C) with H_2O mobile phase and methanol elution buffer from 20% to 90% or a Rezex RHM-monosaccharide ion exclusion column (Phenomenex) (at 40°C) with acetonitrile as the mobile phase. A flow rate of 0.3ml/min was used for both columns.

The Hypersil Gold C18 column separates the sample components by hydrophobicity while the Rezex RHM-monosaccharide ion exclusion column separates sample components by ionic charge. Both columns were used to compare if there would be a clearer change in signal for each of the samples.

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Chromeleon© Dionex ver. 6.80 software was used to analyse the peaks at the following absorbance wavelengths, 190nm, 200nm, 210nm, and 220nm.

2.20. Protein Identification and Structure Analysis

2.20.1. Proteomics

E. coli ArcticExpress (DE3)RIL co-transformed with both, RSF-lodA1 and COLA-lodB1 was cultured for expression. After 72 hour expression, the samples were prepared, and the cell pellet was re-suspended in 50 mM sodium phosphate buffer with a final protein concentration of 5.40 mg/ml. This sample was submitted for LCMS/MS analysis at the Central Analytics Facility at Stellenbosch University.

Furthermore, in cases where multiple bands from the same His-tag purified protein were detected during SDS-PAGE separation, these bands were cut out of the gel and submitted (as gel fragments) for LCMS/MS analysis. This was done to determine whether these different bands comprised of other proteins or if they represented fragments of the purified single protein.

LCMS/MS analysis was performed using either LTQ Orbitrap Velos or Fusion tandem mass spectrometers (both Thermo Scientific, Germany), linear ion-trap mass-spectrometers and the results were analysed against the translated protein sequence for LodA1 and LodB1 as the references. Using tandem-MS (MS/MS) the relative abundance of a protein can be determined. The sample was ionised and produced a retention spectrum. These ionised peptides were measured, and the masses produced were used to determine sequence identity from a database. The identified peptide fragment sequences were then compared to the total AA sequence for the protein of interest. The abundance of the fragments that matched the sample sequence was used to determine relative abundance.

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Chapter 3 Results & Discussion

3.1. Introduction

The genome of *Thalassomonas viridans* includes a "chromid," which is a megaplasmid, separate and independent of the chromosome, and carries a significant number of gene sequences associated with secondary metabolism (Pheiffer *et al.*, 2022). Chromids carry extrachromosomal replicons that may carry additional sequences for proteins under different induction systems to ensure their production when environmental conditions change (Harrison *et al.*, 2010). This section describes the sequence analysis and genome mining of *T. viridans*, and the analysis of the putative *lodAB* operons in *T. viridans* and the potential protein produced from these sequences, followed by heterologous expression of the *lodA* and *lodB* genes.

3.2. Genome Analysis of *Thalassomonas viridans.*

The annotation of the *T. viridans* was performed on multiple platforms (RAST, PROKKA, antiSMASH) and showed the presence of two gene sequences with high similarity (>70%) to the sequence that codes for the protein L-lysine-ε-oxidase (LodA) and its cofactor LodB first isolated from *Marinomonas mediterranea* (Gómez *et al.*, 2006).

The RAST/SEED web server, using the ClassicRAST algorithm (Overbeek, *et al.*, 2005; Aziz *et al.*, 2008) results are graphically represented in Figure **3.1**. The #1 represents the gene sequence for the *lodAB* operon found on the chromosome and #2 represents the *lodAB* operon found on the chromid. The lone *lodB* was located on the chromosome. The gene sequences were named based on their location on either the chromosome or chromid. The resulting expressed proteins are LodA1, LodB1, LodA2, and LodB2, respectively.

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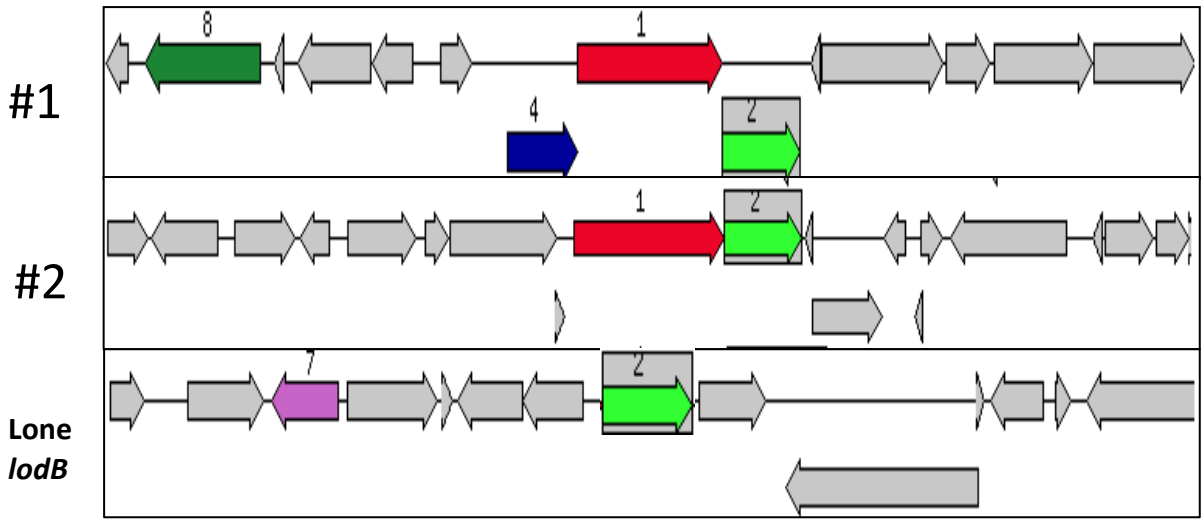

Figure 3.1 - A graphical representation of the occurrence of the *lodAB* operons in *T. viridans*. (Grey arrows are hypothetical proteins or proteins that are unique to the up- and downstream regions for each of the sequence sections generated by the RAST/SEED server. (1) LodA (Sequence for #1 has a 4 NT overlap between *lodA* and *lodB*), (2) Tandem Dehydrogenase flavoprotein/quinoprotein (*lodB*) & additional lone *lodB* open reading frame, not associated with a *lodA*, (4) Is a Putative Membrane Protein, (7) Is a Glycine Cleavage System Transcriptional Activator, (8) Is a Methyl-accepting Chemotaxis Protein.

In *T. viridans* the tandem *lodAB* sequences are annotated as either a putative FAD-dependent oxidoreductase (PROKKA annotation) or dehydrogenase flavoprotein (RAST annotation) similar to the cofactor that produces active LodA/GoxA (Gómez *et al.*, 2010). In *M. mediterranea* the tandem nature of the *lodAB* operon for LodA was first described in the investigation into the mechanism for antimicrobial activity of LodA (Lucas-Elío *et al.*, 2006). In addition, there is a third *lodB* sequence on the chromosome of *T. viridans* that is not associated with a *lodA*-like sequence. The principal similarity of these proteins lies in the conservation of domains that will be described later. The sequence length and corresponding proteins of both LodA and LodB proteins from *M. mediterranea* are shown in Table 3.1. (Lucas-Elío *et al.*, 2006). Full gene sequences for all of the genes can be found in Figure 4.7 in the Appendix.

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Table 3.1 - Sequence analysis and translation of *lodAB* operon in *T. viridans* in comparison to the *lodAB* operon of *M. mediterranea*

Sequence	Position of sequence	DNA length (bases)	Translated protein
lodA #1	Chromosome	2112	703 aa / 77.8kDa
lodB #1	Chromosome	1158	385 aa / 42.9kDa
lodA #2	Chromid	2229	742 aa / 84.1kDa
lodB #2	Chromid	1140	379 aa / 41.6kDa
LodB lone	Chromosome	1137	378 aa / 42.0kDa
M. mediterranea lodA	Unknown	2181	726 aa / 80.1kDa
M. mediterranea lodB	Unknown	1110	369 aa / 41.3kDa

Bacteria of different species (or genera) can exchange genetic material through Horizontal Gene Transfer (HGT) (Burmeister, 2015; Jensen, 2016). This process can result in sequences for specific tailoring enzymes associated with secondary metabolites being shared. It can lead to a bacterium gaining antimicrobial resistance or carrying useful gene sequences, such as plasmids (Burmeister, 2015; Jensen, 2016).

The frequency of HGTs is not well-known, but it presents an immediate opportunity for a cell line to gain a metabolic advantage or face detrimental effects) (Sun *et al.*, 2019). Additional genes acquired through HGT also introduce a burden, as the cell needs to maintain and express them. This can be challenging due to the metabolic requirements or the burden of producing the encoded metabolite (Jensen, 2016). However, HGT has been demonstrated to offer the benefit of antibiotic resistance in some cases (Sun *et al.*, 2019). HGT might also be responsible for an organism carrying multiple copies of a gene sequence (Mærk *et al.*, 2014). These copies are usually preserved if they provide advantages to the organism, possibly by amplifying or diversifying the function of the metabolite. However, gene duplication in

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bacteria is generally unstable (Mærk *et al.*, 2014). The presence of two gene sequences that code for similar proteins could have occurred through HGT to *T. viridans*.

Gene sequences in both structures code for similar proteins as is the case in *T. viridans* where there is a *lodAB* sequence on the chromosome and another on the chromid. It is possible that the chromid may act as a phenotypic carrier, ensuring the bacterium's survival in the variable and competitive marine environment. There may be different modes of induction for each sequence which may help the bacterial colony maintain a competitive advantage under changing conditions.

M. mediterranea also carries additional sequences with low similarity (22.8%) to *lodAB*, but with clearly conserved domains (Campillo-Brocal, *et al.*, 2013). One of these has been characterised as a glycine oxidase (GoxA) with similar characteristics to LodA (Campillo-Brocal, *et al.*, 2013). When multiple copies of a gene encoding similar proteins are maintained by an organism it suggests that these proteins may be important in ensuring a competitive advantage in the changing marine environment and could highlight the importance of carrying two genes for a protein associated with H_2O_2 production.

The presence of the two operons with similarity to those in *M. mediterranea* suggests that *T. viridans* may also produce these types of antibacterial proteins. In addition, structure prediction generated by the UniProt tool predicted 3D conformation and structural similarity to published LodA and GoxA proteins. However, no connection to L-lysine oxidase has been confirmed other than gene sequence similarity through genome annotation.

As LodA is described as an extracellular protein (Gómez *et al.*, 2006), the possible intra- or extracellular location of both LodA proteins from the *T. viridans* was investigated using a number of different platforms, each employing a different technique to determine the probability of a protein being integrated into the cell membrane, intracellular or secreted. The SOSUI-gramN (Imai *et al.*, 2008) web application tool results suggests that LodA1 should be a periplasmic protein while LodA2 should be a cytoplasmic protein. This tool is based on the physiochemical nature of the N- and C- terminals in conjunction with the overall sequence (Imai *et al.*, 2008). When the SignalP (Almagro Armenteros *et al.*, 2019) web application tool is used, neither version of LodA is predicted to have a signal peptide sequence for secretion or transmembrane movement. However, SecretomeP (Bendtsen *et al.*, 2005)

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(http://www.cbs.dtu.dk/services/SecretomeP/), web application predicts that both are non-classically secreted. This platform uses “neural network” type algorithms to calculate the probability of a protein being non-classically secreted by assessing a “feature-profile” derived from the sequence that is then used to classify the protein (Bendtsen *et al.*, 2005).

These predictions do not take into account any post translation modification or interactions with quinone cofactor generated by the LodB proteins (Chacón-Verdú *et al.*, 2014). However, it is in agreement with the same prediction results obtained in the process of characterizing LodA derived from *M. mediterranea* (Lucas-Elío *et al.*, 2006; Gómez *et al.*, 2010). The lack of clear secretion signals indicates that during heterologous expression the protein is not expected to be secreted from the cells and could be extracted from the cells for purification.

3.3. Alignment & Comparison

After annotation of the *lodAB* sequences from *T. viridans*, a number of tools were used for sequence alignment based on both the gene sequences and the amino acid sequence of the protein. In addition, conserved domains were evaluated.

The RAST/SEED web server provides a list of the 12 most similar bacteria used to generate annotations. This annotation system identified *Caulobacter crescentus* CB15 as the closest similarity in extended BGC grouping for the *lodAB* operon on the chromosome (*lodAB#1*) while, the closest similarity for the sequence (*lodAB#2*) from the chromid comes from *Saccharophagus degradans* 2-40. Accession numbers were not provided by the server and no publications were found where LodA-like proteins have been characterised from these organisms (October 2022). See Appendix Table 4.5 for the 12 bacteria this server highlighted as having similarity to either of the *lodAB* sequences.

3.3.1. Gene and protein sequence alignment using BLAST.

Using the amino acid sequence (AA) for each of the LodA proteins the tBLASTn tool was used to search for gene sequences that would code similar sequences. The five sequences most similar to LodA1 and LodA2 are shown in Table 3.2. The sequence similarity for cross-species DNA comparison gave several alignments for *lodA#1* & *#2* (37 and 29, respectively), but all were below 70% identity. Initially all the sequences were identified as ‘hypothetical proteins’, however over the course of the study reassessment of the same data identified these as belonging to a LodA-like superfamily. However, no papers have been published on their

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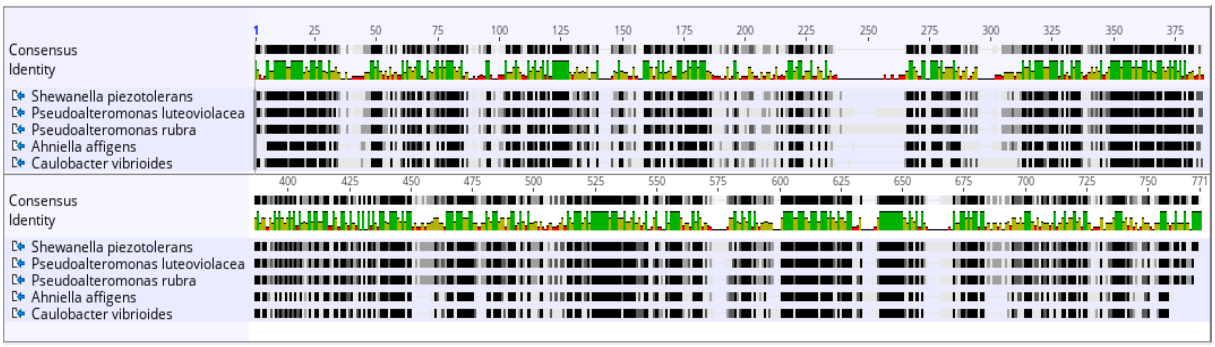
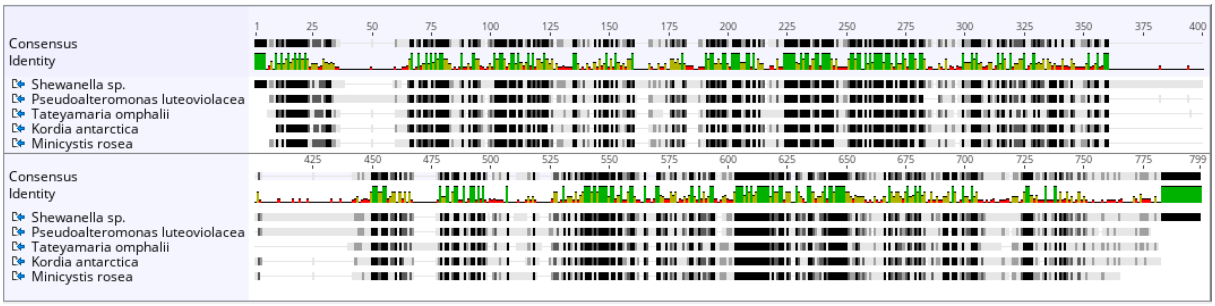
characterisation (Review of annotations - October 2023). The sequences for each of the *lodAB* sequences from *T. viridans*, and the lone *lodB* were analysed to determine similarity and alignment between them. The DNA sequence for *lodAB#1* & *#2* only share 43% identity, and the proteins that they each encode share 25.5% identity. However, there are conserved motifs between the sequences (Figure 3.2). The sequence lengths for all similar sequences vary from approximately 660 to 720 amino acids for LodA1 and 630 to 750 for LodA2.



a



b

Figure 3.2 - A graphical representation of alignment and consensus sequence of the five most similar gene sequences for *lodA#1*(a) and *lodA2* (b).

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Table 3.2 – Top five most similar gene sequences (tBLASTn – September 2020) to *lodA#1* and *lodA#2*

LodA1			
Organism	**Accession**	**% Pairwise Identity**	**% Coverage**
Shewanella piezotolerans	CP000472	71.7	100
Pseudoalteromonas luteoviolacea	CP015416	66.5	99.57
Pseudoalteromonas rubra	CP013612	66.4	99.57
Ahniella affigens	CP027860	49.3	98.01
Caulobacter vibrioides	CP034122	48.7	98.15
LodA2			
Shewanella sp.	CP041614	63.4	99.46
Pseudoalteromonas luteoviolacea	CP015415	47.3	95.83
Minicystis rosea	CP016211	42.0	95.02
Kordia antarctica	CP019288	39.8	95.15
Tateyamaria omphalii	CP019312	37.9	95.69

Using the BLAST Conserved Domain Database (Marchler-Bauer and Bryant, 2004; Marchler-Bauer *et al.*, 2009, 2017), (accessed September 2020) both sequences for LodA1 and LodA2 fall within the LodA-like superfamily and/or LodA-like protein group. The LodB sequences fall under several domain families. LodB1 was specifically grouped with dehydrogenase flavoprotein (FixC). Figure 3.2 shows the overlapping non-specific and super-family hits identified by this analysis. In addition, all LodB sequences had non-specific grouping with geranylgeranyl reductase (GG-red-SF), chloramphenicol-biosynthetic FADH2-dependent halogenase CmlS (halo_ClmS) as well as a distinct FAD-binding-3 domain. Dehydrogenase flavoprotein is associated with carnitine reduction in *E. coli*, but this does require FAD as a

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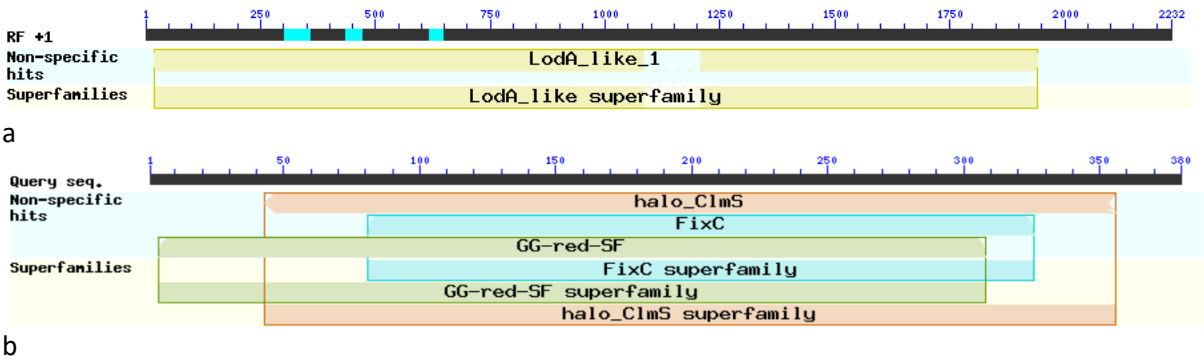
cofactor and is classed as an oxidoreductase (uniprot.org/uniprot/P68644). GG-red-SF is associated with the biosynthesis of cofactors and uses NADP as a ligand generally acting on CH-CH groups of target proteins (uniprot.org/uniprot/Q9CA67). Halo-ClmS is associated with chloramphenicol biosynthesis in *Streptomyces venezuelae* and requires FAD as a cofactor (Podzelinska *et al.*, 2010). LodB2 and the lone LodB were more specifically grouped into the halo_ClmS super-family. In all cases FAD is indicated as a cofactor for the LodB proteins, this is also shown in the Swissmodel results below.

The LodA/GoxA superfamily and LodA-like proteins come from diverse groups but the putative active sites consisting of five residues; **x H D C x** or **[CS] D C [QEH]** respectively are well conserved (Marchler-Bauer *et al.*, 2017). The LodA/GoxA superfamily includes *Flavobacterim*, CFB Group Bacteria (*Cytophaga*, *Fusobacterium*, and *Bacteroides* **-** Gram-negative non-proteobacteria) and cyanobacteria, but the predominant group is γ-proteobacteria (70%) to which *T. viridans* belongs. In *M. mediterranea*, tryptophan 581 and cysteine 516 above are modified by LodB to form the CTQ cofactor for active LodA (Okazaki *et al.*, 2013).

The AA sequences for LodA1 & 2 were analysed using tBLASTp, generated by Benchling (benchling.com – September 2020). This gave greater than 100 hits for similar AA sequences. The top five most similar AA sequences for both LodA1 and LodA2 are shown in Table 3.3. LodA2 had a different distribution in the top 20 results. Again, these sequences have shown well conserved domains.



a

b

Figure 3.3 - Graphic from Conserved Domain Database (ncbi.nlm.nih.gov/Structure/cdd) for both LodA1 and LodA2 sequences (a), and all LodB sequences (b). LodB2 was included in GG-red-Sf and the LodBLone was only grouped with halo-ClmS.

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Table 3.3 – Top five organisms with amino acid sequences for putative proteins similar to LodA1 and LodA2 based on AA coding potential.

LodA1			
Organism	**Accession**	**% Pairwise Identity**	**% Coverage**
Thalassomonas actiniarum	WP_044833915	90.0	99.86
Thalassomonas actiniarum	WP_053042792	84.1	99.87
Shewanella piezotolerans	WP_020910848	71.6	99.86
Shewanella sp.	WP_180961191	69.9	99.57
Shewanella sp.	WP_180961060	69.9	99.57
LodA2			
Thalassomonas actiniarum	WP_053042792	84.1%	99.87%
Reichenbachiella versicolor	WP_109830043	64.0%	99.06%
Shewanella sp.	WP_144046439	63.4%	99.46%
Shewanella sp.	WP_152829712	63.3%	99.46%
Pseudoalteromonas sp.	WP_095364766	53.5%	99.33%

Amino acid alignment of LodA1 & 2 against LodA and GoxA from *M. mediterranea* gave an identity between them of only 7.0%. This consists of 65 identical positions. The proposed domains found in the LodA, GoxA and LodA-like sequences from *M. mediterranea* (Campillo-Brocal, *et al.*, 2015) seem to be conserved in similar regions on LodA1 & 2 sequences as well. In addition, the aspartate (**D**) and cysteine (**C**) residues are conserved per the putative active site for the LodA/GoxA superfamily and LodA-like proteins**; x H D C x** or [**CS] D C [QEH]**. The conserved aspartate (**D**) and cysteine (**C**) residues for both LodA1 and 2 are in positions 561 and 565 respectively. Figure 3.4 shows the conserved domains where the darker text indicates greater similarity, and the green bars indicate identical positions. The active sites for LodA from *M. mediterranea* are 546 [-**D**], 614 [**SH**], 701 [**D**], 705 [**C**], 708 [**GH**].

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The wide distribution of putative proteins with conserved domains similar to LodA and GoxA from *M. mediterranea* suggests that this group of proteins represents a large pool of uncharacterised amino acid oxidases (Campillo-Brocal, *et al.*, 2015).

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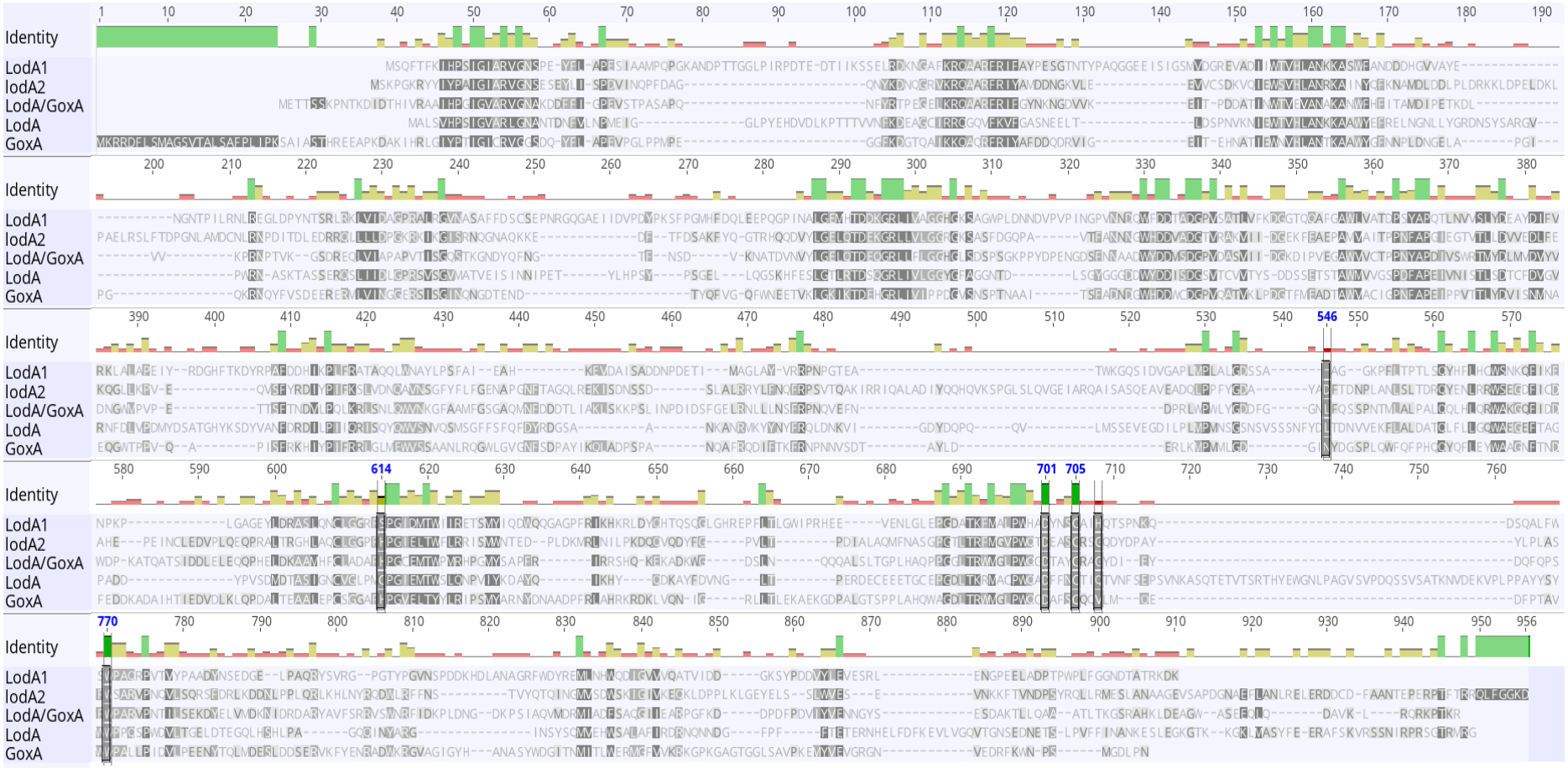

Figure 3.4 - ClustalO alignment of LodA1 & 2, LodA and GoxA from *M. mediterranea* with putative active sites according to the NCBI Conserved Domain Database for LodA-like indicated in blue. (https://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi)

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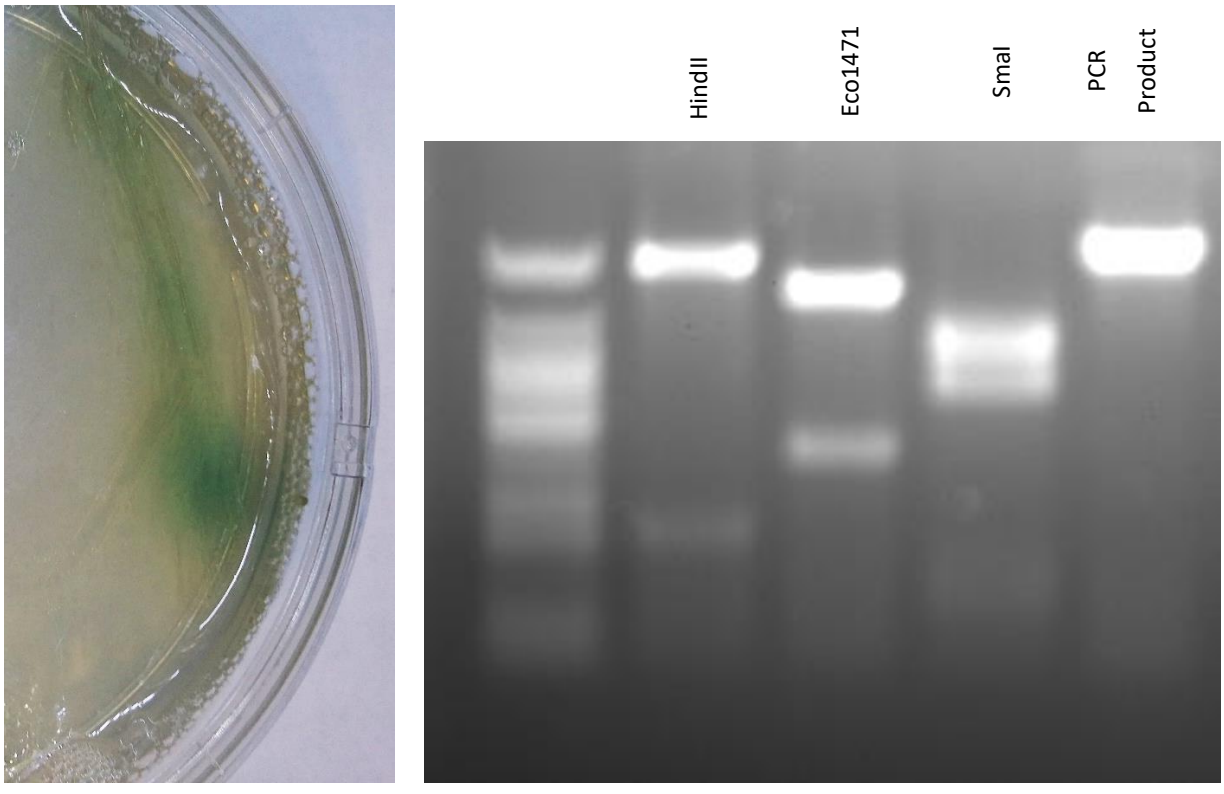
3.4. Culturing *Thalassomonas viridans*.

A freezer stock of *T. viridans* was initially streaked onto Marine Agar plates (Difco). These plates were used for the isolation of a single colony. The culture developed a green/blue colour surrounding the colonies as described by Macián *et al.* (2001)(Figure 3.5a). The *T. viridans* 16S rRNA gene sequence; strain XOM25T, CECT 5083T (GenBank: AJ294748.1) was analysed to determine which restriction enzymes should be used for a diagnostic digest to confirm that the *T. viridans* had been isolated. The three enzymes chosen were SmaI, Eco1471 and HindIII. The 16S rRNA gene PCR product size is approximately 1400 bp, the expected fragment sizes produced by the restriction enzymes are as follows: SmaI produced three fragments (769/605/159 bp), Eco1471 produced two fragments (1125/408 bp) and HindIII also produced two fragments (1278/255 bp). The Amplified Ribosomal DNA Restriction Analysis (ARDRA) performed on the 16S rRNA amplicon (Figure 3.5b) is in good consensus with this. The ARDRA result combined with the presence of the green compound from the same culture suggests that the bacteria is *T. viridans*.



a b

Figure 3.5 - Confirmation of T. viridans. Appearance of green/blue compound on MB agar plates (a) and Amplified Ribosomal DNA Restriction Analysis (ARDRA) performed on the 16S rRNA amplicon and restriction digest: HindIII lane 2, Eco1471 lane 3, SmaI lane 4, PCR product lane 5.

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3.5. Restriction enzyme digest confirmation for each plasmid construct carrying *lodA* and *lodB* sequences.

After transformation of *E. coli* BL21(DE3) with the initial plasmids (pRSF-lodA1, pCOLA-lodB1, pET-lodA2, pET-lodA1.2, pCDF-lodB2, pCDF-lodBL, Table 2.3) received from Biomatik the transformed *E. coli* clones were grown on media with their respective antibiotics. Single colonies of each were then used for plasmid extraction for use in transformation of *E. coli* BL21(DE3) pLysS and *E. coli* ArcticExpress (DE3)RIL, as well as for plasmid identity confirmation and subcloning.

To confirm the identity of the insert for each plasmid, restriction enzyme diagnostic digests were carried out on each of the plasmids (Table 3.4). The restriction enzyme was selected where there would be at least one (1) cut site within the insert and the other/s would be in the backbone of the vector. As a control, a plasmid with the same backbone but without the insert was used. If the fragments generated were of the expected sizes, it would be reasonable to infer that the correct insert was cloned in the plasmid.

Figures 3.6 and 3.7 show the agarose electrophoresis separation of the fragments. Plasmid maps for all plasmids and vectors without inserts are shown in Figure 4.2. In all cases the restriction enzyme digest resulted in the expected bands visible on the agarose gel. This in conjunction with the positive growth with the expected selection media indicates that the inserts were correct for each plasmid.

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Table 3.4 - List of restriction enzymes used in this study for diagnostic restriction digest of the plasmids.

Plasmid/Insert	Enzyme	Expected Sizes
RSF-lodA1	BamHI	5654 / 1485 / 489
RSF-lodA1	HinCII	4206 / 3422
pETDuet	BamHI	Single Cut
pETDuet	HinCII	Single Cut
pET-lodA2	BamHI	4301 / 1619
RSF-lodA1	SmaI	4147 / 1773
pET-lodA1.2	BamHI	5895 / 1619
pET-lodA1.2	PauI	5988 / 1523
COLA-lodB1	MluI	3272 / 1628
COLA-lodB1	HinCII	3479 / 1421
pCDFDuet	MluI	Single cut
pCDFDuet	HinCII	Single cut
pCOLADuet	SmaI	Single cut
pCOLADuet	HinCII	Single cut
CDF-lodB2	SmaI	2758 / 1592 / 506
CDF-lodB2	HinCII	3249 /1607
CDF-lodBL	Eco91I	2185 / 1797 /9 15
CDF-lodBL	HinCII	3681 / 1216

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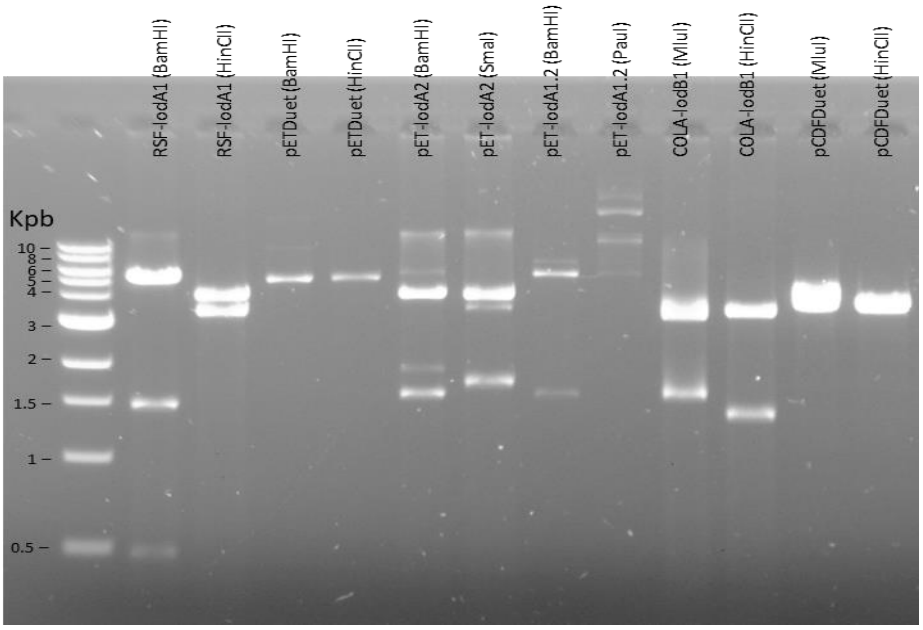

Figure 3.6 - Diagnostic restriction digests of all plasmids used in this study. Lanes 2/3/7 – RSF-lodA1 digested with BamHI, HinCII, SmaI respectively, lanes 4/5 – pETDuet digested with BamHI and HinCII respectively, Lane 6 – pETlodA2 digested with BamHI, laned 8/9 – pET-lodA1.2 digested with BamHI and PauI respectively, lanes 10/11 – COLA-lodB1 digested with MluI and HinCII respectively, lanes 12/13 – pCDFDuet digested with MluI and HinCII respectively.

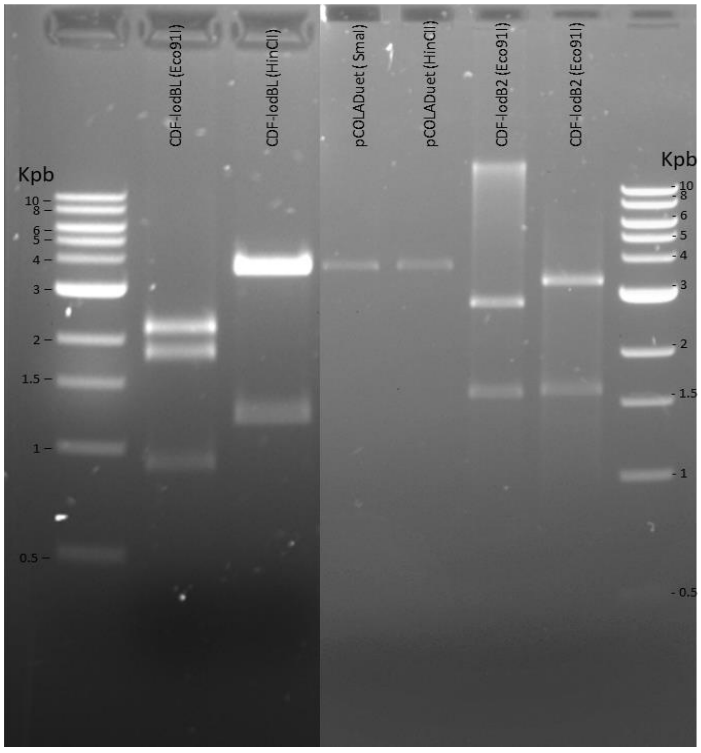

Figure 3.7 – Diagnostic restriction digests of all plasmids used in this study. lanes 2/3 - CDF-lodBL digested with Eco91I and HinCII respectively, lanes 4/5 - pCOLADuet digested with SmaI and HinCII respectively, lanes 6/7 - CDF-lodB2 digested with SmaI and HinCII respectively.

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3.6. Heterologous Expression and purification of LodA and LodB Proteins.

Chacón-Verdú *et al.,* (2014) noted that the molecular mass (MM) of active LodA from *M. mediterranea* heterologously expressed in *E. coli* is 28 Daltons (Da) greater than is calculated by the protein sequence (taking the polyhistidine sequence into account). They proposed that this is due to the CTQ modification of LodA by LodB when they are co-expressed (Chacón-Verdú *et al.*, 2014). It can be expected that the proteins heterologously expressed in this study may show similar results, where the co-expressed LodA should be modified to the active form by LodB. The expected protein sizes from the plasmid inserts are shown in Table 3.5.

Table 3.5 – Plasmid insert translation analysis for each plasmid.

Plasmid	**Translated protein (aa / mw) – (His-tag fused)**
RSF-lodA1	703 aa / 77.8kDa (718 aa / 79.58kDa)
COLA-lodB1	385 aa / 42.9kDa (400 aa / 44.68kDa)
pET-lodA2	742 aa / 84.1kDa (757 aa / 85.88kDa)
CDF-lodB2	379 aa / 41.6kDa (394 aa / 43.38kDa)
CDF-lodBL	378 aa / 42.0kDa (393 aa / 43.78kDa)

Initial expressions (single gene or co-expression of the *lodA* and *lodB*) were carried out for all received plasmids in *E. coli* BL21(DE3). This was followed by expression in *E. coli* BL21(DE3) pLysS and *E. coli* ArcticExpress (DE3)RIL. In the initial 50ml LB expression of the proteins in *E. coli* BL21(DE3) no over expression bands were visible in an SDS-PAGE gel that would indicate over expression of a protein. In the characterization of LodA and LodB from *M. mediterranea* the authors (Chacón-Verdú *et al.*) also noted that there was a deficiency in expressing these proteins in *E. coli* BL21(DE3) and they attributed this to the lack of certain rare codons not supported by this strain (Chacón-Verdú *et al.*, 2014). The reported rare codons that constitute less than 8% of the population for each group in *E. coli* are glycine (GGA), arginine (CGG / AGA / AGG / CGA), isoleucine (AUA), leucine (CUA) and proline (CCC).

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The bias for each of these codons in the *T. viridans* genome versus gene sequence are detailed in Table 3.6. Interestingly, the codons for leucine (CUA) (17% in *M. mediterranea*) and isoleucine (AUA) (12% in *M. mediterranea*) do not appear in the genome of *T. viridans* (Chacón-Verdú *et al.*, 2014). These codon usage biases are higher than the < 8% usage in *E. coli*. This deficiency in codon usage is partly remedied using *E. coli* BL21(DE3) pLysS as an expression host, that provides some of the codons (arginine - CGG & CGA excluded) (Novagen). However, the exclusion of the CGA and CGG codons that constitute 31% and 30% of the arginine codons means that translation efficiency could be negatively impacted and consequently the activity of the expressed enzymes.

Table 3.6 – Percentage bias of each codon in *T. viridans* genome vs. gene sequence for each *lodAB*.

Codon (amino acid)	% bias in genome	% bias in gene sequence
GGA (glycine)	19%	32%
CGG (arginine)	26%	30%
AGA (arginine)	14%	11%
AGG (arginine)	18%	6%
CGA (arginine)	14%	31%
CCC (proline)	20%	24%

Figure 3.8 shows the initial SDS-PAGE analysis for the single expression of LodA1 and LodA2 in *E. coli* BL21(DE3) pLysS in a 50mL LB culture with 1 M IPTG induction (expressed for 24 hours). The band in lanes 4 and 5, which is close to the expected size of LodA1, is visible but not present in either the sample LodA2 nor the negative control (pRSFDuet-1 and pETDuet-1). This band was presumed to be LodA1 given the approximate size. The additional bands at 32kDa are present in all samples, but the identity of the band seen at approximately 27kDa in lanes 4 and 5 was not determined and may be the result of degradation of the LodA1 protein.

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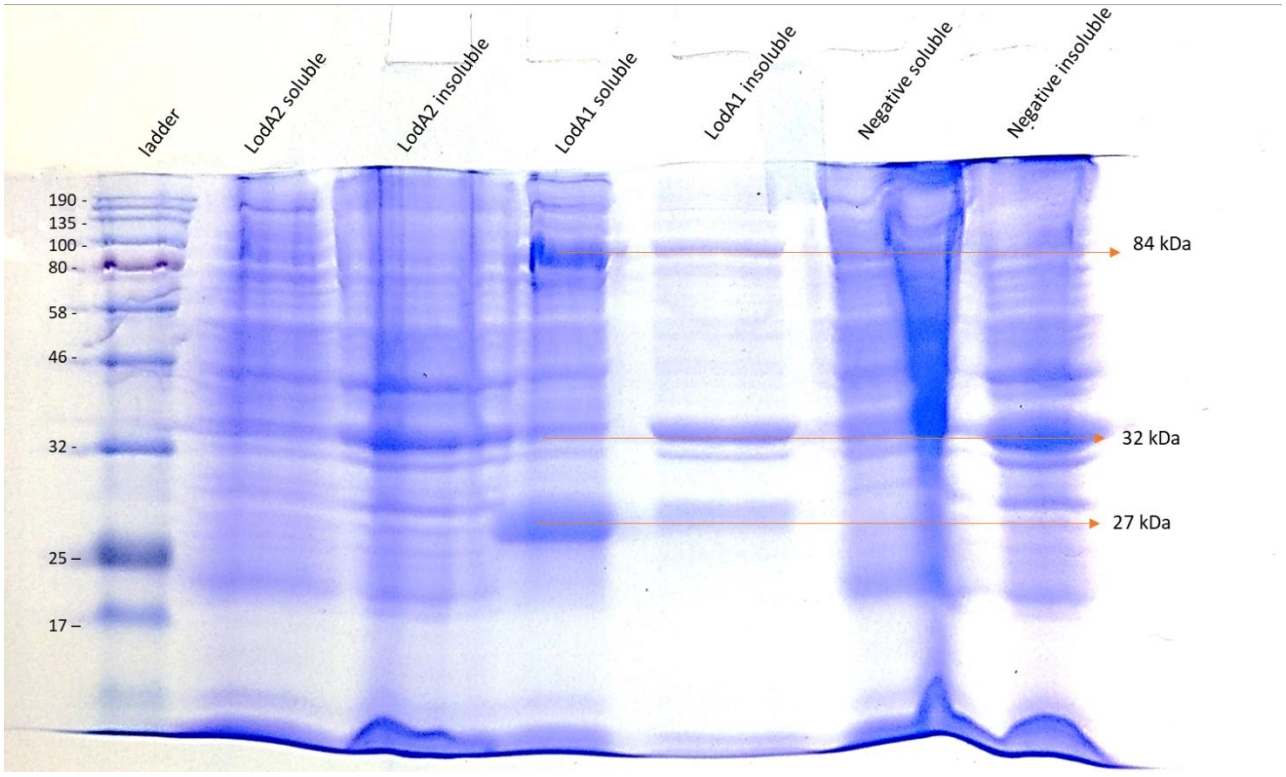

Figure 3.8 - 12% SDS-PAGE analysis for single expression of either LodA1 or LodA2 and negative control (pRSFDuet-1 and pETDuet-1) in *E. coli* BL21(DE3) pLysS. The soluble fraction is the clear supernatant after centrifugation of the lysed cells post-expression. The molecular weight of LodA1 when fused to the 6 histidine tag is 85 kDa.

To further investigate the expression of the LodA proteins, a co-expression experiment with the relevant LodB was carried out in *E. coli* pLysS. Co-expression of LodA with its associated LodB has been shown to be necessary for successful expression of LodA (Chacón-Verdú *et al.*, 2014). Co-expressions of both pairs of LodA and LodB sequences were conducted at room temperature (20°C - 22°C) and 25°C in LB media, with 1M IPTG induction. Post induction, the samples were prepared for soluble and insoluble fractions, SDS-PAGE was used to visualize expressed proteins. There was no significant overexpression of the expected protein sizes in any of the cultures. As a control, *E. coli* pLysS without either expression plasmid was used. *E. coli* BL21(DE3) pLysS was used for the expression strain going forward.

Optimization of expression time and temperature was conducted in 50ml LB media to improve the expression of LodA1 and LodB1. Four time periods post IPTG induction (1 hour, 2 hours, 19 hours, and 24 hours) were tested at three temperature conditions (15°C, 21°C,

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and 28°C). However, there was no significant difference in total protein concentration among the time and temperature points, and no clear overexpression was observed at the expected sizes (Appendix Figure 4.8).

After the revision of various expression protocols alternative rich growth media were explored in addition to increasing the culture volume to 200ml (Sivashanmugam *et al.*, 2009). These changes were made to encourage the over expression of the proteins. Terrific Broth (TB) and ZYM were used in addition to LB media. Co-expression (*E. coli* BL21(DE3) pLysS) of LodA1 and LodB1 was carried out in all three cultures and were incubated at 27°C for 42 hours. The duration was selected as the ZYM5052 protocol calls for longer expression time, and all three cultures needed to be expressed for the same duration. Expression in both TB and LB media conditions was induced using IPTG at 0.4 mM to induce expression when the OD at 600nm was between 0.5 and 0.6. This lower IPTG concentration was also derived from the protocols assessed. At the end of the expression period, sample preparation was conducted. There was a clear difference in the size of the cell pellets when cultured in the different media, the culture grown in ZYM5052 produced a much larger pellet closely followed by TB. Both were significantly larger than the pellet from LB. Furthermore, the soluble fraction samples from TB and ZYM5052 had double the total soluble protein compared to LB (Figure 3.9a). The SDS-PAGE analysis was run using a standard volume (15 μl) for the soluble fraction to compare the total level of protein production (Figure 3.9b). Analysis of the soluble and insoluble fractions shows more intense bands at approximately 84kDa in the soluble fractions (lane 2 & 4) for both TB and ZYM5052. LodB1 would be expected to be at 43kDa.

The rich media had a significant impact on the total protein produced as well as on the expression of soluble proteins. Further expressions for all proteins, either single or co-expressed were conducted in either TB or ZYM5052 at either 200ml or 400mlTB or ZYM5052 cultures. The soluble fraction of the cell free lysate was subjected to His-tag purification.

After co-expression of LodA1 and LodB1 proteins in a TB or ZYM5052 culture, the soluble fraction was subjected to His-tag purification and the fractions were analyzed via SDS-PAGE (Figure 3.10). The His-tag elution fraction had a clear band slightly lower than 95kDa. This is roughly the correct size for LodA1 but there was no band at 43kDa where LodB1 is expected

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to be visible. This is a similar result that was reported in the assessment of the co-expression of LodA and LodB from *M. mediterranea* in *E. coli* (Chacón-Verdú *et al.*, 2014).

Further single and co-expressions were conducted to confirm the production of LodA using 200ml TB media for combinations of LodA and LodB. These were then purified using His-tag and the eluted fractions were concentrated and assessed by SDS-PAGE (Figure 3.11). As can be seen, there are no bands at the expected sizes of the proteins expressed in the single expressions of LodA1 and LodB1 (lanes 2 & 7), as well as no expression of LodA2 co-expressed with LodB2 or LodB1 (Lanes 5 & 8). The pair swap for co-expression was explored to determine if there would be a difference in expression of LodA2. His-tag purified samples from expression with LodA persistently produced a band at approximately 25kDa. The identity of this protein was explored using proteomics. As the expression of LodA2 was unsuccessful after a number of biological repeats, it was decided to focus on expression of only LodA1 and LodB1 going forward.

Single- and co-expressions of LodA1 and LodB1 were conducted in *E. coli* ArcticExpress (DE3)RIL (Agilent, USA). Conditions used were per the standard protocol recommended the supplier. Each expression in LB media was incubated at 12°C for 24h post induction with IPTG (1.0 mM). Low temperature expression systems like *E. coli* ArcticExpress (DE3)RIL assist where incorrect protein folding, and codon bias are expected to be responsible for poor heterologous protein expression. This is achieved by enabling low-temperature heterologous expression of proteins where the rates of production are slowed. There was no clear over expression of any of the proteins.

The single and co-expressions in *E. coli* ArcticExpress (DE3)RIL were repeated using TB and an induction time of 24 hours with IPTG (0.4 mM). The soluble fractions were then His-tag purified (Figure 3.12, lane 8) and run on the same gel as the soluble and insoluble fractions. Despite the significant band at the approximate size expected for LodA1 in the soluble fraction for single expression of LodA1 (Figure 3.12, lane 5) there was no corresponding protein isolated in the His-tag elution fraction. There was however a clear band at the expected size of LodA1 for the co-expression His-tag elution fraction.

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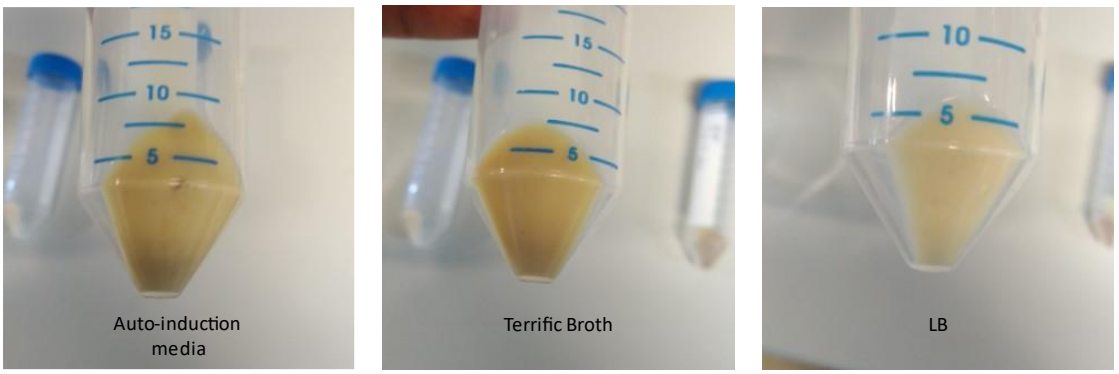

a

OD = 2.295
11.2 mg/ml

OD = 2.222
10.34 mg/ml

OD = 2.068
5.45 mg/ml

Soluble Fraction Protein Concentration

b

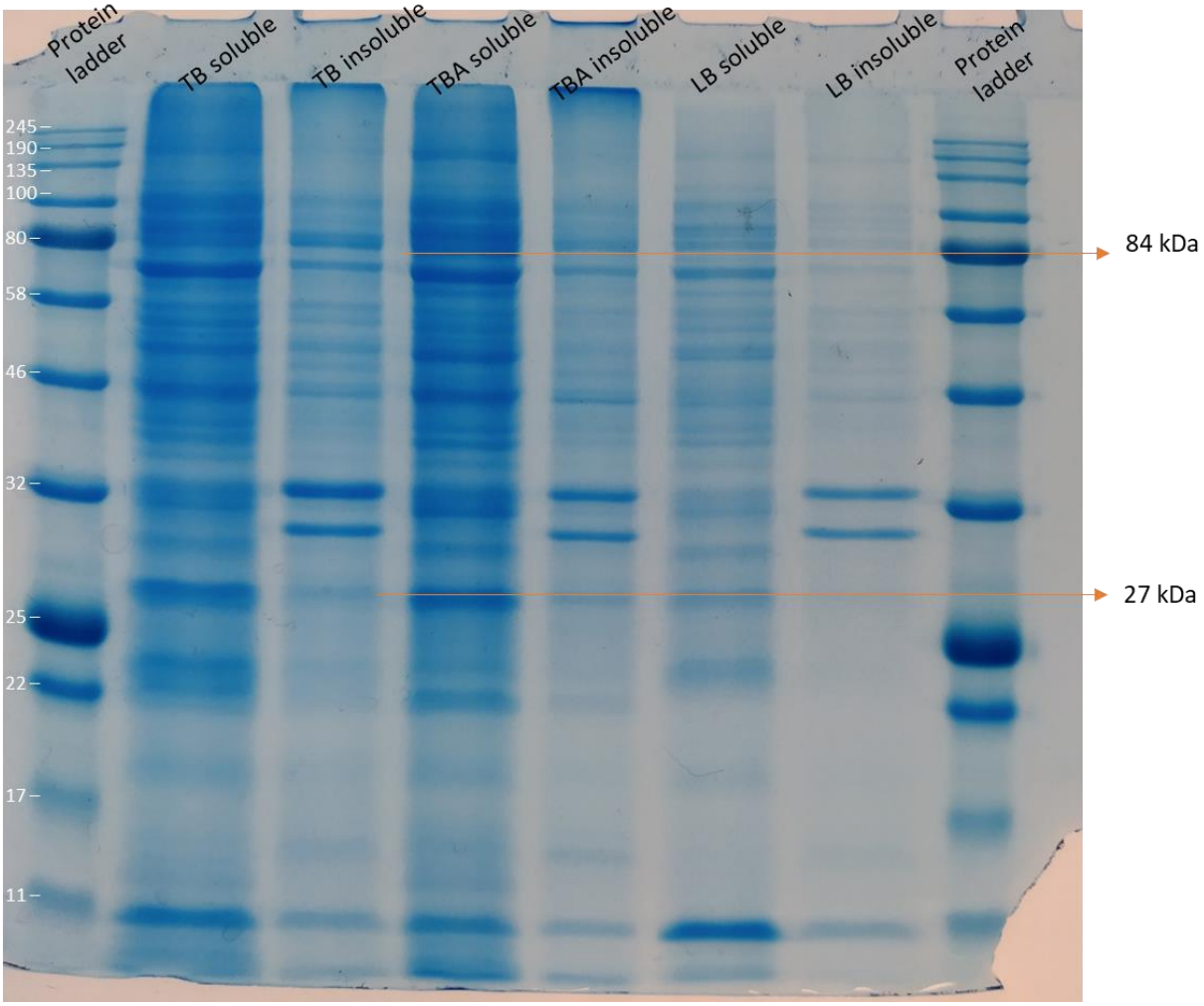

Figure 3.9 - 12% SDS-PAGE analysis for co-expression of LodA1 and LodB1. (a) Total culture pellet size and soluble protein concentration for co-expressed LodA1 & LodB1 at 27°C in *E. coli* BL21(DE3) pLysS. (b) SDS-PAGE of soluble & insoluble fractions from cultures prepared using different growth media. Lanes 2, 4 and 6 are the soluble fractions and lanes 3, 5 and 7 are the insoluble fractions (ZYM5052 = auto-induction media, TB = Terrific Broth, LB = Lysogeny Broth).

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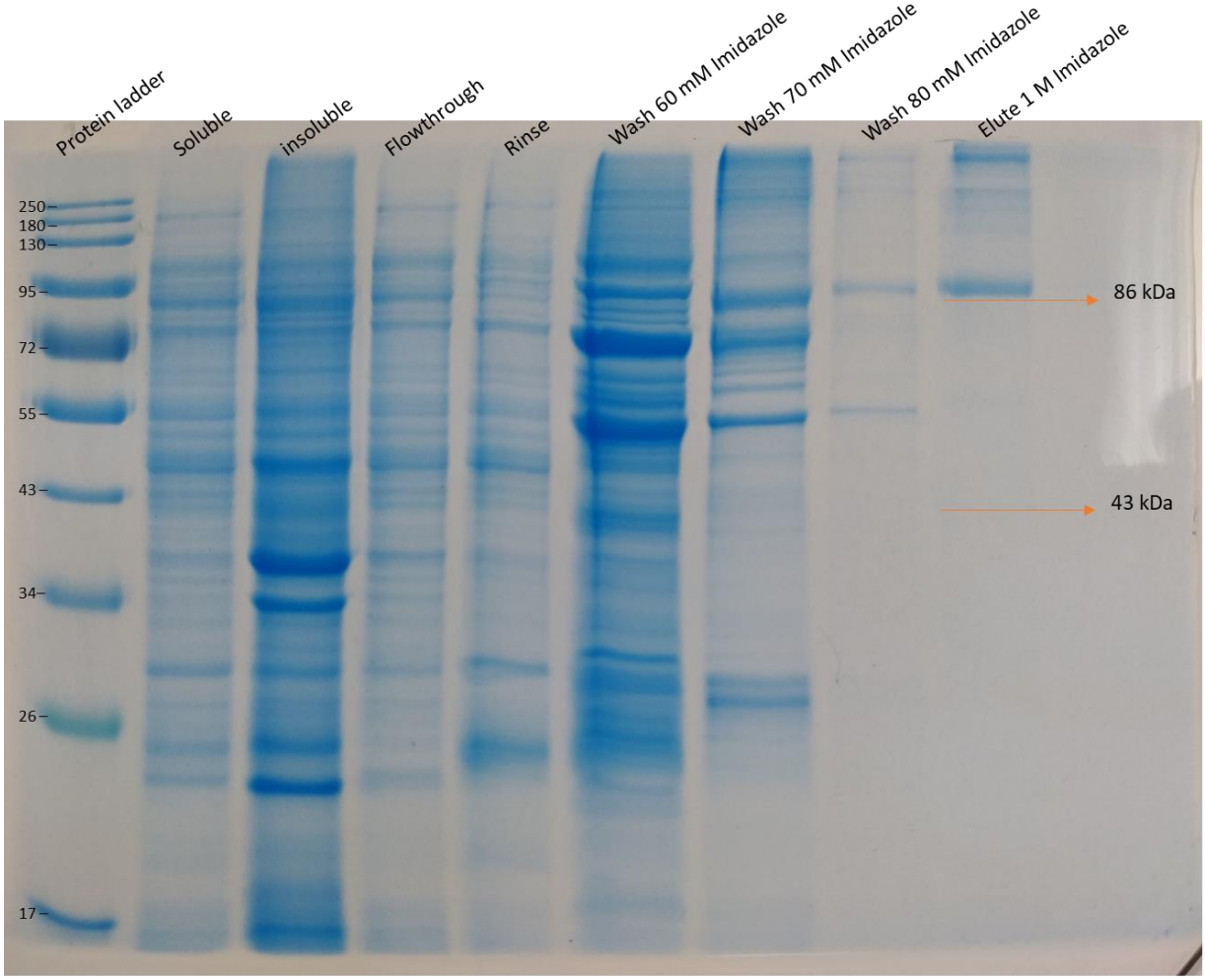

Figure 3.10 - 12% SDS-PAGE analysis of His-tag fractions for co-expressed LodA1 and LodB1 in *E. coli* BL21(DE3) pLysS grown in 200ml TB medium. Lane 1 is the protein ladder, lanes 2 and 3 are the soluble and insoluble fractions respectively from sample preparations. Lanes 4 to 9 are fractions from each step in His-tag purification.

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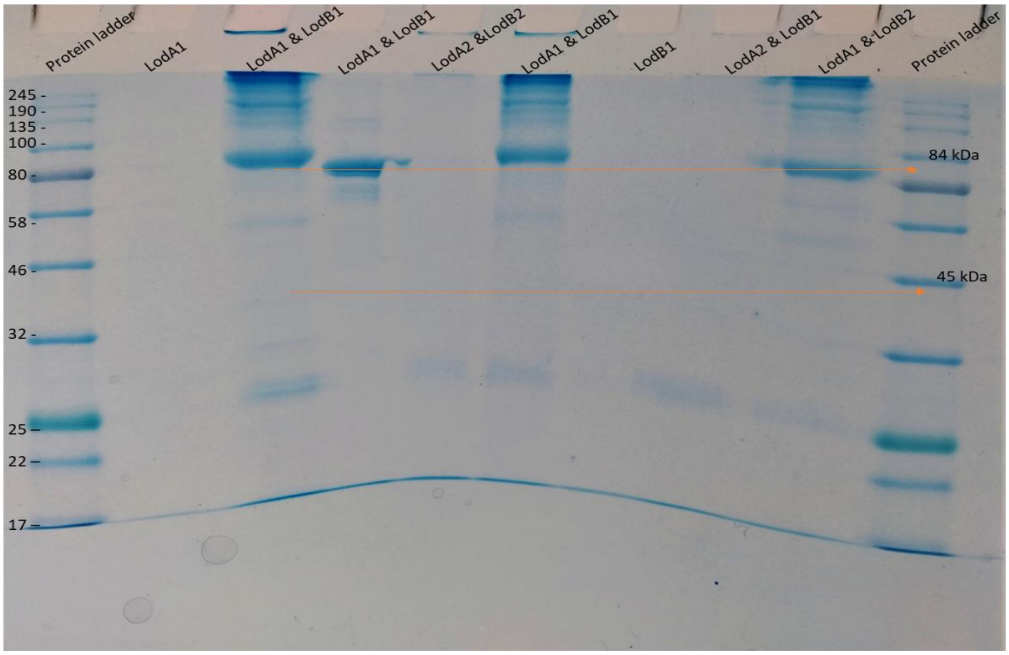

Figure 3.11 - 12% SDS-PAGE for of His-tag elution fractions for single and co-expressed LodA and LodB in *E. coli* BL21(DE3) pLysS from 200ml TB culture. Lanes 2 and 7 were single expressions of LodA1 and LodB1 respectively, lanes 3, 4 and 6 were biological repeats of co-expression of LodA1 and LodB1, lanes 5 was a co-expression of LodA2 and LodB2, lanes 8 and 9 were co-expressions where the LodA and LodB pairs were swapped.

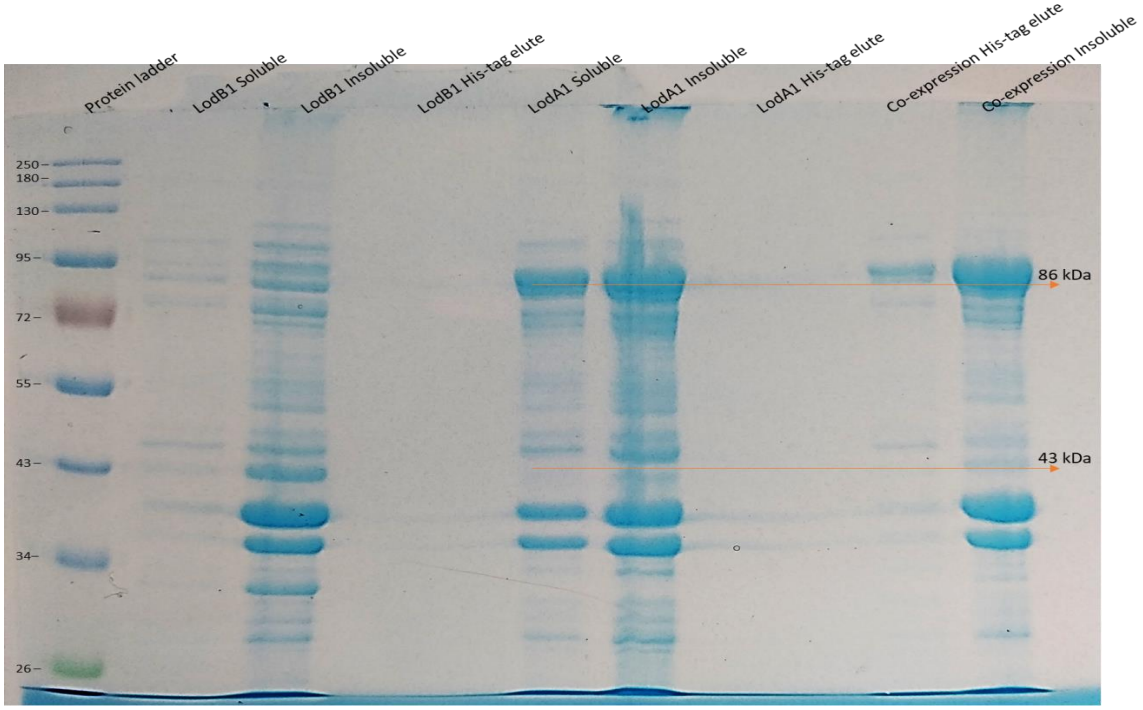

Figure 3.12 - 10% SDS-PAGE analysis for single and co-expression of LodA and LodB protein expressions in *E. coli* ArcticExpress (DE3)RIL. Soluble and insoluble fractions (lanes 3, 6 and 9) are post-induction. In lane 8 the His-tag elution fraction a band at approximately the expected size for LodA1 (43kDa) is observed.

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More severe denaturing conditions were used to investigate the identity of the multiple bands frequently observed at approximately 180kDa and larger in the SDS-PAGE gels for purified samples of co-expressed LodA1 and LodB1 (e.g. Figure 3.11, lanes 3, 6 and 9). The same His-tag elution samples in Figure 3.12 were treated with 1 M β-mercaptoethanol and heat treated at 95°C for 30 minutes. These samples were then analysed using SDS-PAGE (Figure 3.13). The bands that were previously visible at approximately 180kDa and larger were no longer present, while new bands between 26kDa and 34kDa were observed (Figure 3.13, lane 3). The expected 86kDa band in lane 4 was no longer present, but a band was not present between 26kDa and 34kDa.

These results suggested that LodA1 is not being properly formed and may be an aggregation that is dissociated by the increased denaturing conditions. To isolate and analyse the bands, samples from Figure 3.13, lane 3 were rerun on a 12% SDS-PAGE gel to better resolve the bands. The band at approximately 30kDa was submitted for LCMS/MS analysis. The result was then analysed against the translated protein sequence for LodA1 and LodB1 as the references.

Overall, the low level of expression of any of the LodA proteins and no detectable expression of the LodB proteins was concerning and additional composition and structural analysis was conducted. LCMS/MS analysis was conducted on both the total soluble fraction of co-expression (in *E. coli* BL21(DE3) pLysS) of LodA1 and LodB1 as well as on the band of approximately 30kDa produced by aggressive denaturing in SDS-PAGE analysis.

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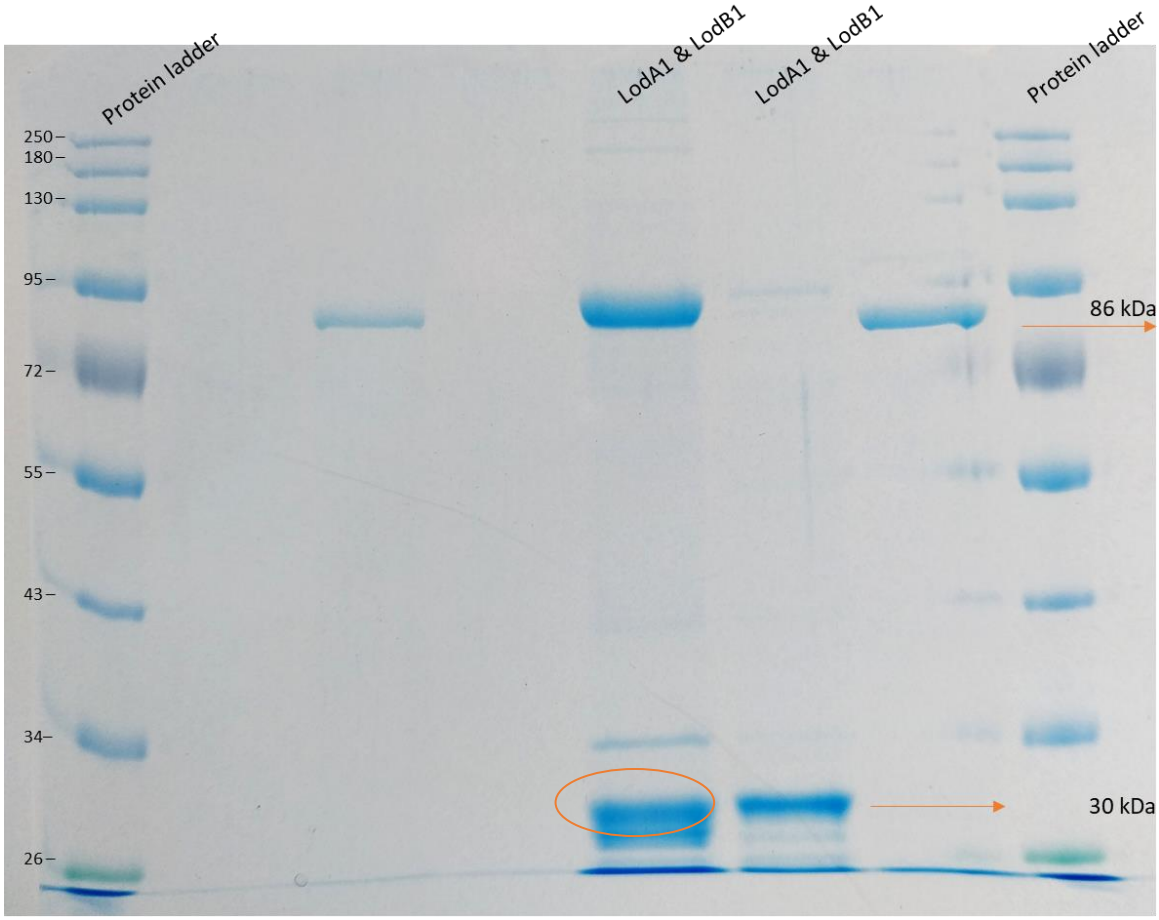

Figure 3.13 - 10% SDS-PAGE of purified co-expressed LodA1 and LodB1 protein in *E. coli* pLysS. Lanes 3 and 4 were biological repeats of co-expression of LodA1 and LodB1. A technical repeat of lane 3 was conducted on a higher percentage gel to resolve the bands better. The band indicated in orange at approximately 30kDa was excised and submitted for proteomic analysis to determine its possible identity.

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3.7. LCMS/MS analysis of the total soluble protein and selected SDS-PAGE band.

A total soluble fraction of co-expressed LodA1 and LodB1 (*E. coli* BL21(DE3) pLysS in TB was submitted for LCMS/MS. The identified peptide fragment sequences were then compared to the AA sequence for LodA1 and LodB1. The abundance of the fragments that match the sample sequence was then used to determine relative abundance of LodA1 and LodB1 in the total soluble protein fraction.

From the data generated (Table 3.7) for the total soluble protein fraction identified, LodA1 was identified with an 87% coverage for its AA sequence including the polyhistidine tag. The coverage is an indication of the proportion of the identified AA fragments that align with the AA sequence of LodA1. The total abundance of LodA1 was 0.82% of the total soluble protein fraction.

The coverage for fragments from LodB1 was only 20%. This suggests that LodB1 is present but only constitutes 0.03% of the total soluble protein fraction. This accounts for the very low levels of purified protein obtained and explains why LodB1 was not visible in SDS-PAGE analysis. Figure 3.14 shows the coverage of the peptide fragments for both LodA1 and LodB1 against their respective AA sequences.

The LCMS/MS analysis (Figure 3.15) of the band excised at approximately 30kDa upon SDS-PAGE analysis after denaturing of His-tag purified sample (Figure 3.13) confirms that there is some degradation of LodA1. Although the excised band had 100% identity to LodA, it only covered 14% of the total amino acid sequence.

Table 3.7 – LCMS/MS analysis data of co-expressed LodA1 and LodB1.

Identified Proteins (3)	Molecular Weight including His-tag fusion	Percentage of Total Spectra	Exclusive Spectrum Count	Exclusive Unique Peptide Count	Exclusive Unique Spectrum Count	Amino Acid Coverage
LodA1	86kDa	0.82%	246	122	196	87%
LodB1	44kDa	0.033%	10	7	10	20%
LodA1 – SDS Fragment			16	11	16	14%

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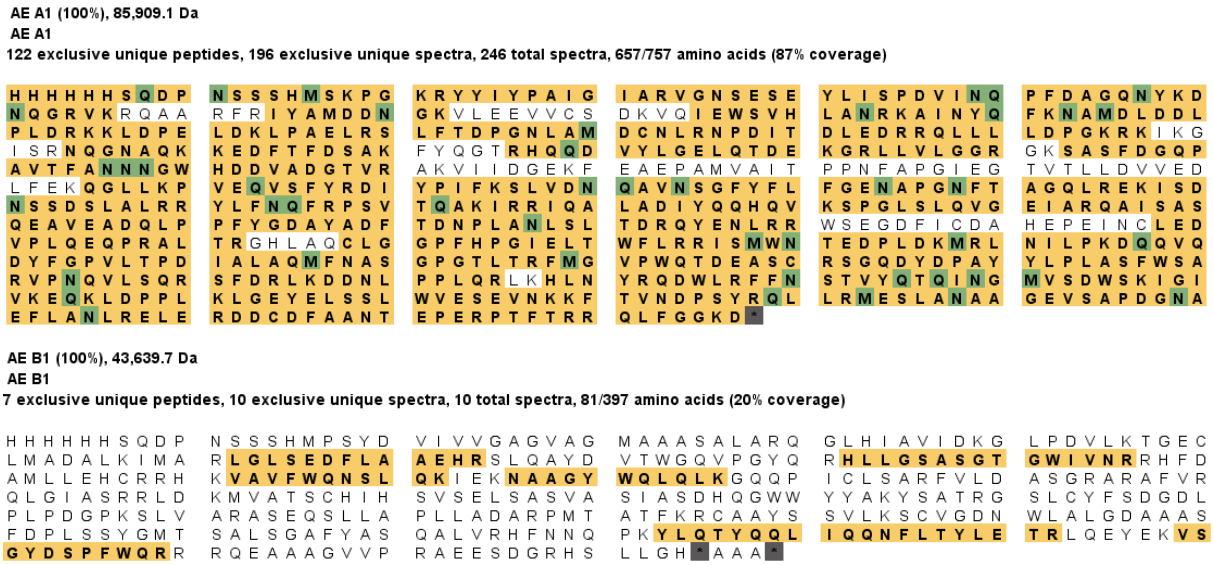

Figure 3.14 - Visual representation of distribution of peptide fragments generated in LCMS/MS analysis of co-expressed LodA1 and LodB1. The highlighted sections represent the largest peptide fragments aligned to the AA sequence for each LodA1 and LodB1.

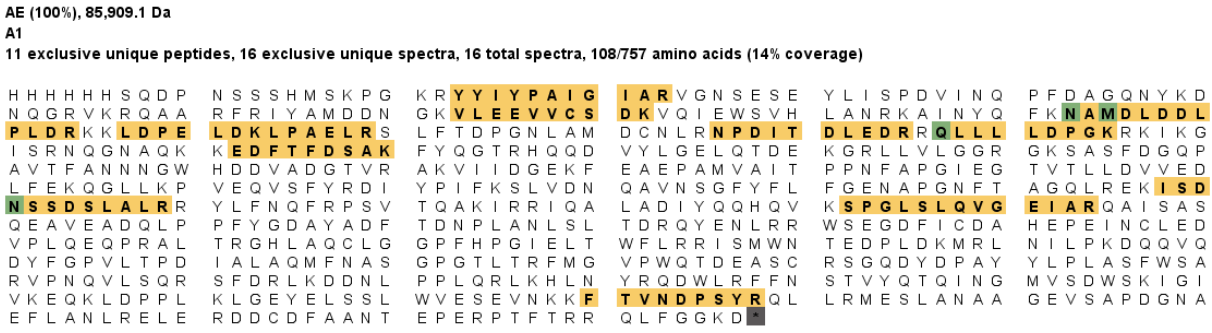

Figure 3.15 - Visual representation of distribution of peptide fragments generated in proteomic analysis of the excised band at approximately 30kDa produced in SDS-PAGE of strongly denatured His-tag purified sample of co-expressed LodA1 and LodB1. The highlighted sections represent the largest peptide fragments aligned to the AA sequence for each LodA1.

3.8. Activity Assays

Samples from the His-tag elution fractions from the heterologous expression samples were subject to buffer exchange to potassium phosphate buffer (50 mM pH 7.0) and concentrated using centrifugal filter devices. Given the products released by the action of LodA as an amino acid ε-oxidase (Section 1.3.2), two assays were compared, one (1) to follow the production of H_2O_2, and one (1) for the production of NH_4^+. Both the H_2O_2 and the NH_4^+ assays were miniaturised to 96-well format to reduce the sample volume required per assay as well as to increase the number of assays that can be conducted.

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3.8.1. Phenol-hypochlorite – Ammonia Detection Assay

This assay measures total ammonia in a sample and in the original protocol (Weatherburn, 1967) the total assay volume is 10ml. In this study this assay was conducted in a 96-well plate. To test the detection limits of the assay, a standard curve was created using ammonium chloride (0.005mM to 1mM NH_4Cl) and absorbance at 600nm was measured using a Spectrostar Nano spectrophotometer (BMG Labtech, Germany), Figure 4.9 in the Appendix**.** The linear range between ammonium chloride concentration and absorbance was between 0.005 to 0.400mM. Any activity that produces NH_4^+ or NH_3 would be seen as an increase in absorbance (Makhongela *et al.*, 2007).

The positive control for this assay was heat-treated cell free extract from *E. coli* heterologously expressing amidase (3.4mg/ml) with acetamide as the substrate (Cameron, 2002). The graph in Figure 3.16 shows the results of the positive control for this assay. There is a clear increase in absorbance over the duration of the assay that would be expected for the increase in NH_4^+ concentration caused by the deamination of acetamide by the amidase. One (1) unit (U) of activity is defined as the amount of enzyme that catalyses the production of 1μmol of NH_4^+ per minute under standard assay conditions. The calculated enzyme units (U) for amidase as a substrate was 15.54 U and the enzyme activity was calculated as 303 U/mg. The published enzyme units and activity for this enzyme heterologously expressed in *E. coli* is 30.9 U and 148 U/mg for a reaction containing 25mM acetamide (heat treated cell extract) (Makhongela *et al.*, 2007). The Km value for this amidase was not calculated using different substrate concentrations as the sensitivity of this assay was negatively affected by the background of the amino acid substrates (Figure 3.17).

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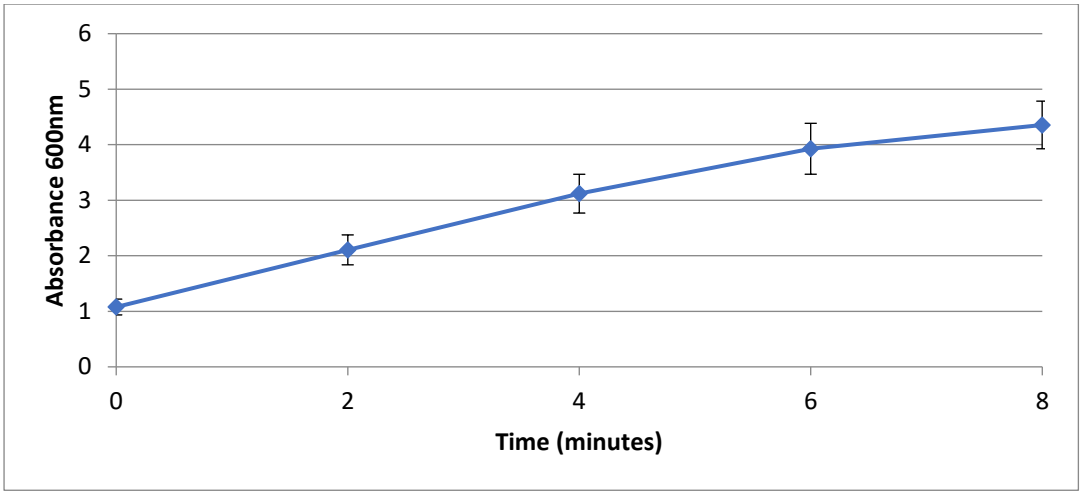

Figure 3.16 - Phenol-hypochlorite ammonia detection assay results using amidase and acetamide as the substrate (0.1 mM). The assay was conducted over 8 minutes with aliquots taken at 2 minute intervals. The t = 0 value is not zero as the absorbance readings are corrected against the blank (no enzyme).

The blank (enzyme free) absorbance value for each of the amino acid substrates at 4 mM was investigated to check for background values. The absorbance values generated for each substrate was higher than that of acetamide used for the assay control (Figure 3.17) and may be due to free ammonia in the substrates. This intensity of background was expected to mask any enzymatic activity of LodA1 and therefore, was deemed to not be a suitable assay. The alternative assay was assessed, which detects H_2O_2 production using phenol red and horseradish peroxidase.

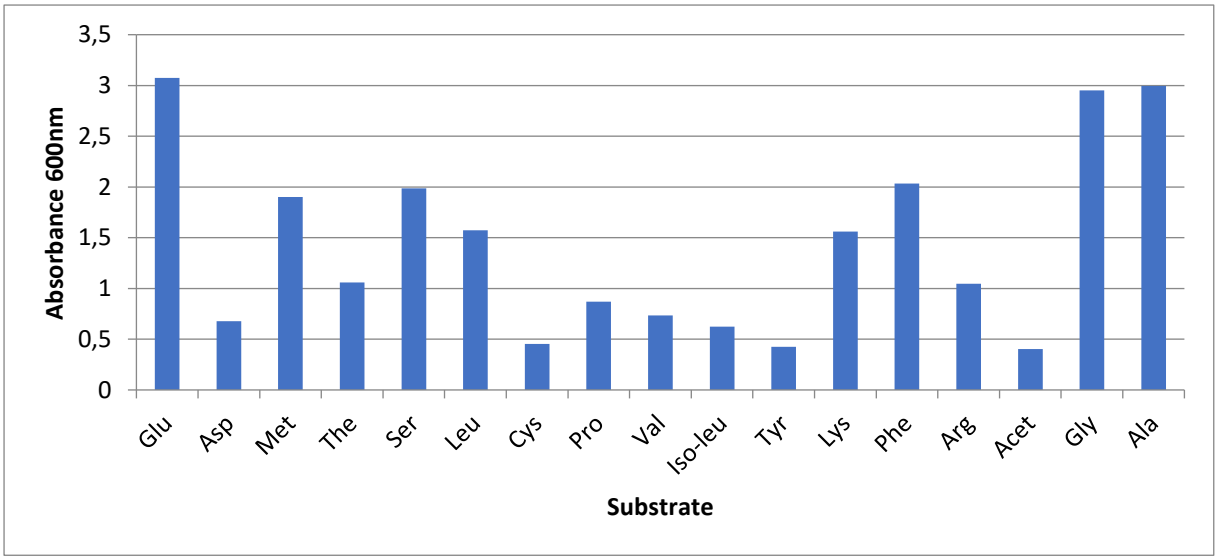

Figure 3.17 – Background absorbance produced by the available substrates used in the phenol-hypochlorite assay.

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3.8.2. Phenol Red/Horseradish Peroxidase (PR/HRP) – Hydrogen Peroxide Detection Assay

Detection of H_2O_2 released by the expected oxidation of the substrate was used to test for enzyme activity and to perform kinetic assays (Kusakabe *et al.*, 1979, 1980; Amano *et al.*, 2015). The method used was based on the assay described by Pick and Keisari, 1980, with some modifications. Firstly, the reaction is "stopped" by the addition of a sodium hydroxide solution, causing an increase in the pH to approximately 12. In this study, the pH adjustment step assay was excluded to allow for continuous kinetics measurements to be taken. Secondly, the absorbance recommended in the original assay that had the most significant absorbance change for a given H_2O_2 concentration was 610nm where the pH adjustment step had resulted in a colour change (Pick and Keisari, 1980). In this study, an absorbance at 557nm was selected for the assays as it had the most significant shift in colour change for an increase in H_2O_2 concentration (Figure 3.18). This colour shift is read as a decrease in absorbance at 557nm for a given increase in H_2O_2. As can be seen in Figure 3.18, the absorbance at 610nm does not offer a significant colour shift when used without the pH adjustment. The relationship between absorbance at 557nm and an increase in H_2O_2 concentration at pH 7.2 can be seen in the standard curve (Figure 4.11), where there is a decrease in absorbance for a given increase in H_2O_2 concentration.

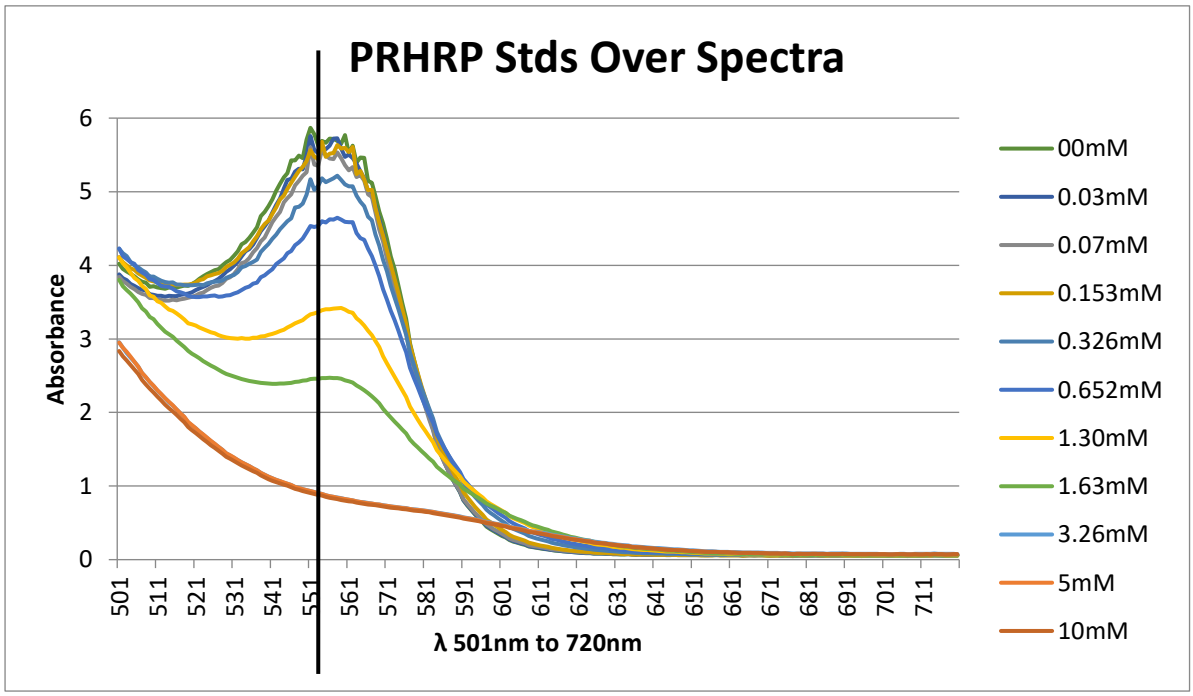

Figure 3.18 - Absorbance spectra for PR/HRP assay standard curve of H_2O_2 concentration, 0.0mM to 10mM.

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Lysine α-oxidase (LαO) from *Trichoderma viride* was used as a positive control for the production of H_2O_2 using this modified assay (Kusakabe *et al.*, 1979, 1980; Amano *et al.*, 2015). Positive activity assays were conducted with 1.69μg/ml LαO against various concentrations of lysine. The stated specific activity from the supplier is $\geq$ 20 U/mg. The activity curves for lysine concentrations of 2.075mM, 4.15mM and 16.6mM can be seen in Figure 3.19. The overall profile of the reactions was generally similar to each other, and the average ΔmM H_2O_2/min for the duration of each reaction was 0.74, 0.82 and 0.76 respectively. The variation in time zero (0) could be missed reaction due to delay in data acquisition after the reactions are initiated and the spectrophotometer reading is initiated (closing the spectrophotometer and the reading head moving into position). The initial 20 seconds of each reaction was used to calculate the activity units.

The average enzyme units calculated from these reactions for 1.69μg/ml LαO against various concentrations of lysine was 8.936 U/ml, and the specific activity was calculated as 52.878 U/mg. Previous publications for this LαO have given the specific activity as 66.2 U/mg (Kusakabe *et al.*, 1980).

The non-zero start value is due to nature of reading using the 96 well format on this spectrophotometer and missing the absolute start of the reaction. In addition, even though HRP (horseradish peroxidase) was at a concentration based on original assays, this could have also altered the specific activity results. Additionally, the purity of the substrate may have had an impact on the enzyme activity. Thus, this assay data was not ideal for the calculation of reaction rates as the reading delay misses some of the initial reaction.

The 96 well plate format in conjunction with this specific spectrophotometer gives a clear indication that the H_2O_2 production can be detected but may be too sensitive to variations in reading conditions. In addition, the delay between starting the reaction and the device being able initiate reading the wells may have resulted in missing the initial reaction. The LαO may be highly active within the first 10 seconds so by the time the enzyme has been added to all the rows and start recording, the initial rates could have been reached. This could be partly compensated for by reducing the enzyme concentration to reduce the reaction rate and would need to be confirmed on a different microplate reading spectrophotometer. However,

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this assay set up was determined to be suitable to assay the heterologously expressed LodA for lysine oxidase activity.

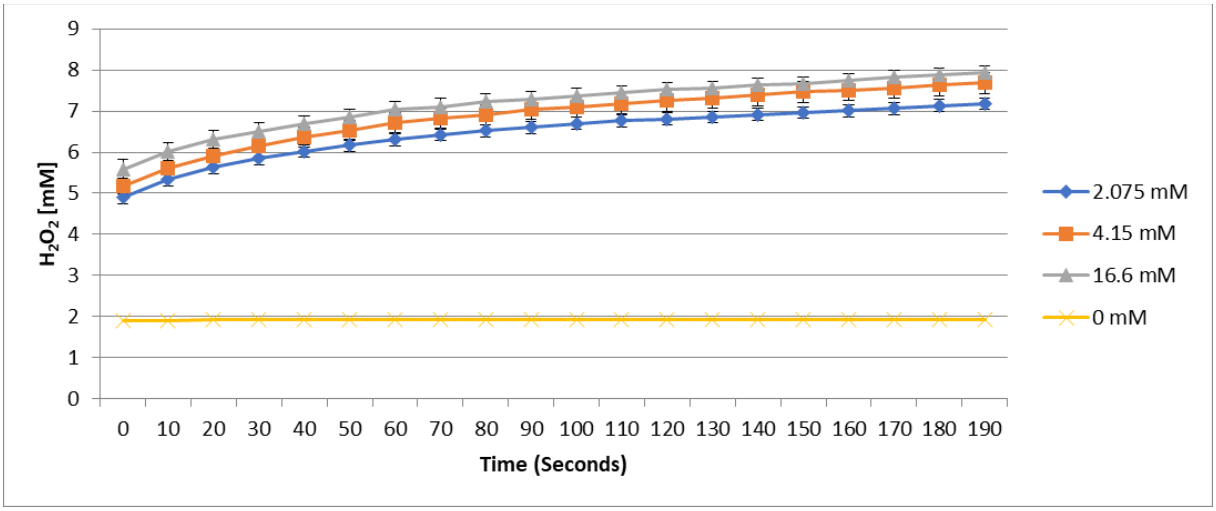

Figure 3.19 – Graph for product (H_2O_2) produced for PR/HRP assay for LαO (1.69µg/ml) against concentrations of lysine (2.075mM, 4.15mM and 16.6mM) at 37°C over 3 mins.

Purified LodA1 co-expressed with LodB1 in *E. coli* BL21(DE3) pLysS was assayed against 25mM glycine and lysine (the two expected substrates) at room temperature (Figure 3.20) as per the positive control. There is no reduction in absorbance for either of these substrates and no activity can be calculated from these results. The data is a combination of the averages from technical repeats of biological repeats.

To investigate if other substrates are the target of LodA1, end point reactions were conducted on alternative substrates, glutamine, methionine, leucine, phenylalanine, tryptophan and asparagine. These all have an α-amine group. In all these instances there was no substantial difference between the initial reading and the reading at 10 minutes, yielding results similar to what is seen in Figure 3.20 (data not shown).

Assessment of the standard deviation for individual assays showed that the readings from wells near the centre of the plate had smaller deviations compared to those that were conducted in wells on the edges (Medema and Fischbach, 2015; Navarro-Muñoz *et al.*, 2020). This edge effect could. The small assay size means that they are more significantly affected by small changes in the assay solution. In addition to the changes in chemistry due to volume changes, differences in temperature across the plate may influence the absorbance readings.

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Finally, variations in the meniscus of the solution in the well may change the effective path length, and thus alter the absorbance reading.

Overall, this modified phenol red/HPR colourimetric assay provided an effective method for detecting H_2O_2 generated during the oxidation of lysine by LαO. However, no such activity could be detected for purified LodA1 using this assay.

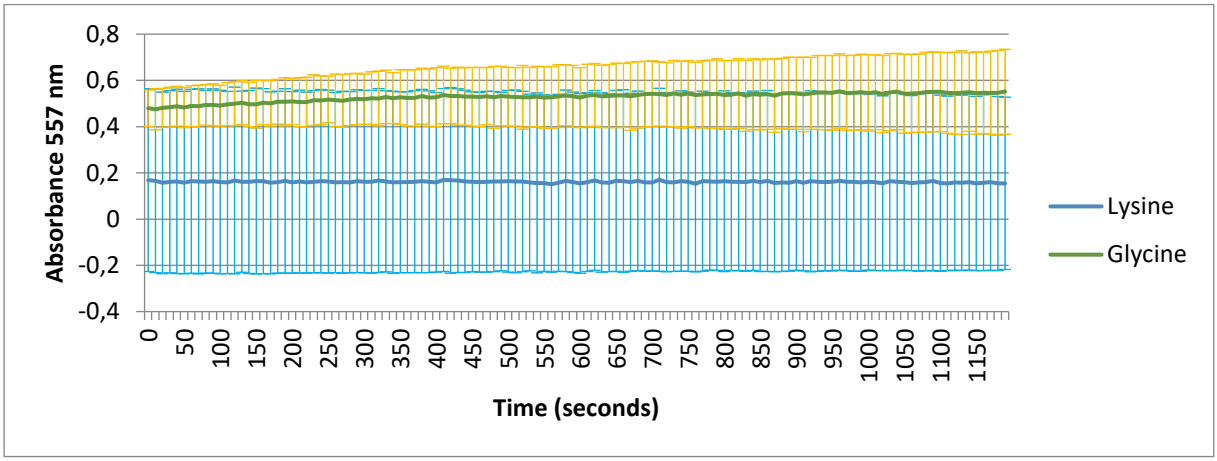

Figure 3.20 – Absorbance at 557nm for PR/HRP assay for biological repeats of purified LodA with lysine (blue) or glycine (yellow) as substrate. All readings were calculated from subtracting the blank reading for each assay.

The apparent structural similarity of LodA1 from *T. viridans* (based on DNA sequence) to LodA isolated from LodA isolated from *M. mediterranea*, suggested that the activity of the enzyme could be similar (Swiss model, 2022). At the time of writing, none of the other similar proteins identified when using the BLAST platform were characterized as enzymes other than LodA-like superfamily and/or LodA-like protein group. A characteristic of this group is the production of H_2O_2 and NH_4^+ as well as the corresponding keto acid (Gómez *et al.*, 2006a; Yu and Qiao, 2012) and this was the basis of the choice of assay. An additional factor in the choice of substrate was that only lysine and glycine had been identified as substates for two characterised enzymes in the superfamily.

It is possible that LodA1 does not act on the free amino acids or that it has a non-oxidative action that does not produce H_2O_2 during the reaction. However, at the time of analysis, no similarity to proteins characterized enzymes with transaminase or dehydratase activity was identified. No similarity in sequence was indicated that LodA1 belonged to any of the known peptidase groups of enzymes.

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3.8.3. Substrate/Product Shift Assays

To investigate if LodA1 was acting on other free amino acids as substrates, HPLC analysis was conducted on solutions with only LodA1, only the substrate and both together. The solutions were prepared and incubated in the same conditions at the same time. Samples of each were then loaded onto the HPLC machine. LαO was used as a positive control to confirm if either a substrate peak intensity shift occurred or if additional retention peaks appeared indicating substrate modification.

The amino acids used as substrate (Table 2.5) were incubated with and without purified enzymes, either LαO or LodA1 (Matsuda and Asano, 2010b; Pena *et al.*, 2016) that was co-expressed with LodB1 in *E. coli* BL21(DE3) pLysS.

The chromatograms of lysine (4.15 mM) that was incubated with LαO at 37°C as a positive control for either 1 or 2 hours are shown in Figure 3.21. The primary differences in the chromatograms are that significant peaks appear at approximately 3.30 minutes (arrow A), 3.8 minutes (arrow B), 5.00 minutes (arrow C) and 6.60 minutes (arrow D). These are only present in the samples where LαO was incubated with lysine. Peaks B and D have a different intensity for the 1- and 2-hour incubation periods. The 1-hour incubation has a larger peak than that for the 2 hour incubation at B, but the 2 hour incubation has a larger peak at D (a later retention). This could indicate a secondary spontaneous reaction of the products that occurs over time. Without the use of tandem mass spectroscopy and suitable standards, the identity of these peaks cannot be determined.

The results of the positive control using LαO incubated with lysine suggests that this method of end point assay and analysis is effective at detecting changes in substrate and production of product due to enzymatic activity of LαO.

Biological repeats of purified LodA1 from a co-expression with LodB1 (in *E. coli* BL21(DE3) pLysS) were incubated with each of the substrates (Table 2.5) at 25°C overnight. This was done to allow for any activity to take place. The activity of the expressed LodA1 was expected to be low, given the lack of results in the H_2O_2 assay. The chromatograms for each of the substrates are found in the Appendix, Figures 4.8a-s. Assessing the results of these assays showed that there is no evidence of enzymatic activity on these substrates. No additional peaks at later retention times were produced as was the case for LαO. As there were no

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changes in the chromatogram that would indicate that the either LodA1 does not act on free amino acids or the expressed LodA1 is not in an active form.

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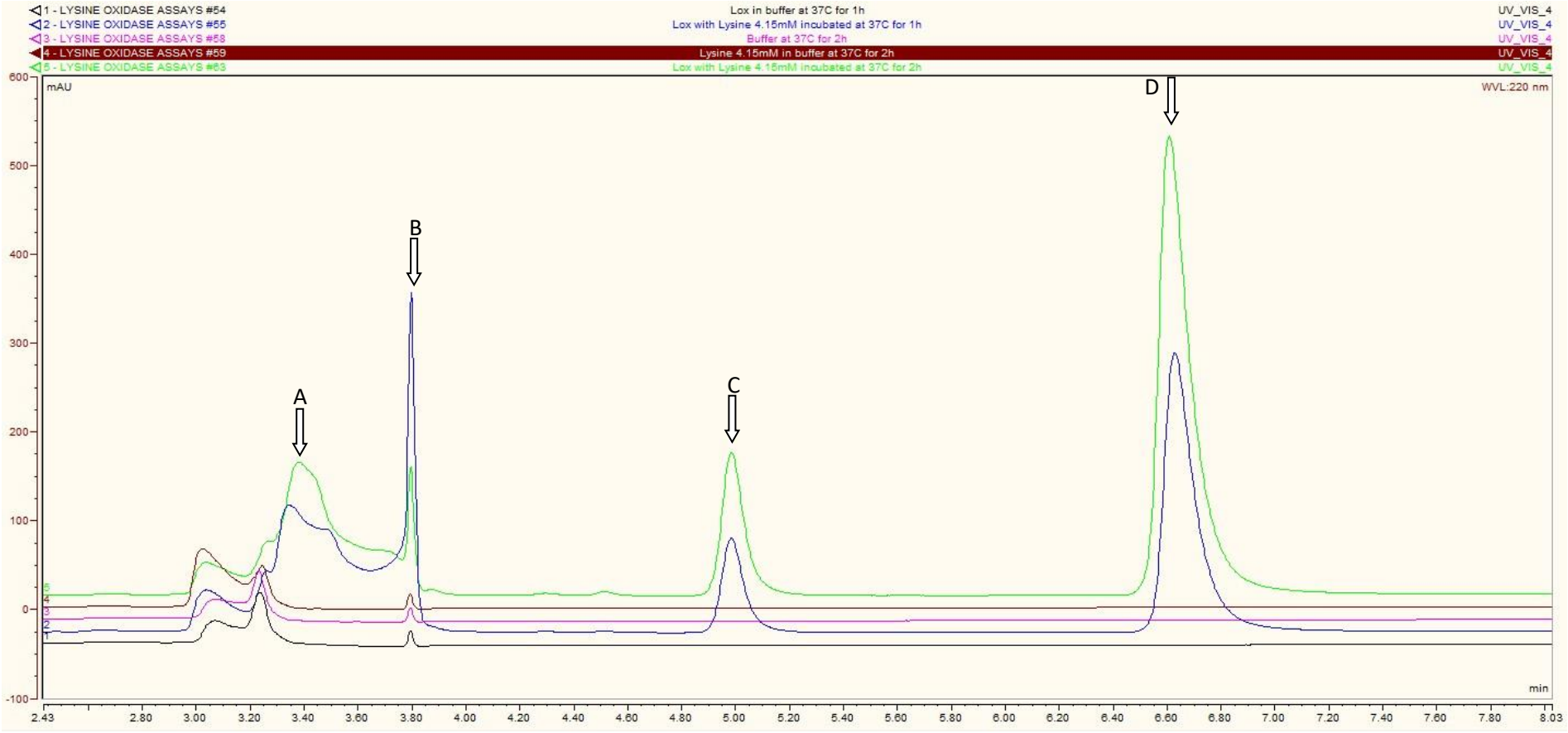

Figure 3.21 - Chromatograms at an absorbance of 220nm for *T. viridans* LαO and against various lysine (4.15 mM) that was incubated at 37 °C for either 1 or 2 hours. The primary differences in the chromatograms are that significant peak shifts at approximately 3.30 minutes (arrow A), 3.8 minutes (arrow B), 5.00 minutes (arrow C) and 6.60 minutes (arrow D). Chromatograms are numbered from bottom to top. 1- (black) LαO, 2- (blue) LαO incubated with lysine for 1 hour, 3- (pink) buffer, 4- (red) lysine, and 5- (green) LαO incubated with lysine for 2 hours.

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3.9. Conclusion

Heterologous expression of LodA1 was successful as seen in the SDS analysis as well as confirmation using LCMS/MS. However, the other proteins, LodA2 and the LodB proteins were not successful, and they could not be resolved based on SDS-PAGE analysis. LCMS/MS analysis indicated that LodB1 was present, but it only contributed 0.03% of the total protein in the sample and thus was not visible in SDS-PAGE analysis. To overcome the low concentrations of expressed protein, large culture volumes were processed, and the purified samples were concentrated as far as possible (approximately 100µg/ml) to visualise the protein and for activity assays. The increased concentration did not improve the resolution of LodB1.

The LCMS/MS analysis of the unexpected smaller molecular weight bands that were resolved in SDS-PAGE analysis of purified LodA1 (co-expressed with LodB1) that had been subject to extreme denaturing conditions showed that these were fragments from LodA1. This could indicate that there is some issue in the synthesis process for the protein.

As LodA was expected to be toxic to the cell due to the production of H_2O_2, some difficulty in expression was expected. However, there are a number of papers detailing the expression of proteins from the same family using very similar techniques with *E. coli* (Gómez *et al.*, 2006; Matsuda and Asano, 2010; Molina-Quintero, Lucas-Elío and Sanchez-Amat, 2010; Sehanobish *et al.*, 2015). However, there were no instances where the transformed or induced host suffered toxicity. Some cultures showed no sign of lysis even days later, particularly in the auto induction media, where one would expect the prolonged enhanced expression to cause greater rates of cell death. Despite expression and purification of LodA, an active form of the enzyme was not produced.

Activity assays using positive controls indicated that the choice of assays were suitable for the detection of the products (NH_4^+ and H_2O_2). Additionally, HPLC analysis showing that the products of LαO activity on lysine are resolvable. This indicates that had there been some form of activity from LodA1 on any of the amino acids tested, additional peaks would have been seen in the chromatograms.

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Chapter 4 General discussion and conclusion.

Antibiotics are a fundamental tool in the control of microorganisms – both in the fight against pathogenic infections and in the preservation of products. The widespread use of antibiotics has resulted in the development of antibiotic resistance in bacteria, highlighting the importance of discovering novel compounds that are able to counteract those bacteria that have already developed some antibiotic resistance (Bérdy, 2005). The pursuit of new antibiotic secondary metabolites (SMs) and antimicrobial peptides/proteins (AMPs) depends on innovative methods to investigate new sources for the identification of indigenous products. Genome mining provides an alternative approach for researchers, helping us stay a step ahead in the face of increased prevalence of antibiotic resistance (Bhushan *et al.*, 2019).

The microbial diversity of the marine environment is at the forefront of exploration for novel compounds (Carroll *et al.*, 2020; Stonik, Makarieva and Shubina, 2020). The complexity and diversity of the marine environment and the subsequence range of secondary metabolites that can be produced requires the mining and annotation of large genomes (compared to traditional culturing and metabolite isolation of SMs). Genome mining and annotation may be a more efficient way to identify potential target SMs and AMPs for antibiotic replacements. However, those identified will be based on their similarities to already characterised SMs and AMPs - possibly limiting the identification of truly novel compounds. In addition, the heterologous expression of these SMs or AMPs for characterisation is complicated due to the time required to determine the most suitable host as well as to optimise conditions needed for heterologous expression. Such was the case where *Rhizobium leguminosarum* was used for the expression of polytheonamide-type peptides in place of *E. coli* (Bhushan *et al.*, 2019).

The hurdles that need to be overcome to isolate and characterise these SMs include the costs of genomic analysis and DNA synthesis, time for culturing and assay development as well as and limitations of the process to heterologously express the target.

The examination of gene and amino acid sequences for proteins similar to LodA indicates that this is a broad family of enzymes, seemingly preserved across numerous bacterial species. In addition to the conserved domains, the *lodA* gene sequences possess a tandem sequence related to *lodB*, which produces the protein responsible for creating cystine tryptophylquinone in active LodA. Considering that the only enzymes from this family that

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have been characterized are LodA and GoxA from *M. mediterranea*, there could be an alternative activity to the amino acid oxidase activity described in LodA. The analysis revealed that beyond the conserved domains, as outlined in section 3.3, the proteins share minimal similarity. These differences could suggest an alternate function for the proteins. It's plausible that the enzymes derived from *T. viridans* carry out a different metabolic activity, although none are immediately apparent at a sequence similarity level.

This study has highlighted numerous difficulties in the search for, and characterisation of, novel AMPs from the vast number of AMPs that are produced by underrepresented microorganisms such as *T. viridans*. Even though there are examples where a synthetic gene sequence has been used for the production and expression of, it is still a time and resource consuming task to isolate and characterise novel AMPs.

Future work on these proteins could be focused on the codon bias for *T. viridans* towards the two codons that are excluded by in the *E. coli* BL21(DE3) pLysS system (arginine - CGG & CGA) as this may be the potential cause of the low level of protein expression as well as the lack of activity in the protein that is produced. In addition, the *lodA* and *lodB* could be co-expressed from a single plasmid such as pRSFDuet. This may mitigate a potential copy number mismatch where LodB is not produced at high enough levels to maintain the formation of active LodA. As described by Chacón-Verdú *et al.*, (2015), both enzymes are stabilised by the other and using a single plasmid may improve the expression of both LodA and LodB as well as the LodA expressed in an active form.

An alternative expression host may be better suited to the production of LodA proteins such as yeast or *Rhizobium leguminosarum* as used for the expression of polytheonamide-type peptides (Bhushan *et al.*, 2019). These alternative hosts may provide more suitable codon bias as well as post-translational modification machinery and excretion systems that may enable the transfer of the enzyme out of the cell. For some of the other non-snake venom associated LAAOs *E. coli* was not the ideal expression host and the lack of post-translational modification was noted as the possible cause, although specifics were not given (Yu and Qiao, 2012).

In conclusion, the process from genome mining to identify potential SMs through to the heterologous expression and characterisation of the SM, has been tested. The genome of

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T. viridans was analysed to determine the presence of AMPs that could be characterized by heterologous expression. The sequences for two LodA-like proteins were chosen to be synthesized and were inserted into plasmids for expression in an *E. coli* system. The expressed proteins were then purified and subjected to activity assays to characterise their activity. Activity assays conducted on the purified LodA1 (co-expressed with LodB1) did not show any activity employing different analytical methods, whereas the positive control in each case confirmed that the assays were feasible.

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Appendix

Table 4.1 – Culturing and Expression Media used in this study.

	1 Litre final volume
LB Medium (Lysogeny Broth)	
Tryptone	10g
Yeast extract	5g
NaCl	5g
LB Agar (Lysogeny Agar)	
Tryptone	10g
Yeast extract	5g
NaCl	5g
Bacteriological Agar	
Marine Minimal Media (MMM)	
Sodium chloride	20g
Magnesium sulfate heptahydrate	7g
Tris base	6.1g
Magnesium chloride pentahydrate	5.3g
Potassium glutamate	2g
Calcium chloride	1.25g
Potassium chloride	0.7g
Dipotassium phosphate	75mg
Iron(II) sulfate heptahydrate	25mg
Copper sulfate pentahydrate	5mg
ZYM5052 auto-inducing complex media	
Tryptone	1%
Yeast extract	0.5%
glycerol	0.5%
α-lactose monohydrate	0.2%
glucose	0.05%
Ammonium Chloride	50mM
Sodium hydroxide	40mM
Disodium phosphate	25mM
Monopotassium phosphate	25mM
succinic acid	20mM
Sodium sulfate	5mM
Magnesium sulfate	2mM
Iron(III) chloride	0.1mM
Tryptone	1μM

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Table 4.2 – Solutions for alkaline lysis isolation of plasmid DNA.

Plasmid Midi-Prep / Alkaline Lysis	
Solution 1	
Glucose	1M
Tris-HCl	1M
EDTA	0.5M
H2O	
Solution 2	
NaOH	10M
SDS	10%
H2O	
Solution 3	
Glacial Acetic acid (100%) (60.05g/mol)	2M
Potassium Acetate (98.15g/mol)	3M
H2O	

Table 4.3 – Preparation of stacking gel (4%) and separating gel for SDS-PAGE used in this study.

	4%	
dH20	3.075ml	
0.5M Tris-HCl, pH 6.8	1.250ml	
20% (w/v) SDS	0.025ml	
Acrylamide/ Bisacrylamide (30%/ 0.8% w/v)	0.670ml	
10% Ammonium Persulfate (APS)	0.025ml	
Tetramethylethylenediamine TEMED	0.01ml	
	10%	12%
dH20	4.1	3.4
1.5M Tris-HCL, pH 8.8	2.5	2.5
20% (w/v) SDS	0.05	0.05
Acrylamide/ BisacrylamidE (30%/ 0.8% w/v)	3.3	4.3
10% Ammonium Persulfate (APS)	0.05	0.05
Tetramethylethylenediamine TEMED	0.01	0.01

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Table 4.4 – Buffer components for His-Tag purification.

His-Tag Buffers		
8X Binding Buffer (pH 7.9)		1x
NaCl	4M	0.5M
Tris-HCl	160mM	20mM
Imidazol	40mM	5mM
8X Wash Buffer (pH 7.9)		1x
NaCl	4M	0.5M
Tris-HCl	160mM	20mM
Imidazol	480mM	60mM
4X Elute Buffer (pH 7.9)		1x
NaCl	2M	0.5M
Tris-HCl	80mM	20mM
Imidazol	4M	1M
4X Strip Buffer (pH 7.9)		1x
NaCL	2M	0.5M
Tri-HCl	80mM	20mM
EDTA	400mM	100mM
8X Charge Buffer		1x
NiSO4	400mM	100mM

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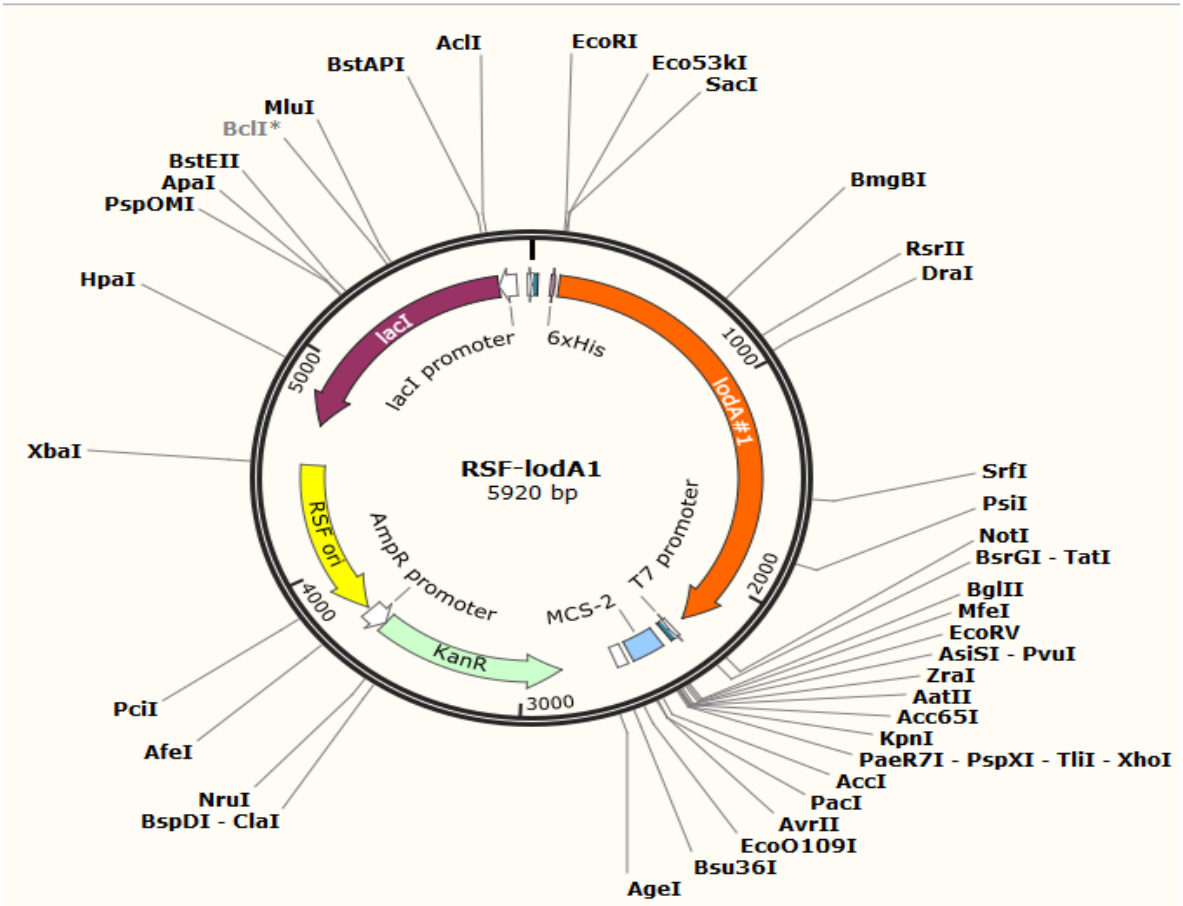

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Figure 4.1 – Plasmid map and insert sequence for plasmid RSF-lodA1

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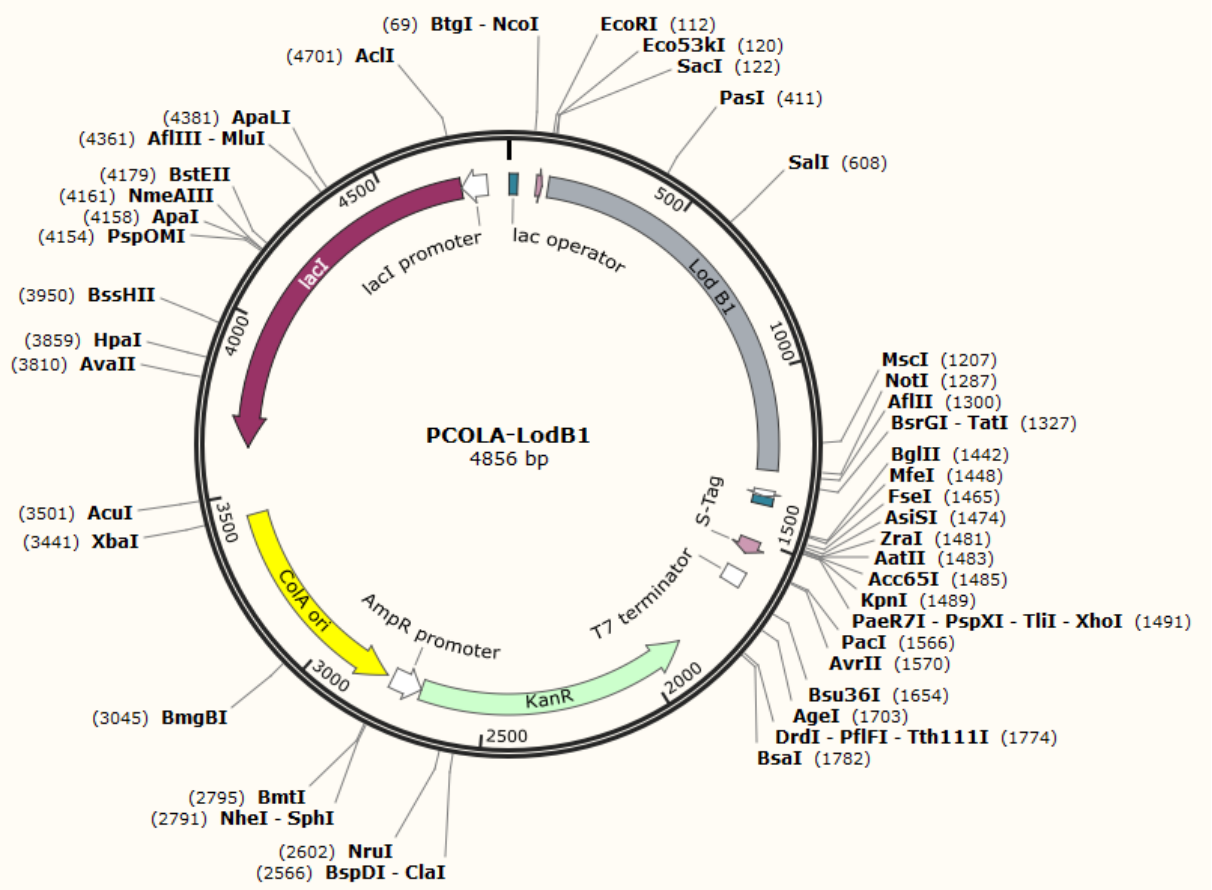

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Figure 4.2 – Plasmid map and insert sequence for plasmid COLA-lodB1

https://uwcscholar.uwc.ac.za/home

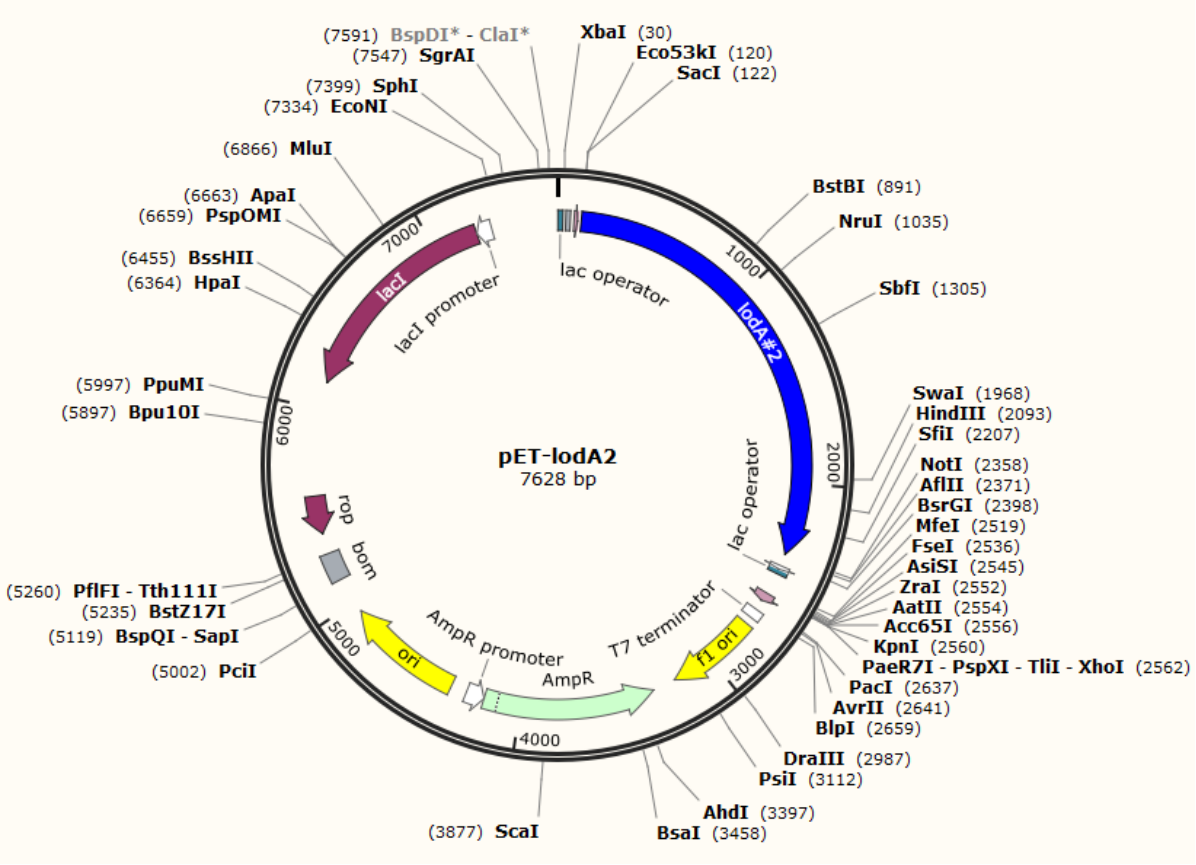

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Figure 4.3 – Plasmid map and insert sequence for plasmid pET-lodA2

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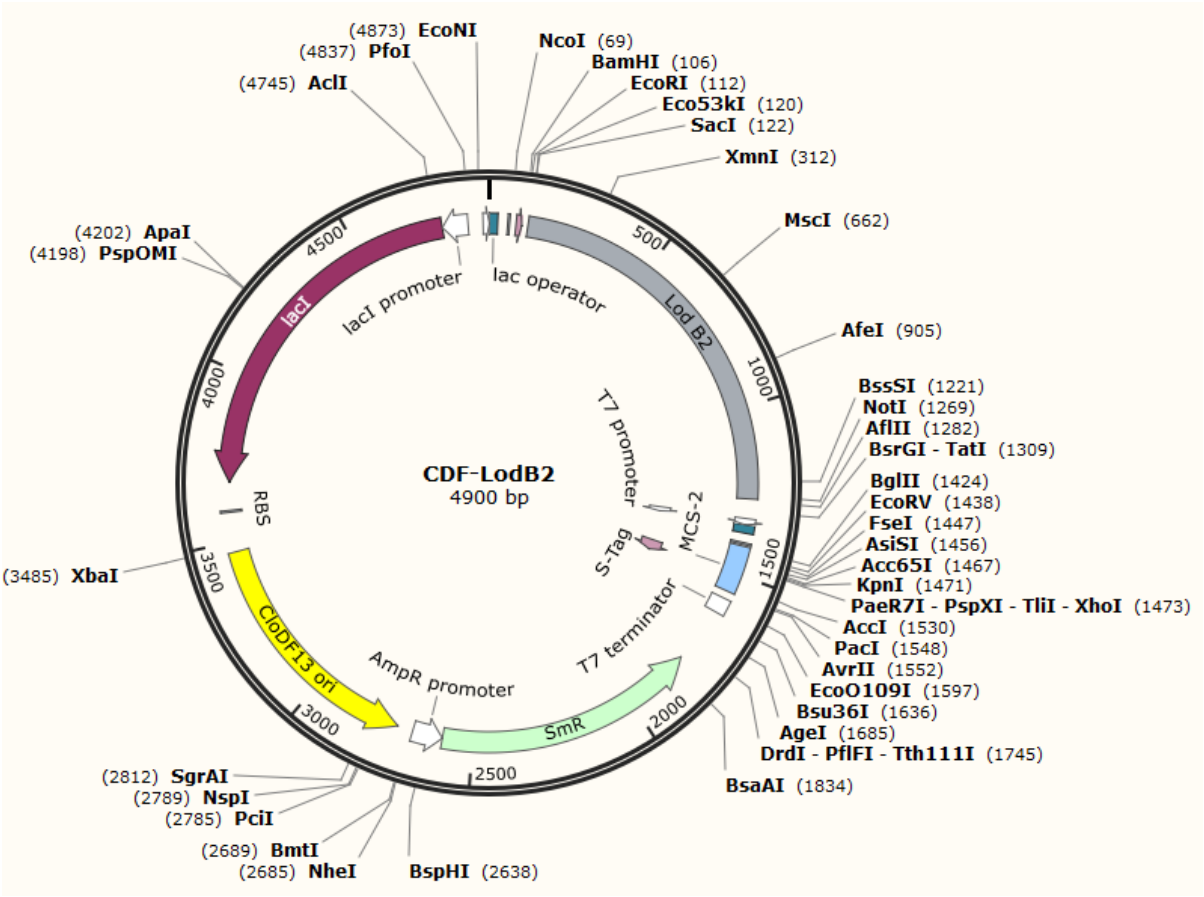

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Figure 4.4 – Plasmid map and insert sequence for plasmid CDF-lodB2

https://uwcscholar.uwc.ac.za/home

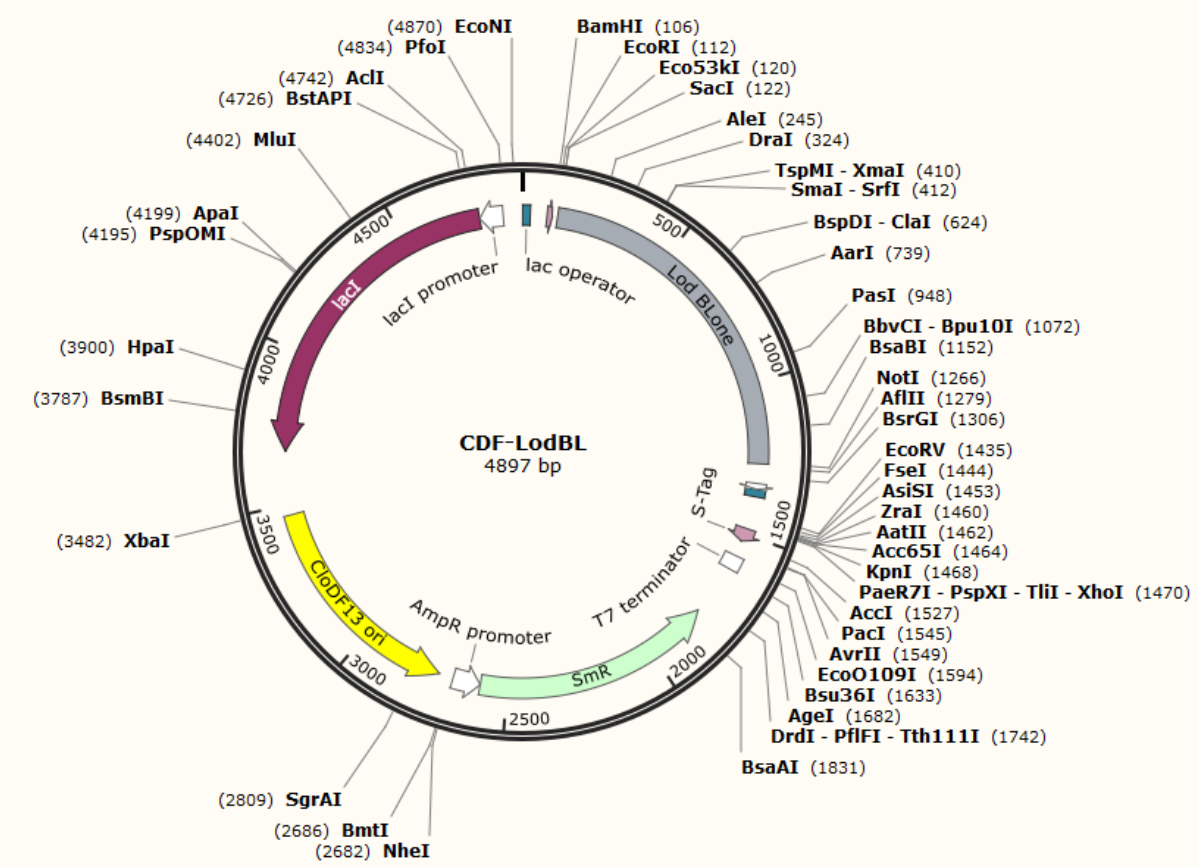

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Figure 4.5 – Plasmid map and insert sequence for plasmid CDF-lodBL

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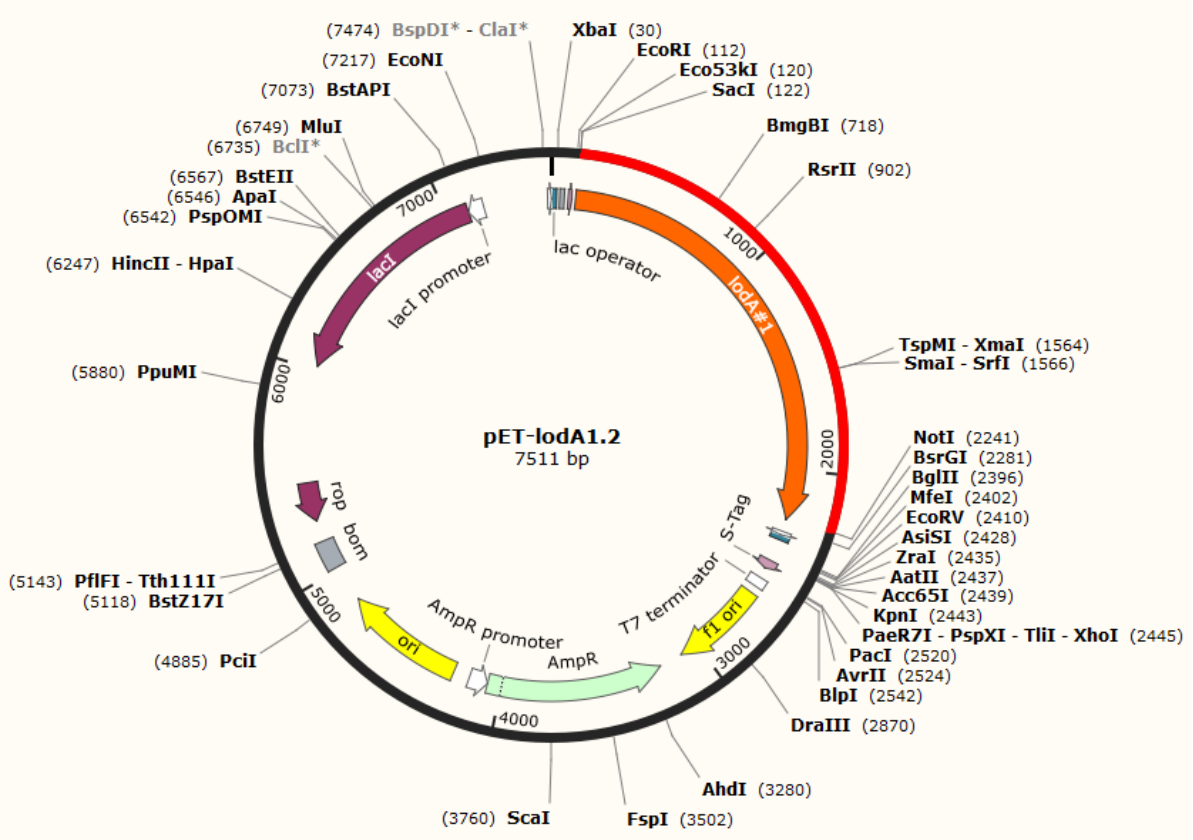

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Figure 4.6 – Plasmid map and insert sequence for plasmid pET-lodA1.2 (subcloned fragment insert from RSF-lodA1)

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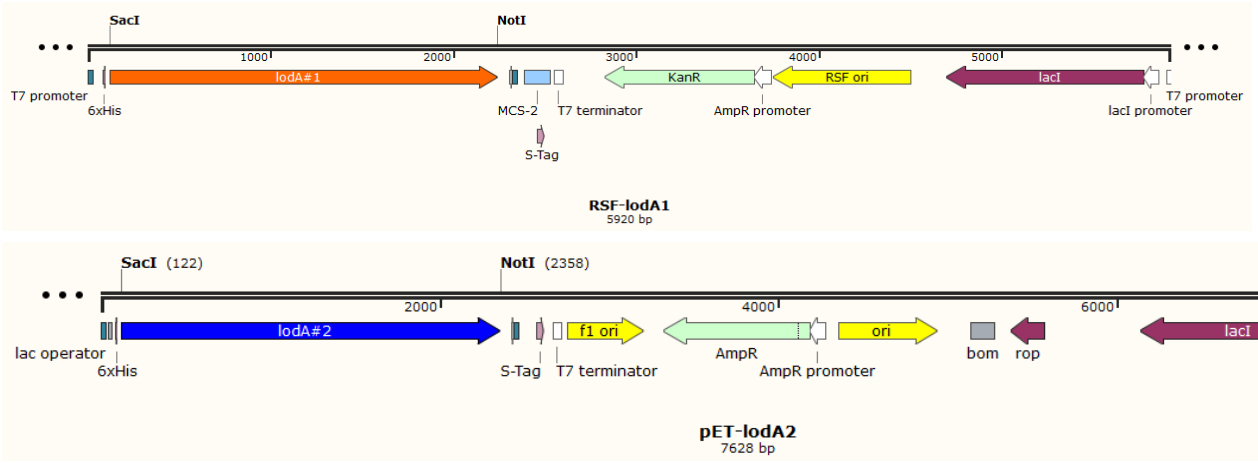

Each above plasmids digested with SacI and NotI restriction enzymes at start and end of insert.

lodA1 fragment and pET-lodA2 backbone purified from agarose separation.

lodA1 fragment and pET- backbone Treated with T4 polymerase to remove overhang from cut

lodA1 fragment and pET- backbone combined with T4 ligase to reform plasmid (pET-lodA1.2).

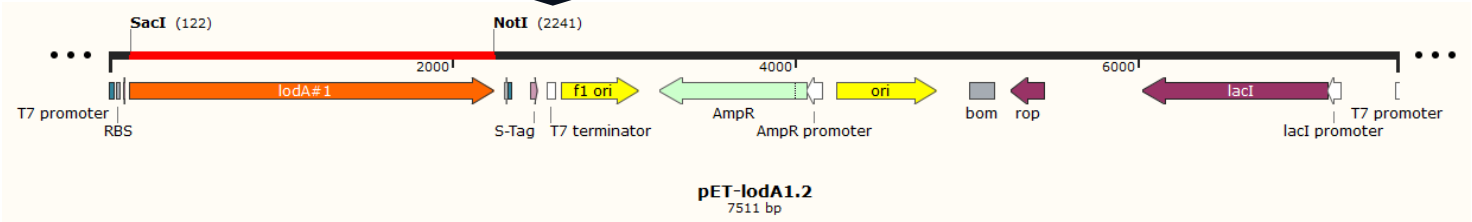

Figure 4.7 - Schematic diagram of plasmid pET-lodA2 and RSF-lodA1 and sub-cloning sequence for form pET-lodA1.2. The section highlighted in red corresponds to insert LodA#1 with restriction sites *SacI* and *NotI*.

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>lodAB1 T. viridans
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>lodAB2 T. viridans
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>lodAB *M. mediterranea*

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>goxAB *M. mediterranea*

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Figure 4.8 – Gene sequences for *lodAB1*, *lodAB2* and *lodBL* gene sequences from *Thalassomonas viridans*, and *lodAB* and *goxAB* gene sequences from *Marinomonas mediterranea.*

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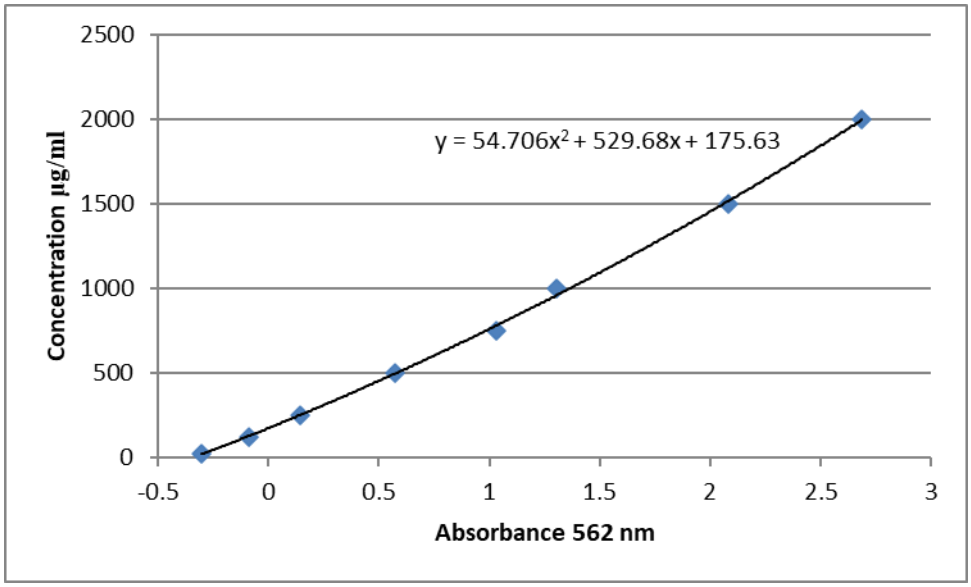

Figure 4.9 – Standard curve for protein concentration assays. Standard curves were plotted using MS Excel with absorbance on the dependant (X) axes and the standard protein concentration was plotted on the variable (Y) axes. A polynomial trend line was then added to the data set. The concentration of the unknown samples was then solved for Y, where Y = ax2+bx+c (Thermo Scientific, 2012).

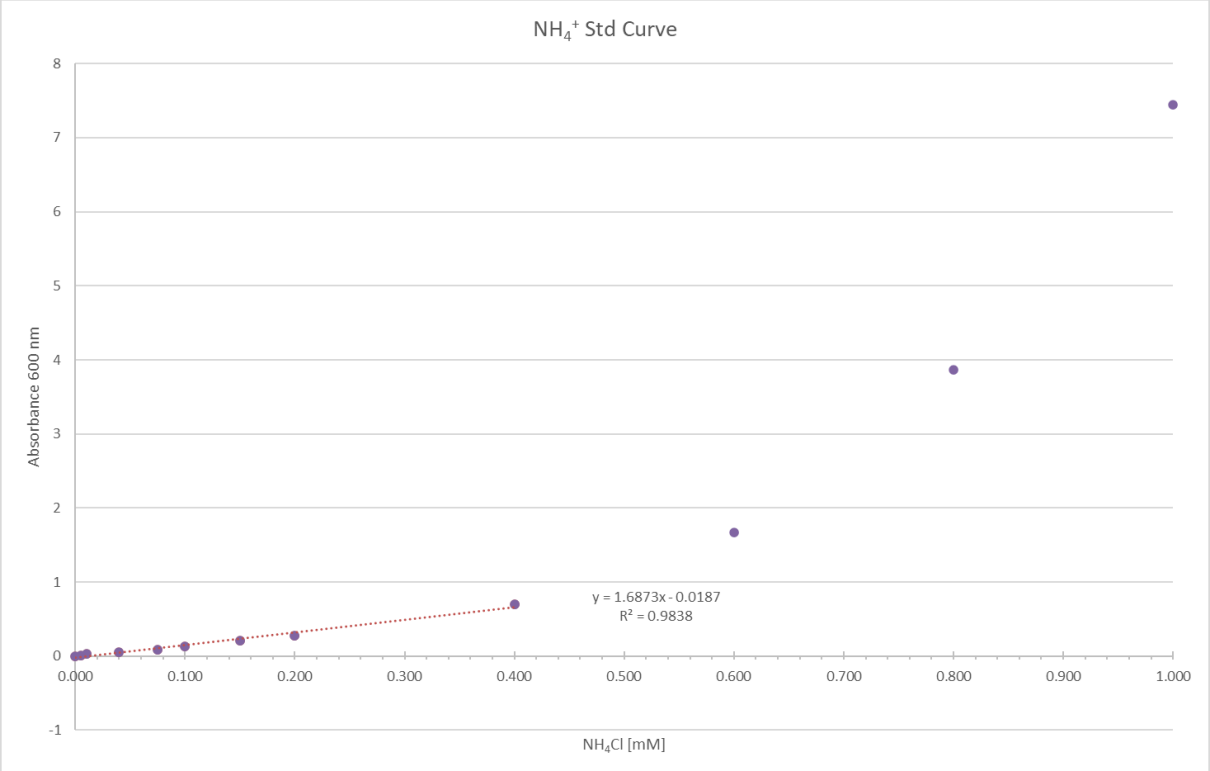

Figure 4.10 - Standard curve of ammonia for phenol-hypochlorite assay. The standard curve was developed using 0.005 mM to 1.0 mM ammonium chloride in potassium phosphate buffer (pH 7.0). The linear range of the standard curve is 0.005 mM to 0.4 mM ammonium chloride.

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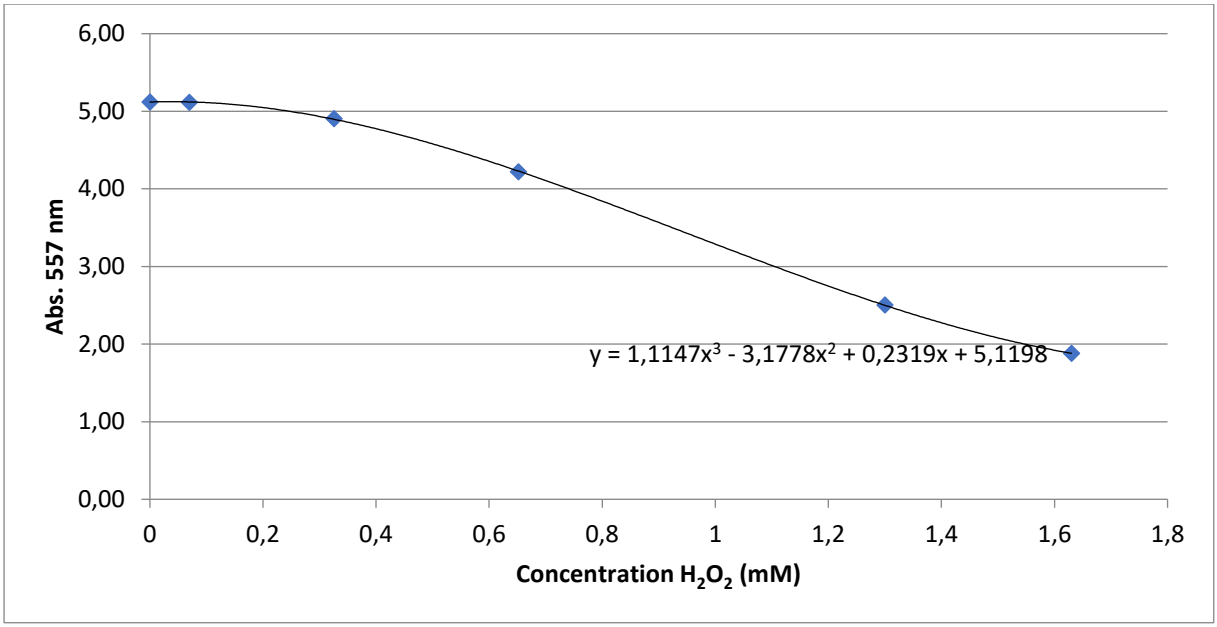

Figure 4.11 - Standard curve for hydrogen peroxide Phenol Red/Horseradish Peroxidase assay. The standard curve was developed using 0.005 mM to 1.63 mM H_2O_2 in potassium phosphate buffer (10 mM pH 7.0).

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Table 4.5 – list of organisms with similar sequences to *lodAb* from *T. viridans* generated by RAST/SEED server.

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1	Caulobacter crescentus CB15	fig|190650.1.peg.554
2	Cellvibrio japonicus Ueda107	fig|498211.3.peg.227
3	Magnetococcus sp. MC-1	fig|156889.7.peg.322
4	Chromobacterium violaceum ATCC 12472	fig|243365.1.peg.3268
5	Marinomonas sp. MWYL1	fig|400668.6.peg.3797
6	Pseudoalteromonas tunicata D2	fig|87626.3.peg.939
7	Pirellula sp. 1	fig|243090.1.peg.2349
8	Hahella chejuensis KCTC 2396	fig|349521.5.peg.2781
9	Cellvibrio japonicus Ueda107	fig|498211.3.peg.3632
10	Delftia acidovorans SPH-1	fig|398578.3.peg.3517
11	Cyanothece sp PCC 8801	fig|41431.3.peg.2779
12	Saccharophagus degradans 2-40	fig|203122.12.peg.317
	lodAB#2	fig|6666666.540131.peg.6041
1	Delftia acidovorans SPH-1	fig|398578.3.peg.3517
2	Cellvibrio japonicus Ueda107	fig|498211.3.peg.3632
3	Hahella chejuensis KCTC 2396	fig|349521.5.peg.2781
4	Cyanothece sp PCC 8801	fig|41431.3.peg.2779
5	Chromobacterium violaceum ATCC 12472	fig|243365.1.peg.3268
6	Magnetococcus sp. MC-1	fig|156889.7.peg.322
7	Pseudoalteromonas tunicata D2	fig|87626.3.peg.939
8	Marinomonas sp. MWYL1	fig|400668.6.peg.3797
9	Caulobacter crescentus CB15	fig|190650.1.peg.554
10	Cellvibrio japonicus Ueda107	fig|498211.3.peg.227
11	Saccharophagus degradans 2-40	fig|203122.12.peg.317
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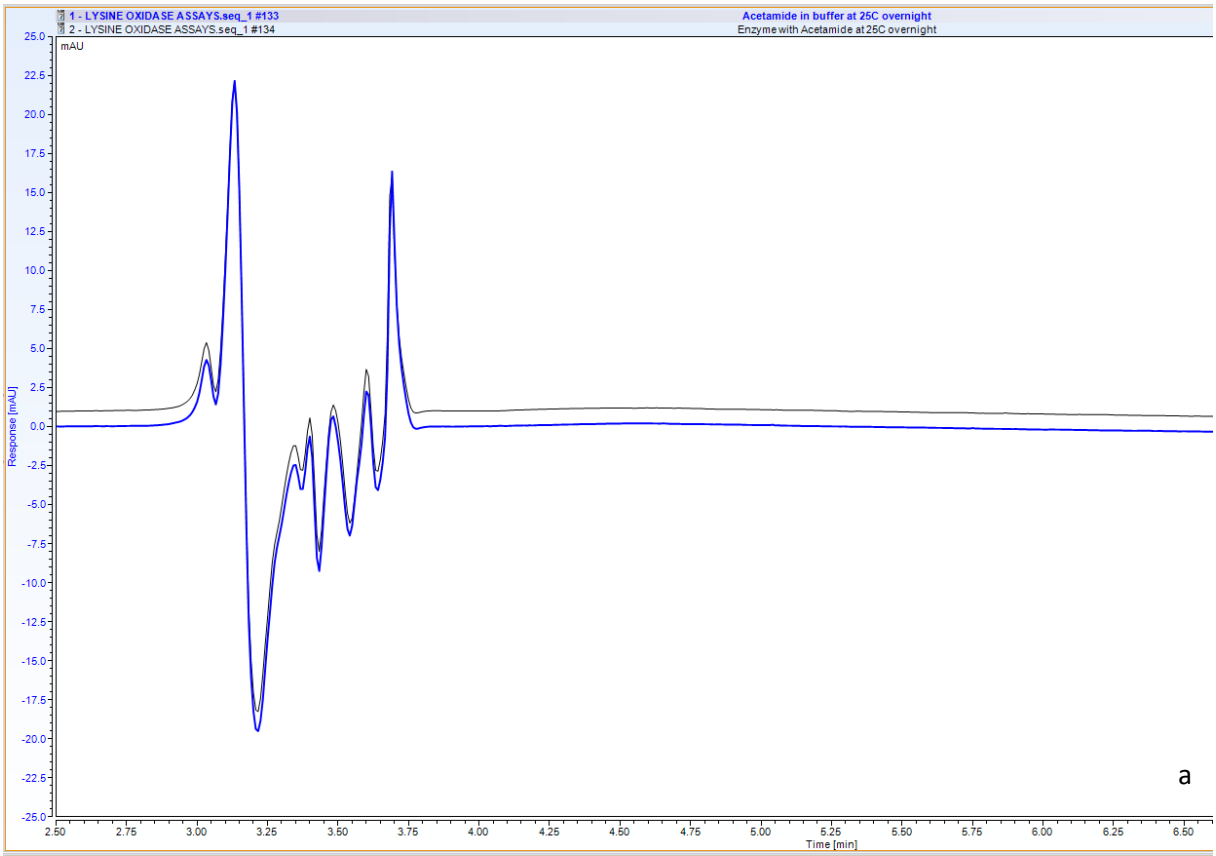

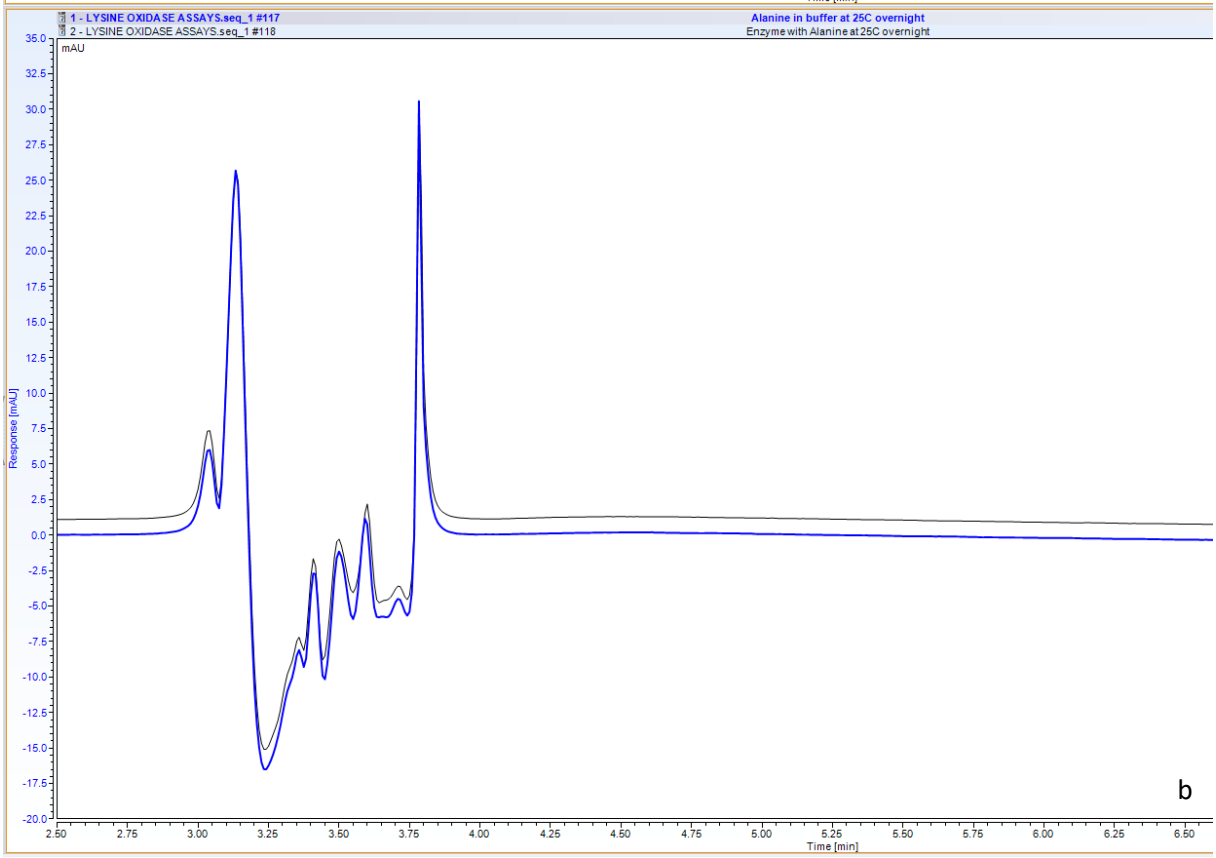

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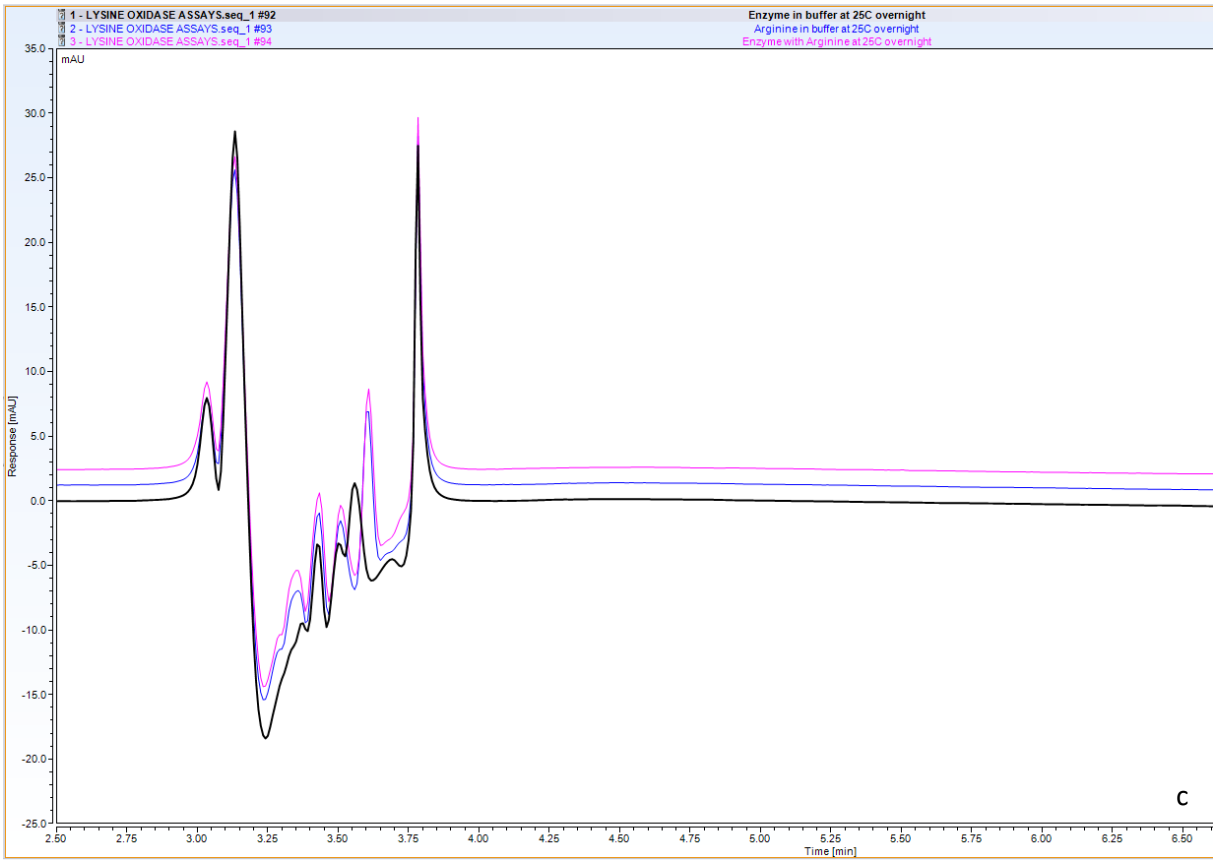

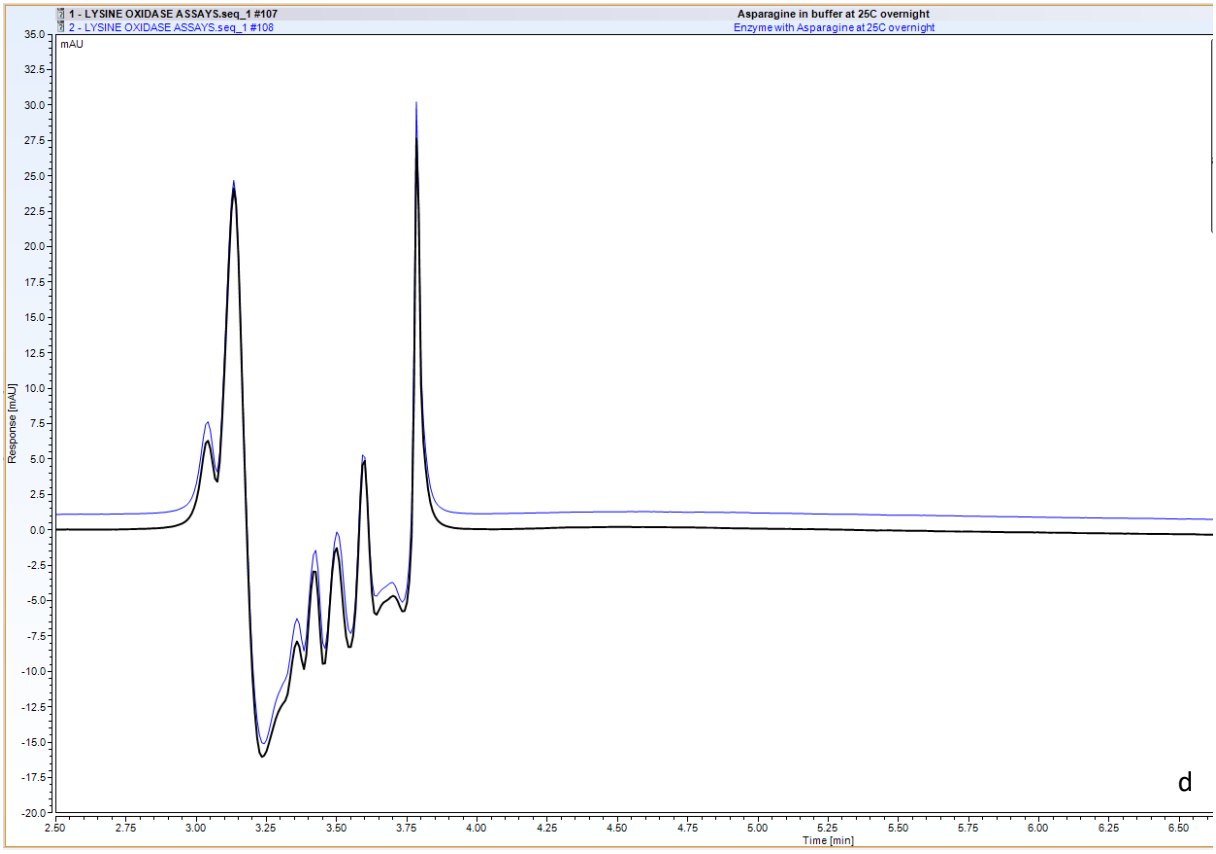

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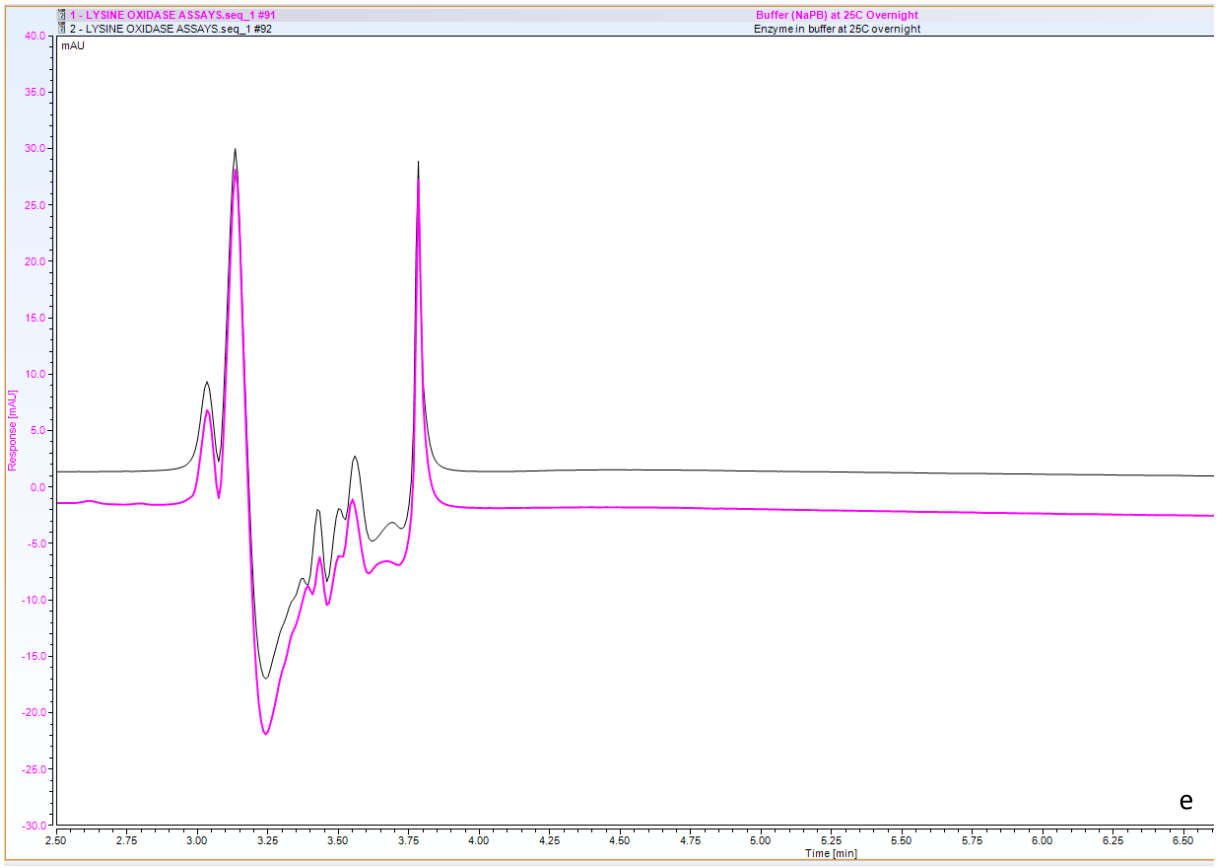

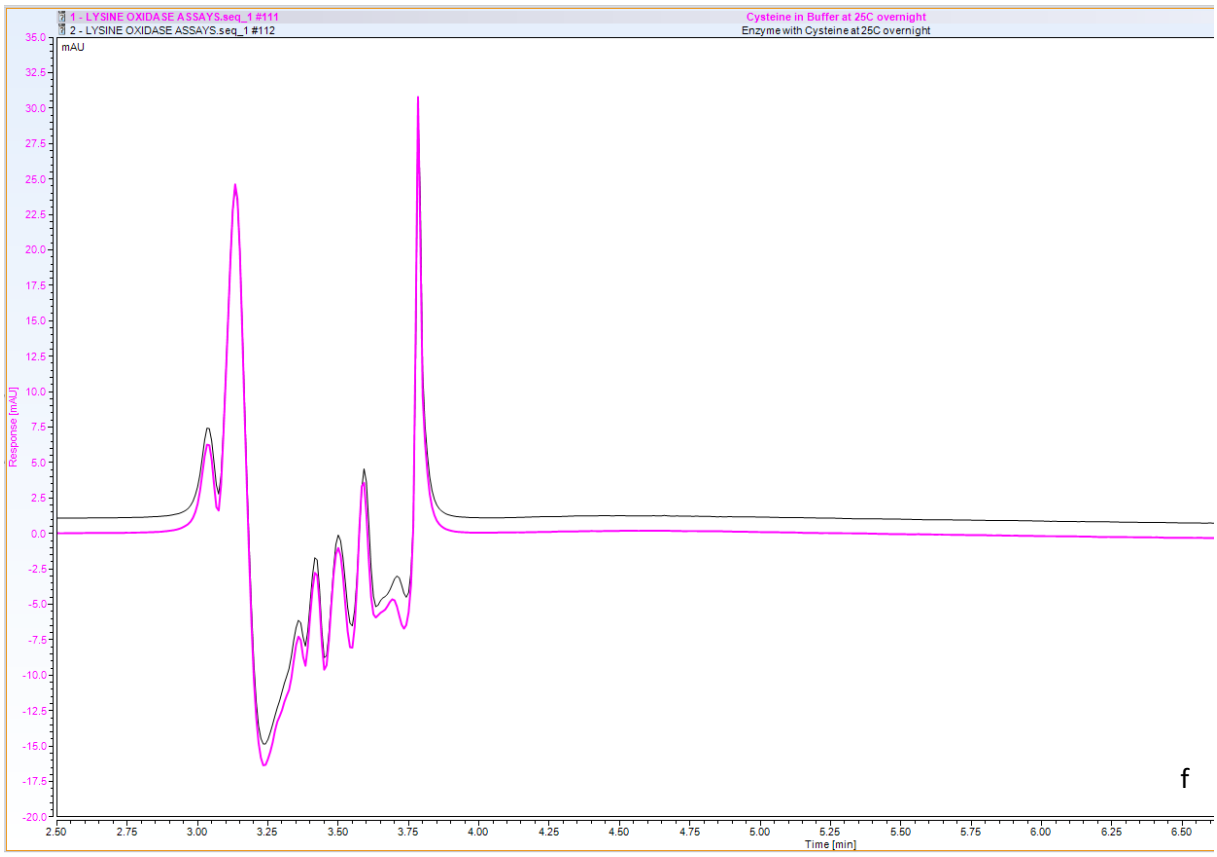

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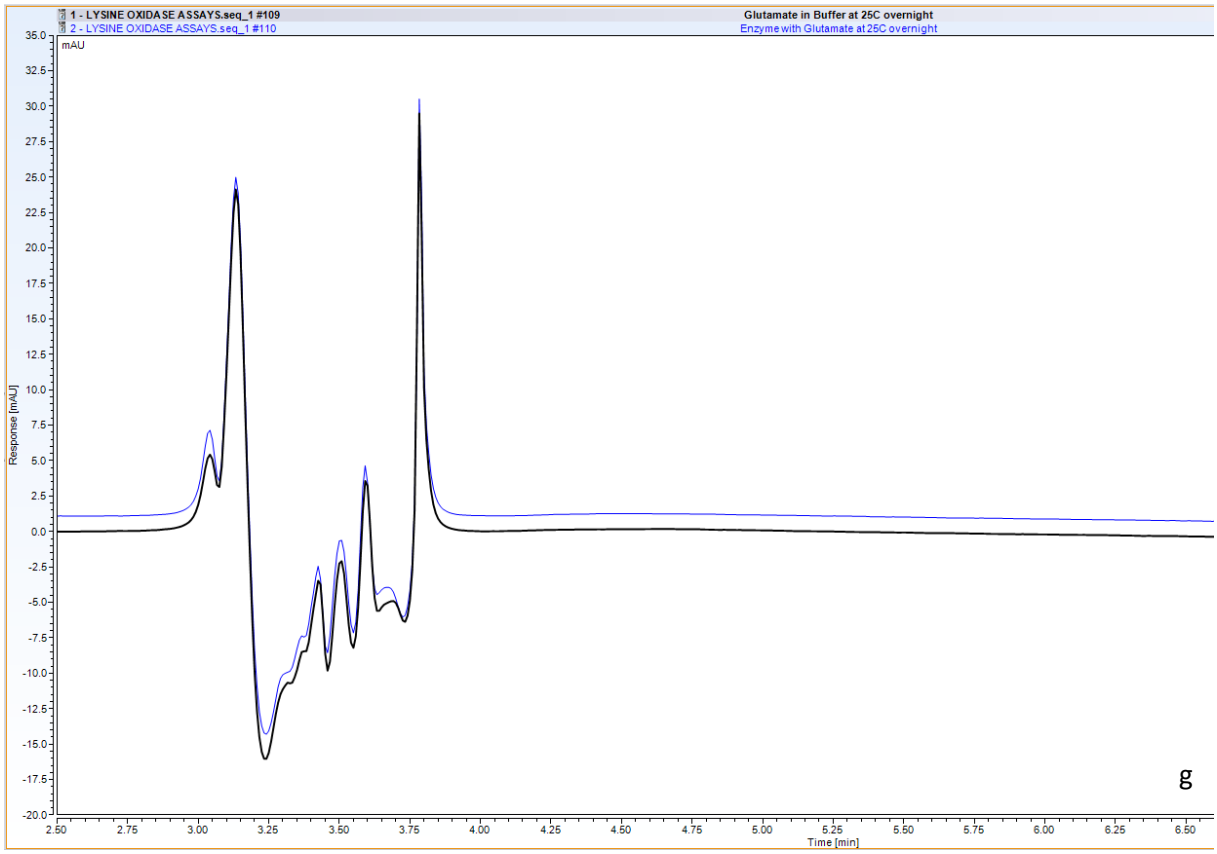

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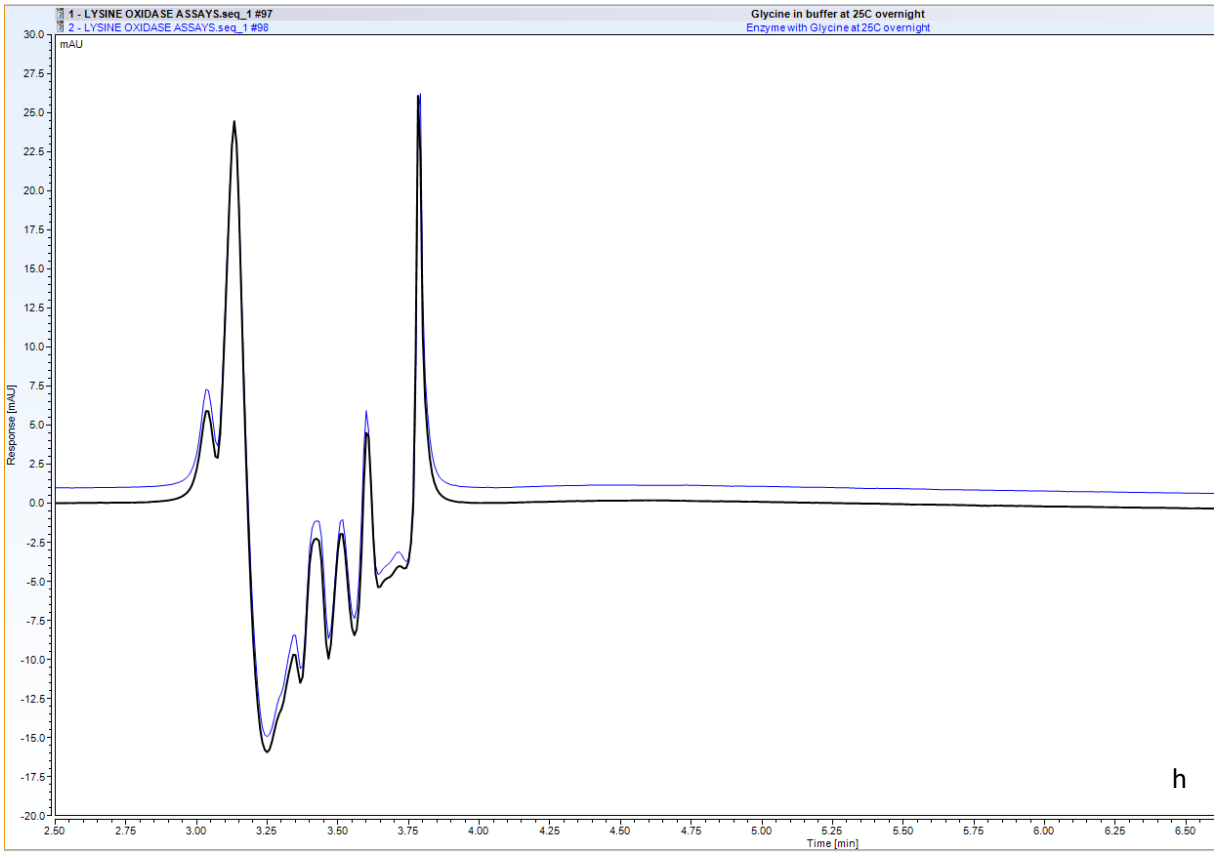

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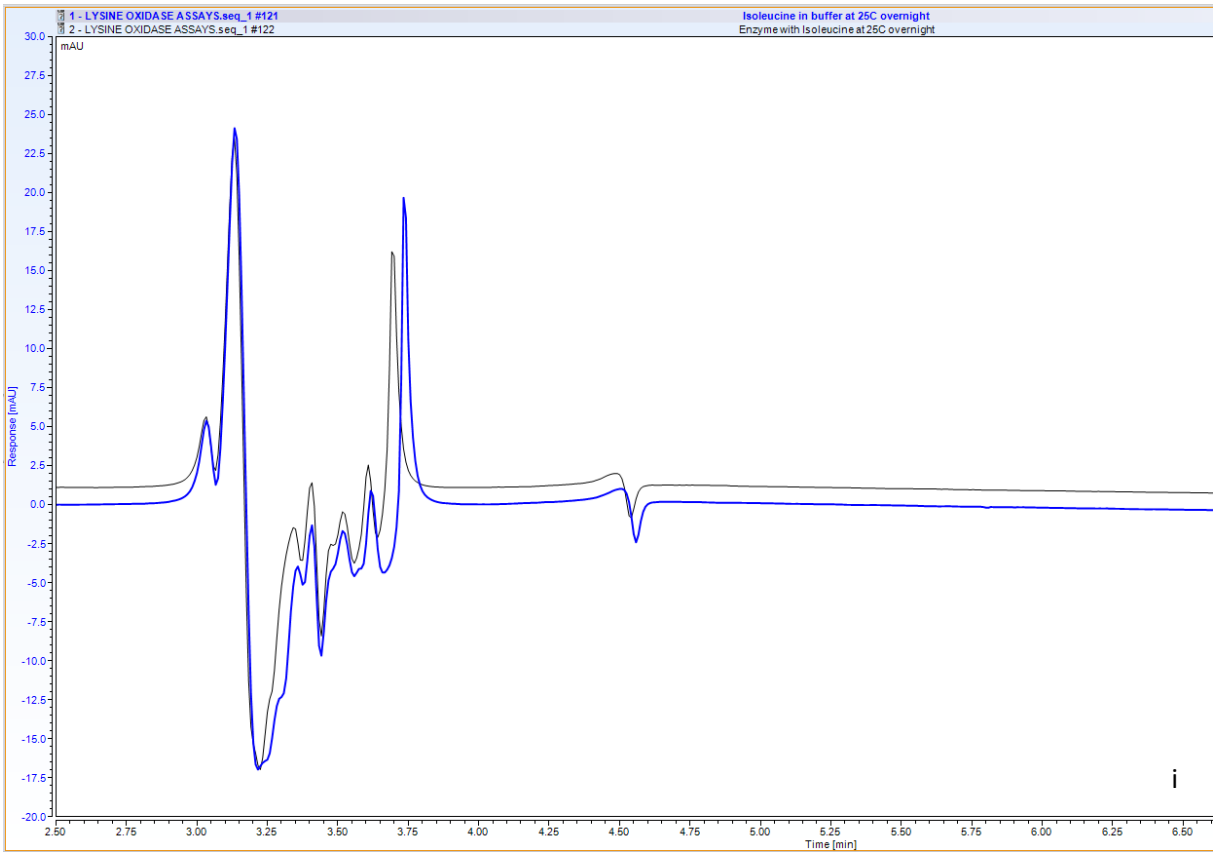

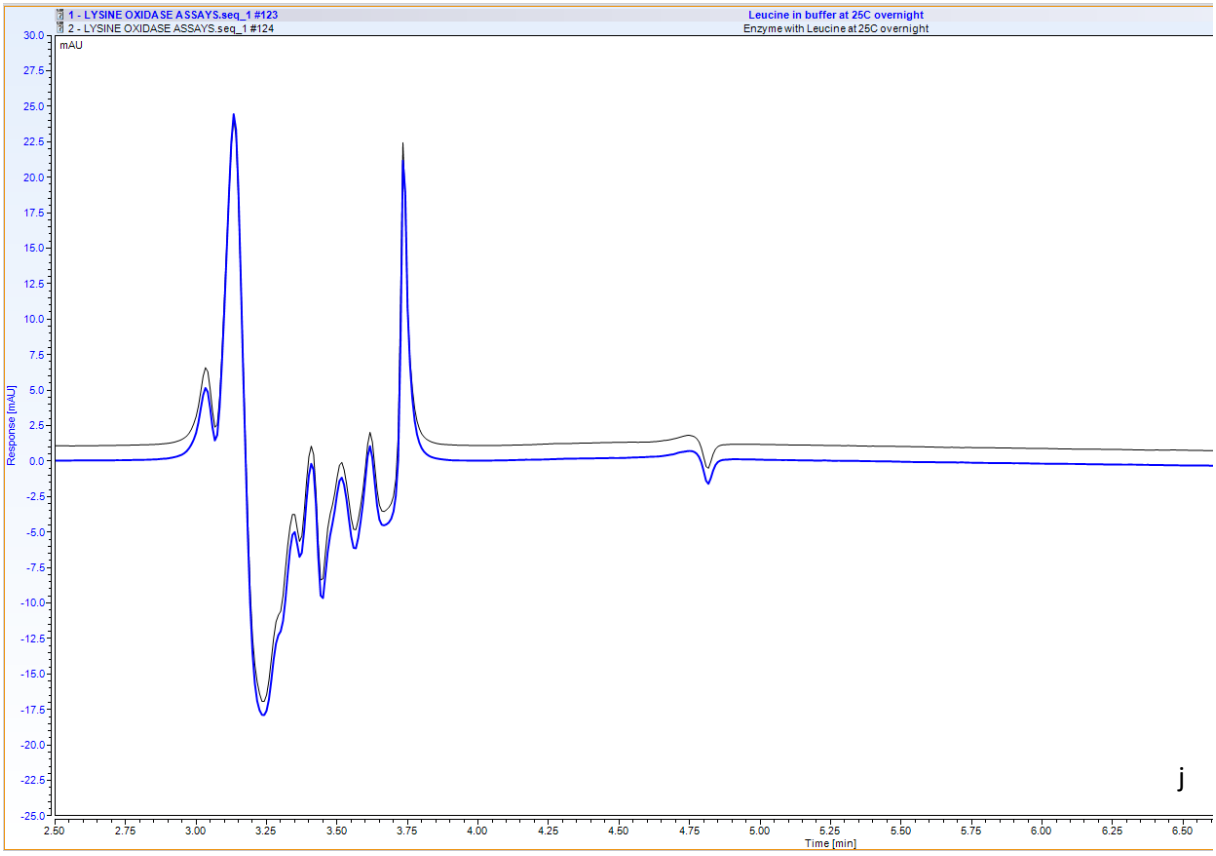

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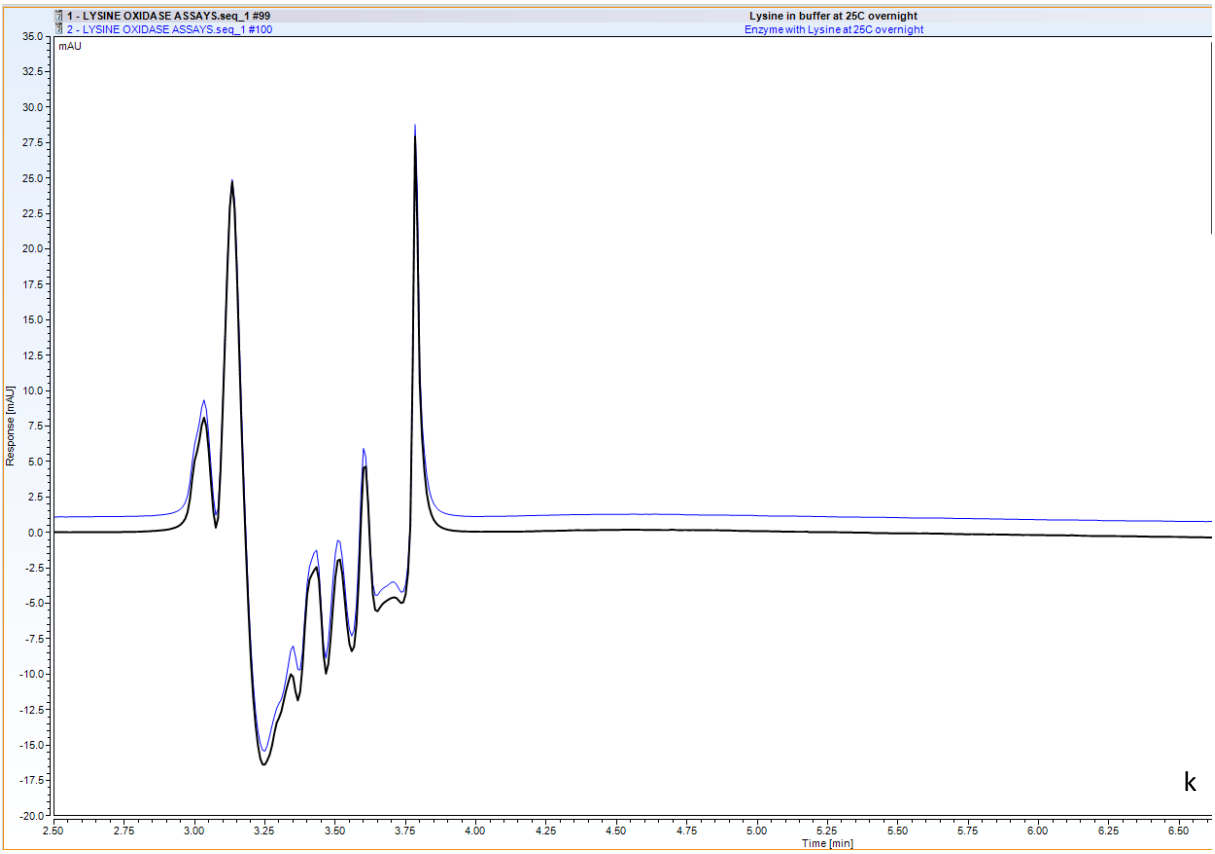

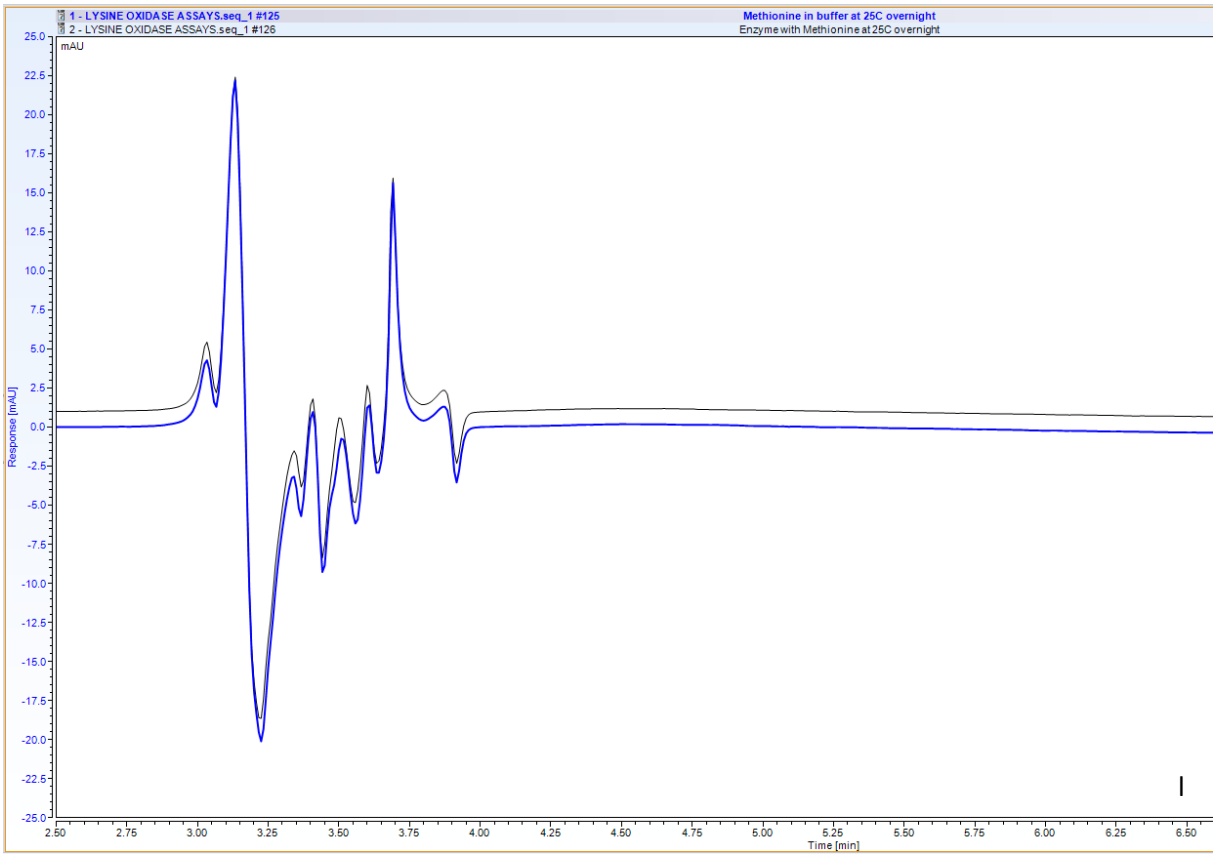

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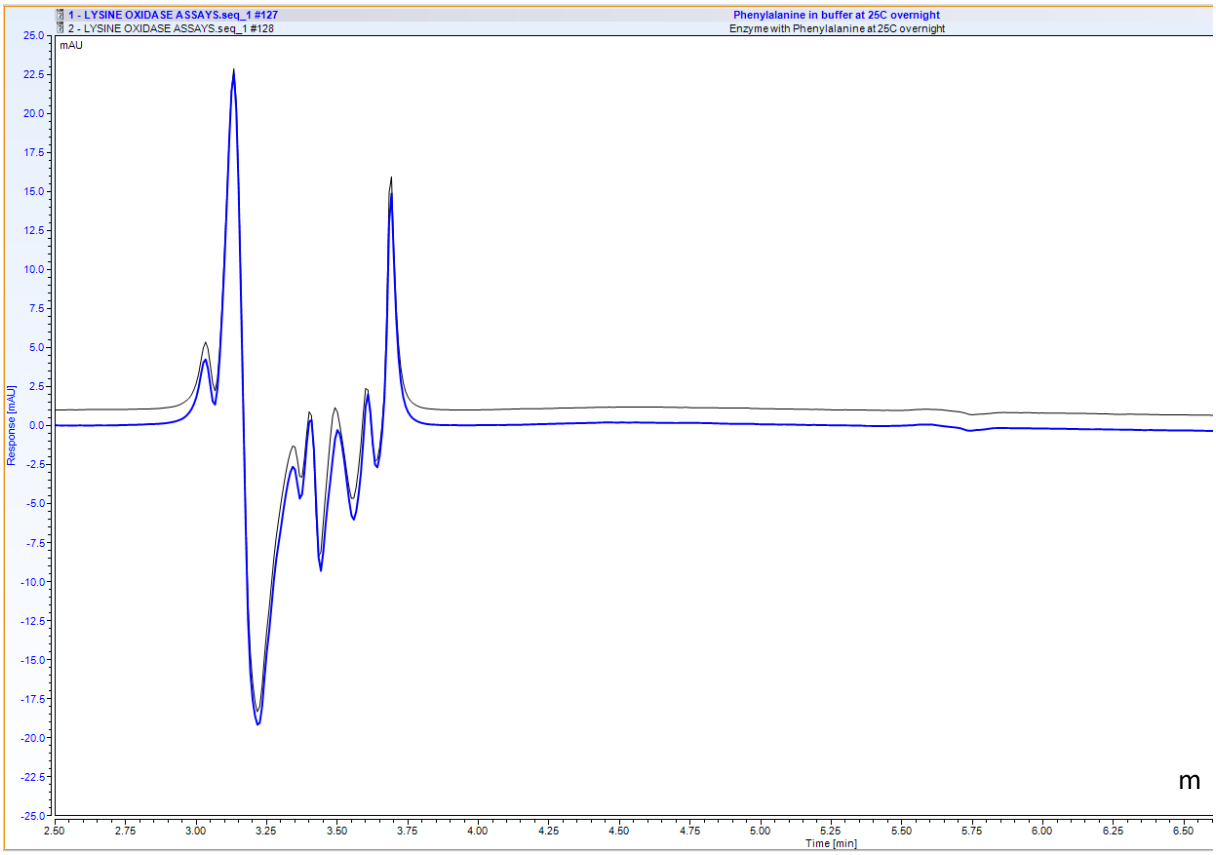

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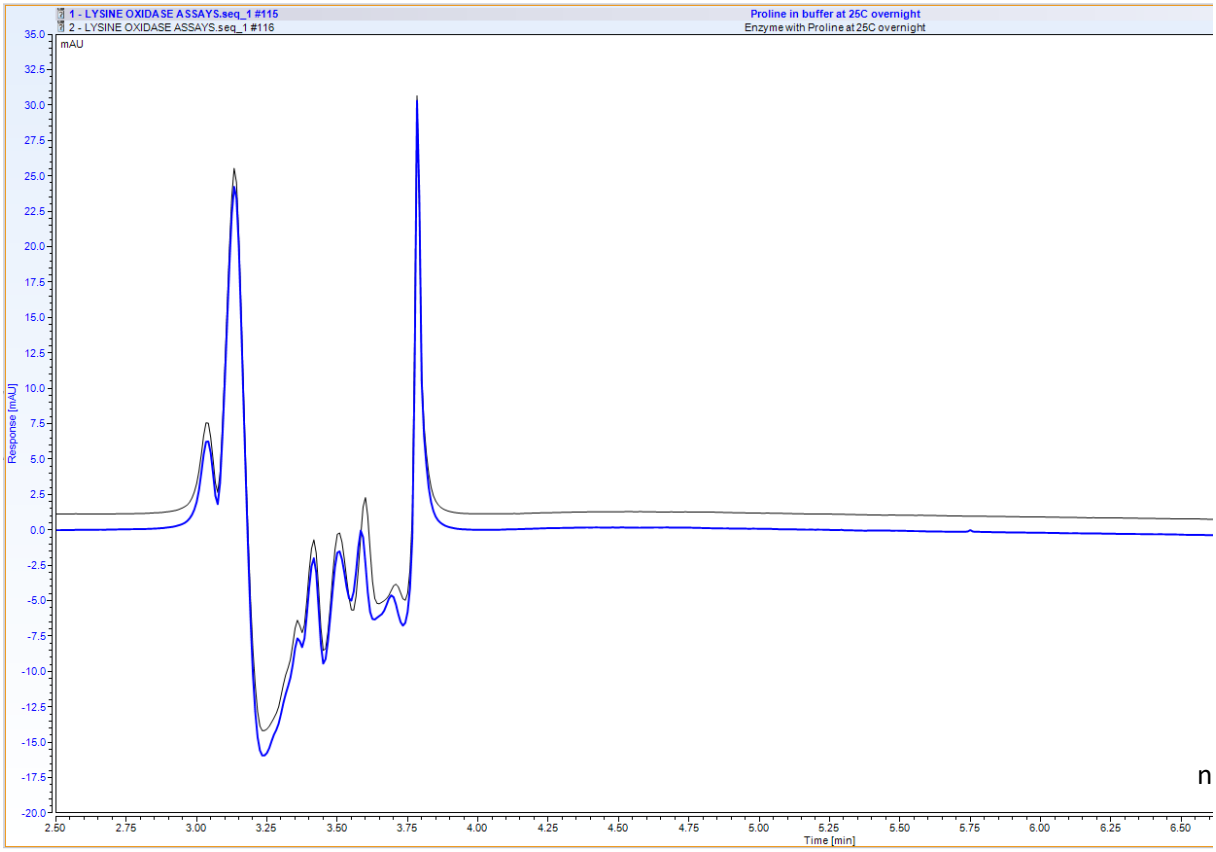

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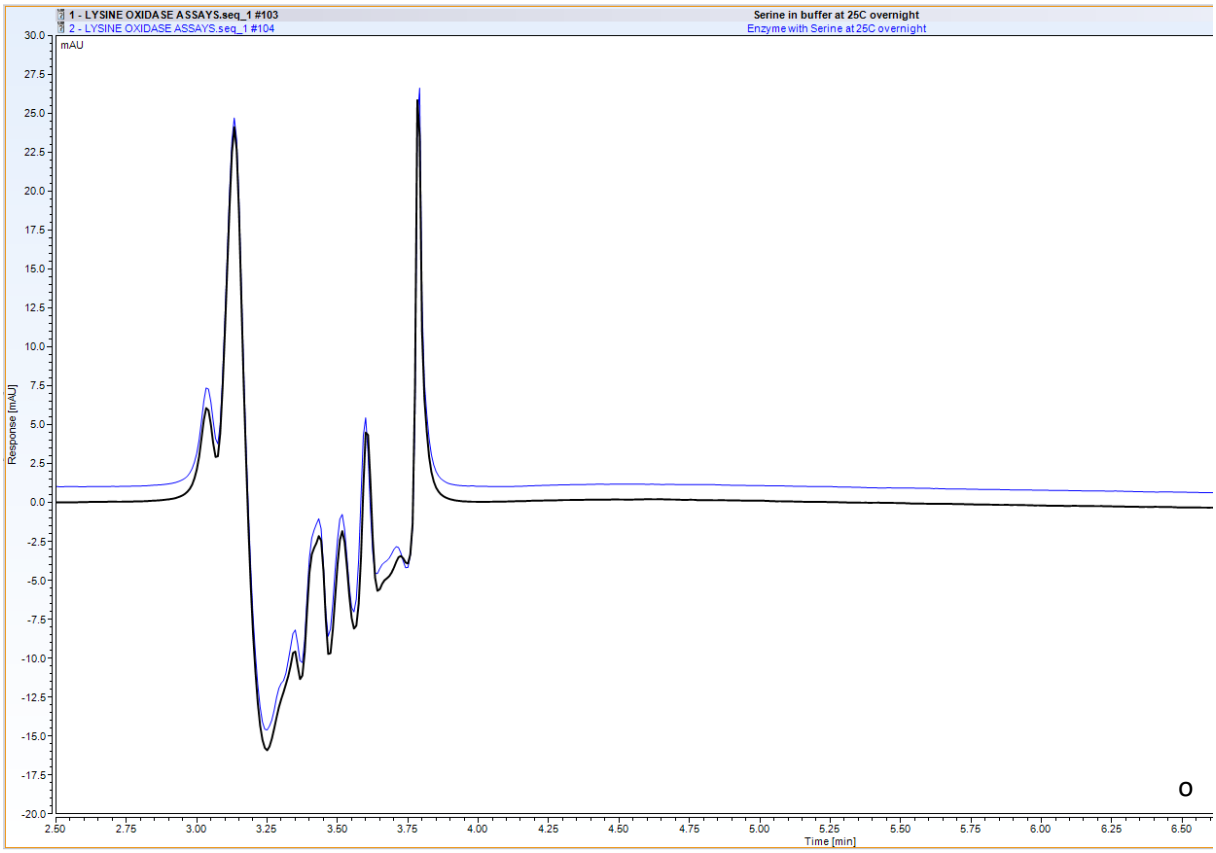

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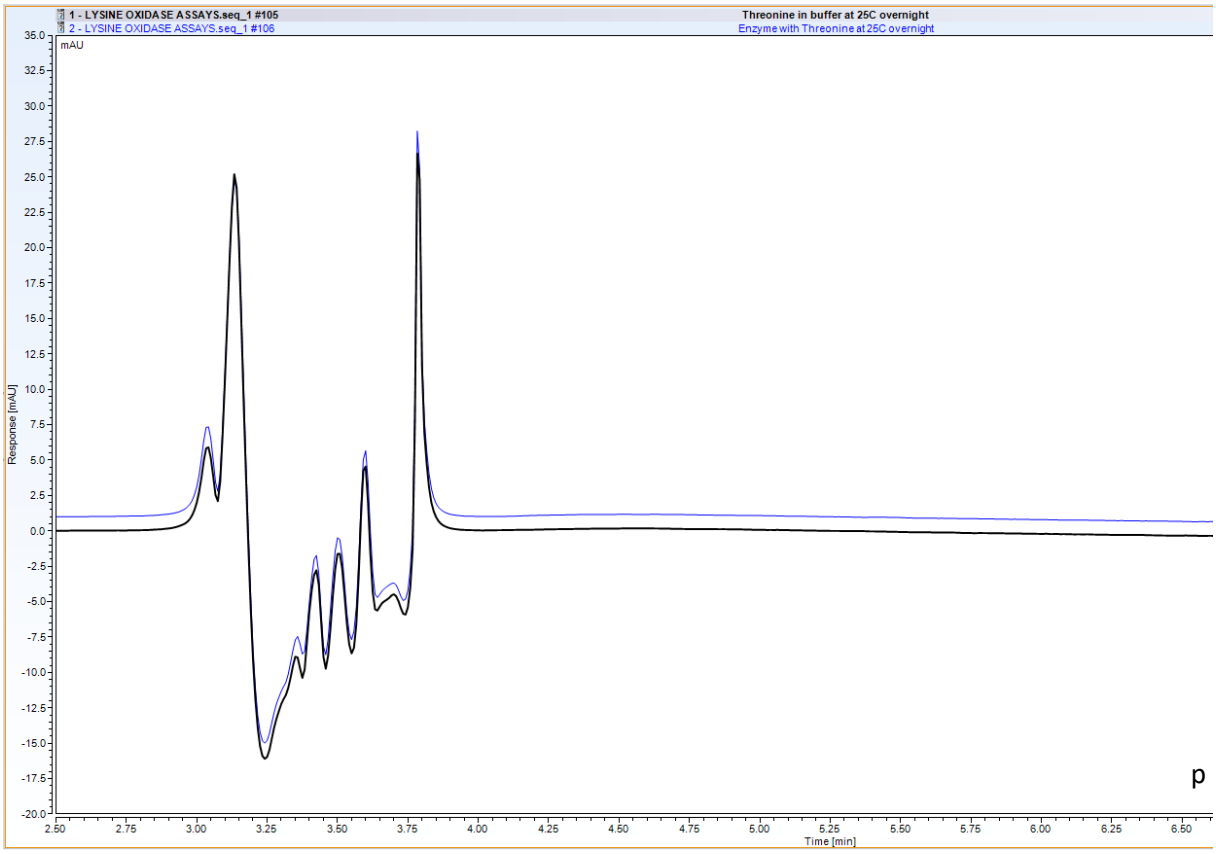

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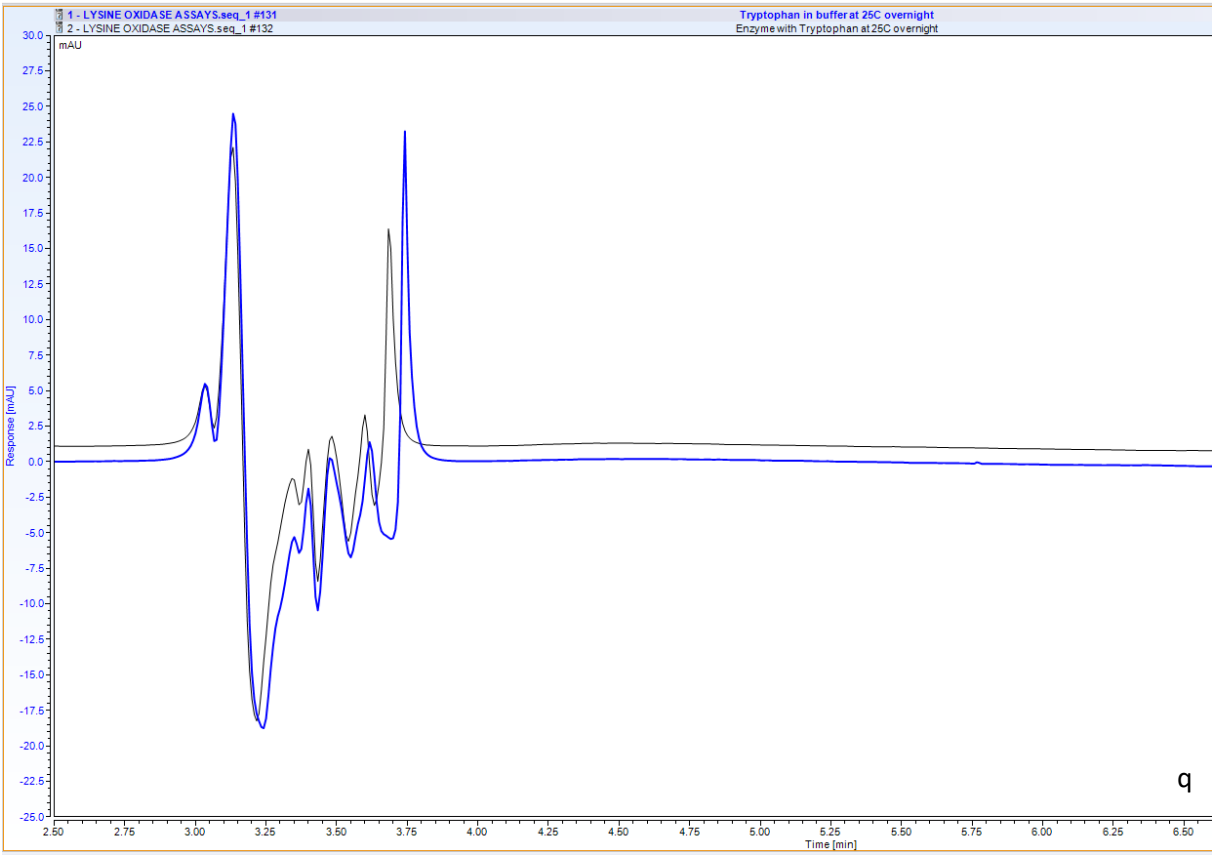

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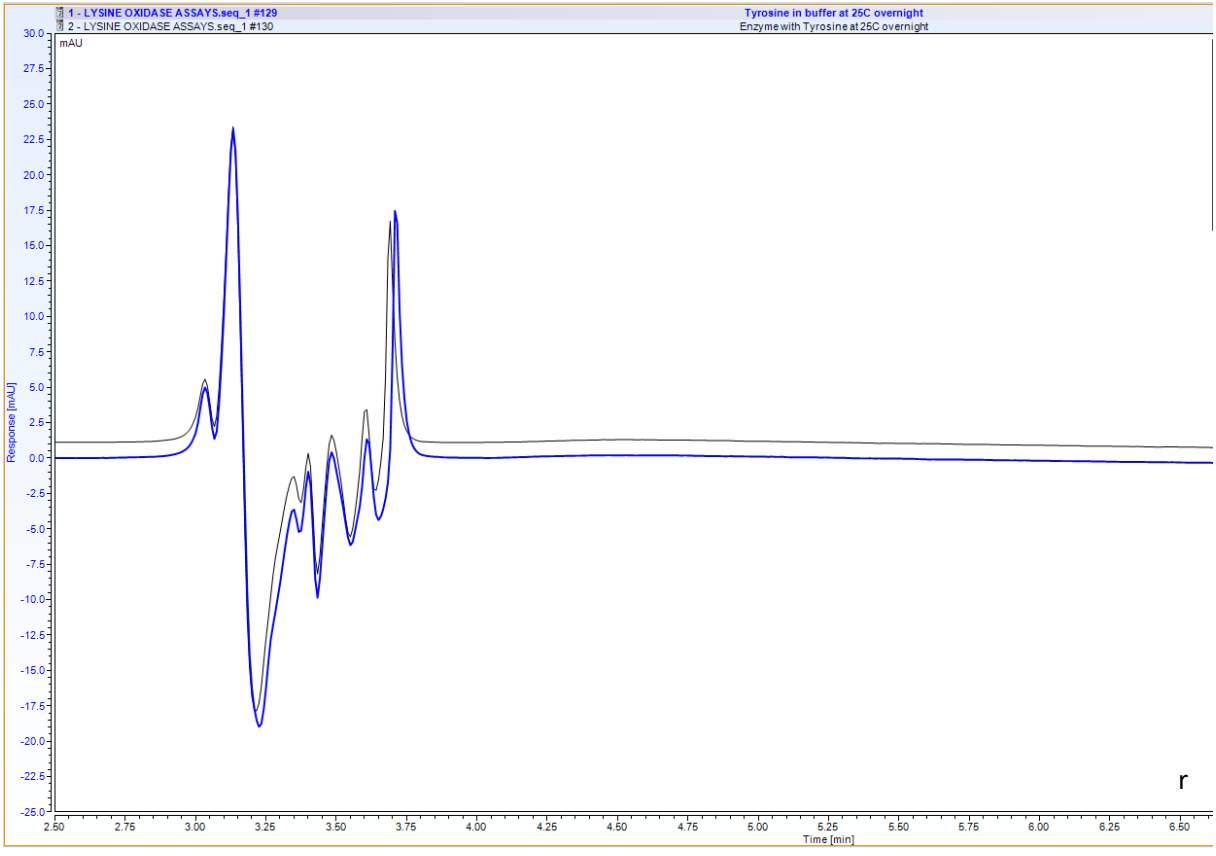

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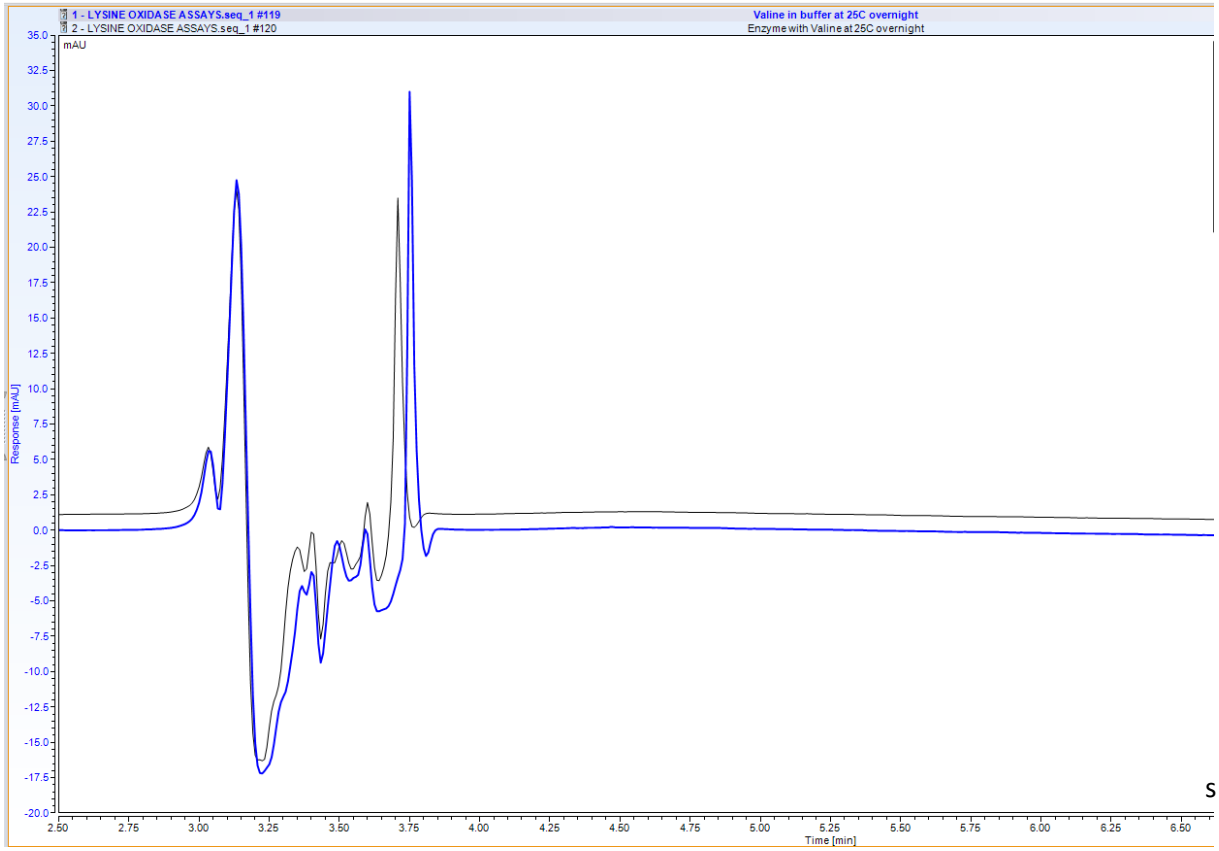

Figure 4.9 – Retention chromatograms for AA substrates with and without LodA1. a – Acetamide, b – Alanine, c – Arginine, d – Asparagine, e – Buffer & LodA, f – Cysteine, g – Glutamate, h – Glycine, I – isoleucine, j - Leucine, k – Lysine, l – Methionine, m – Phenylalanine, n – Proline, o – Serine, p – Threonine, q – Tryptophan, r – Tyrosine, s – Valine.

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