The characterisation of

Shewanella algae

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A thesis submitted in fulfilment of the requirements for the degree

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from

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UTS

Certificate of Original Authorship

I certify that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree except as part of the collaborative doctoral degree and/or fully acknowledged within the text.

I also certify that the thesis has been written by me. Any help that I have received in my research work and the preparation of the thesis itself has been acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

This research is supported by an Australian Government Research Training Program Scholarship.

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Date: 13th October 2017

Table of Contents

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List of Publications

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Publications in Review

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Conference Proceedings

BacPath 13: Molecular Analysis of Bacterial Pathogens, Victoria 2015
Poster presentation
'The development of a proteogenomic pipeline to identify bacterial virulence factors'

The Australian Society for Microbiology, Annual Scientific Meeting, Canberra 2015
Oral presentation
'*The development of a proteogenomic pipeline to characterise the type VI secretory system of Gram-negative bacteria'*

The Australian Society for Microbiology Benton Dickson Awards, Sydney 2015
Oral presentation
'The development of a proteogenomic pipeline to characterise the type VI secretory system of Gram-negative bacteria'

Launch of AusGEM, Sydney 2014
Poster presentation
'Uncovering potential virulence factors of the emerging human pathogen Shewanella algae'

"Proteomics and Beyond" Symposium, Sydney 2014
Poster presentation
'Uncovering potential virulence factors of the emerging human pathogen Shewanella algae'

The 19th Lorne Proteomics Symposium, Victoria 2014
Poster presentation
'Investigations into the type VI secretory system of an emerging bacterial pathogen, Shewanella algae, using a proteogenomic approach'

BacPath 12: Molecular Analysis of Bacterial Pathogens, Queensland 2013
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'Development of a proteogenomic approach to characterise the Type VI secretory system (T6SS) of Gram-negative bacteria'

The 5th Congress of European Microbiologists (FEMS) Conference, Germany 2013
Poster presentation
'The secreteomes of indigenous Vibrio cholerae from Sydney water reveal pathogenic characteristics similar to O1 serotype Vibrio cholerae'

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'The secretomes of indigenous Vibrio cholerae from Sydney water reveal numerous pathogenic characteristics'

The Australian Society for Microbiology, Annual Scientific Meeting 2012
Poster presentation
'Shewanella algae: A wolf in sheep's clothing'

List of Figures

List of Tables

Abbreviations

1D-SDS-PAGE	One dimensional - sodium dodecyl sulphate - polyacrylamide gel electrophoresis
2D-SDS-PAGE	Two dimensional - sodium dodecyl sulphate - polyacrylamide gel electrophoresis
A5	Andrew and Aaron's Awesome Assembly pipeline
BHI	Brain heat infusion
BLAST	Basic local alignment search tool
CDS	Calibrated dichotomous sensitivity
DNA	Deoxyribose nucleic acid
E. coli	*Escherichia coli*
emPAI	Exponentially modified protein abundance index
HCl	Hydrochloride
Hcp	Hemolysin co-regulated protein
HMM	Hidden Markov models
LB	Luria Bertani
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LGT	Lateral gene transfer
m/z	Mass-to-charge ratio
MS	Mass spectrometry
MSHA	Mannose sensitive haemagglutinin
NSAF	Normalized spectral abundance factor
OD	Optical density
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Propidium iodide
RAST	Rapid annotation subsystems technology

rpm	Revolutions per minute
S. algae	*Shewanella algae*
S. oneidensis	*Shewanella oneidensis*
S. putrefaciens	*Shewanella putrefaciens*
S. woodyi	*Shewanella woodyi*
SA1	Sydney strain of *Shewanella algae* SA1
SA2	Sydney strain of *Shewanella algae* SA2
SDS	Sodium dodecyl sulphate
SVM	Support Vector Machine
T6SS	Type VI secretion system
TBP	Tributylphosphine
TCBS	Thiosulfate citrate bile salts agar
V. cholerae	*Vibrio cholerae*
VgrG	Valine-glycine-repeat protein G

Abstract

The genus *Shewanella* comprises an extremely diverse group of facultative anaerobes that are widely distributed in freshwater and marine environments, including intertidal and benthic zones, their sediments and oil field wastes throughout the world [1, 2]. They are Gram-negative bacilli that are 1 - 2 μm in length and 0.4 - 0.7 μm in width which are motile via a single polar flagellum, exhibit un-paralleled respiratory diversity, and have robust sensing and regulatory systems which allow them to survive environments with low temperatures (less than 4°C), high salt concentrations and an extensive range of barometric pressures [3, 4]. These features lend themselves to phenotypic and physiological differences within the genus, but also have elicited interest in their use in biotechnology, including for bioremediation and microbial fuel cells [5, 6].

There are 63 species that comprise the *Shewanella* genus [7], and a handful of these are known to cause disease in humans and animals. The main species associated with human infection is *Shewanella algae* (*S. algae*) [1, 8], which naturally resides in aquatic environments and has been isolated from marine and freshwater sediments, oil fields, animals, marine life (including fish, sea lions, echinoderms, birds and poultry), and from human clinical material as the causative organism of diseases such as otitis media, cellulitis, septicemia and increasingly gastroenteritis [9-15]. To date, there have been limited studies investigating the mechanisms of pathogenicity and antibiotic resistance of *S. algae*.

The work presented in this dissertation has sought to address a number of gaps in knowledge regarding the pathogenesis of the emerging human pathogen *S. algae* using a systems biology approach. *S. algae* has the ability to cause mono-microbial infections in humans, ranging from infections of the skin and soft tissues, to blood borne and enteric infections. This thesis presents the first genome sequences of *S. algae* isolated from Sydney, Australia, and the first proteomic investigations which, combined, identify the presence and expression of potential virulence in this emerging human pathogen.

This dissertation has linked the *S. algae* genotype to the phenotype, giving a more holistic understand of the bacterium which is crucial to understanding any roles it has in pathogenesis. We identified a range of genes encoding putative virulence factors in *S. algae*, including toxins, haemolysins, adhesins, secretion systems, proteases and genes required for biofilm formation and motility/chemotaxis. Furthermore, the investigation into the expression of these proteins, via the differential growth media in the proteome and secretome, have highlighted that many of the genes encoding for these virulence factors require specific conditions for their expression.

Chapter One

An overview of the thesis

Chapter 1 – An overview of the thesis

1.1 Introduction

Infectious diseases are a major threat to public health, with it being estimated that deaths caused by multidrug resistant bacteria will overtake that of cancer by 2050 [16]. One quarter of deaths worldwide are the result of a pathology incurred by an infectious agent [17]. Bacteria cause disease by expressing distinct sets of virulence factors, which allow them to adhere, colonise, and invade target host cells, and evade the host immune system [18]. The rapid speed at which bacteria can evolve gives them an adaptive advantage, allowing them to acquire new virulence traits (often via horizontal gene transfer mechanisms), colonise new environmental and host niches, and withstand selection pressures imposed by antibiotics and various arms of the immune response.

How bacteria interact with one another and the environment they live in, as well as identifying their mode of virulence, is crucial to having a holistic understanding of bacterial pathogenesis. Therefore, taking a systems biology approach to understand bacterial pathogenesis is beneficial, as it aims to take an integrative, cyclical, and interdisciplinary approach combining different methods and techniques to investigate biological areas in order to gain a more holistic understanding of the problem area, with the notion that 'the sum is greater than the individual parts' (i.e. at a systems level, rather than an individual protein level) [19-22]. This systems biology approach has proved successful in aiding the understanding of bacterial pathogens and the host-pathogen interaction [23-25].

Whilst there are numerous successful methods to investigate aspects of the bacterial lifestyle, studying them under standard laboratory conditions can only give so much information. To date, no method realistically reflects the *in vivo* growth mode of bacteria and the challenges presented to them by their host. It is known that bacteria can have alternative lifestyles, such as living in different cellular morphologies like L-forms [26], viable but non-culturable states [27, 28], small colony variants [29, 30] or living in biofilms, which create a nutrient rich environment allowing for microorganisms to form layered multicellular communities [31]. Studying bacteria in a liquid culture under standard laboratory conditions does not reflect the challenges presented to bacteria in the environment or their host. Therefore, there is a need to develop novel strategies to study the bacterial lifestyle which are more reflective of real-life and infection situations.

Emerging bacterial infectious diseases are defined as being newly appeared in a population, or are infectious agents that have re-emerged and are increasing in incidence, geographic location or host range [32]. Many Gram-negative bacteria are known to be pathogenic to humans, animals, marine life and foods. Human pathogens of clinical importance include *Escherichia coli*, *Pseudomonas aeruginosa* and members of the *Klebsiella* and *Acinetobacter* species, however there are also emerging human pathogens being recognised, including *Shewanella algae* (*S. algae*). *S. algae* are Gram negative facultative anaerobes first isolated from red algae in 1990. Being a member of the *Shewanellacea* family, *S. algae* are known to have extremely diverse respiratory capabilities that allow them to proliferate in different environmental niches. Current interest in *S. algae* focuses on extorting this respiratory capability for use in

bioremediation and microbial fuel cells. However, *S. algae* are one of four species in the *Shewanellacea* genus, known to cause disease in humans and animals. They represent >80% of all infections cause by this genus [1] and are known to cause infection of the skin, soft tissue, gastrointestinal tract and blood [33]. In addition, there are now reports of multidrug resistant *S. algae* strains being isolated from patients [12, 34-36]. Evidence is lacking as to how *S. algae* causes disease in human hosts, with only one genomic study speculating on potential virulence factors and antibiotic resistant genes present in *S. algae MARS 14* [36]. Taken together, this suggests the need to further investigate the mechanisms of *S. algae* pathogenesis, particularly before it is adopted widely in the biotechnology industry.

1.2 Aims of the thesis

The aforementioned section outlines a number of key areas that require further investigation. From these areas, a set of aims was constructed to address within this dissertation. The overarching aim of this thesis is to investigate the potential virulence factors in *S. algae* (Figure 1-1). Specifically, these aims are:

- **Aim one**: Collation of case studies of infections caused by S. *algae* to determine the disease aetiology of this emerging pathogen.
- **Aim two:** Genomic analysis of *S. algae* SA1 and SA2 genomes, including genome sequencing, phylogeny and bioinformatic analysis to identify the genes involved in *S. algae* pathogenicity and antimicrobial resistance.

- **Aim three**: Investigate the expression of putative virulence factors in the *S. algae* differential proteome.
- **Aim four**: Investigate the expression of putative virulence factors in the *S. algae* differential secretome.

This thesis will present the first genome sequences of *S. algae* strains isolated from Sydney, Australia, and the first to perform proteomic studies investigating the differential expression of potential virulence factors; linking genotype to phenotype which is the core to understanding bacterial pathogenesis. The strains used in this thesis, *S. algae* strains SA1 and SA2, were isolated from the Georges River in Sydney as part of a project investigating environmental *Vibrio cholerae* (*V. cholerae*) in Sydney water. *S. algae* strains were co-isolated on thiosulfate-citrate-bile salts (TCBS), a *Vibrio* selective media containing bile salts, agar alongside *V. cholerae*. The ability of *S. algae* to grow on this media allowed us to speculate that it had the potential to grow in conditions such as the human gastrointestinal tract. To investigate the pathogenic mechanisms of *S. algae* SA1 and SA2, a systems biology approach was taken, combining whole genome sequencing with proteomic studies to investigate the presence and expression of putative virulence factors.

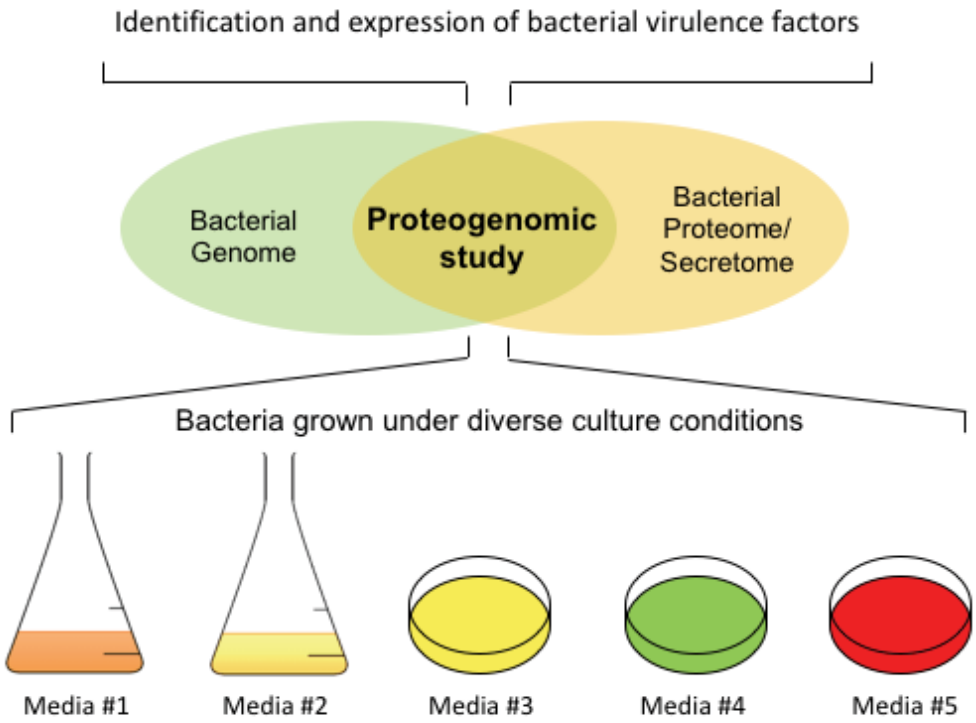

Figure 1-1 A diagram representing the overall aims of the project.

The *S. algae* genome was sequenced and potential virulence factors identified. The expression of these virulence factors was confirmed via proteomic studies using diverse, infection relevant culture media to mimic the *in vivo* growth conditions presented to the bacteria by the host.

1.3 Dissertation organisation

Following this chapter, this dissertation is comprised of six further chapters:

- Chapter 2: *Shewanella algae* – an emerging human pathogen

  This chapter aims to provide background information detailing what is currently known about *S. algae* and its role as an emerging human pathogen, which serves to answer the first aim of this dissertation. This chapter has been formatted for publication in Clinical Reviews in Microbiology.

- Chapter 3: The draft genome sequence of *Shewanella algae* SA2

  This chapter provides the draft genome sequence for *S. algae* SA2, and identifies genes involved in pathogenesis and the resistance to antimicrobials and heavy metals. This chapter aims to answer aim two of this dissertation and is in Gut Pathogens.

- Chapter 4: Differential expression of virulence factors in *Shewanella algae* SA1

  This chapter provides the draft genome sequence of *S. algae* SA1, identify genes involved in pathogenesis and the resistance to antimicrobials and heavy metals, and investigates the expression of these genes under different growth conditions. This chapter serves to answer aims two and three of this dissertation and has been formatted for publication in Emerging Infectious Diseases.

- Chapter 5: Analysis of the *Shewanella algae* SA1 secretome identifies novel virulence factors

  This chapter investigates the expression of putative virulence factors in the *S. algae* SA1 secretome under different growth conditions at different time points. This aims to address aim four of the dissertation and has been formatted for publication in the Journal of Proteome Research.

- Chapter 6: Identification of a novel *qnrA* allele, *qnrA8*, in environmental *Shewanella algae*.

  This chapter briefly discusses the identification of a novel *qnrA* allele found in *S. algae* SA1 and SA2. This chapter was recently accepted as a Correspondence in the Journal of Antimicrobial Chemotherapy.

- Chapter 7: General discussion and concluding remarks

  This final chapter to the thesis contains the general discussion and recommendations for future directions.

- Supplementary material

  All supplementary materials for this thesis are provided on a CD attached to the back cover of this thesis, as many of the supplementary materials are too large for printing in the dissertation.

Chapter Two

Shewanella algae – an emerging human pathogen

Chapter 2 - *Shewanella algae* - an emerging human pathogen

2.1 Compound abstract

Rationale: To date, the breadth of diseases caused by the emerging human pathogen *S. algae* and the mechanisms of pathogenicity and resistance to antibiotics remain largely unknown. This chapter aims to shed light on the diseases caused and identify any known mechanisms of pathogenesis and resistance to antibiotics.

Methods: We searched and collated all documented case studies to determine the aetiology of *S. algae* diseases. We specifically looked for the location, age, and gender of the patient, any co-morbidities or co-infecting organisms, the treatment regime, outcome, resistance to antibiotics, and potential risk factors associated with the disease state.

Results: We have summarised all published case studies on *S. algae* associated with human infection, highlighting that *S. algae* is capable of causing mono-microbial disease in humans. *S. algae* is increasingly resistant to multiple antibiotics and has been shown to accept and transfer antimicrobial resistance genes by lateral gene transfer. With the reported incidence of *S. algae* infections on the rise and the increasing levels of resistance it displays to antimicrobials, the pathogenic potential of this organism is underreported and should be thoroughly investigated before it is adopted widely in the biotechnology industry.

2.2 The genus *Shewanella*

The bacterial genus *Shewanella* is comprised of a large group of facultative anaerobes that are widely distributed in marine and freshwater environments. *Shewanella* species are metabolically diverse and have been isolated from extreme environments where they experience high pressure, temperature, pH and salinity [3, 4, 37]. These organisms have been investigated for their role in bioremediation [6, 38] and as candidate bacteria for research on microbial fuel cells [39-41]. However, several species within the *Shewanella* genus are also known to be pathogenic to humans, animals and marine life. The incidence of their isolation as the causative agents of disease is rising, especially for *Shewanella algae* (*S. algae*), and isolated strains increasingly carry novel antibiotic resistance genes that can find their way into other members of the Enterobactericeae [42, 43]. Here we review what is known about infections caused by *S. algae* and highlight its role as an emerging human pathogen.

2.2.1 Background and taxonomy

Members of the family Shewanellacea belong to the Gammaproteobacteria, a class of proteobacteria comprised of facultative anaerobic and fermentative Gram-negative bacteria [44]. Other members of the Gammaproteobacteria include medically and environmentally significant bacterial families such as Vibrionales, Pseudamonadales and Enterobacteriales [45], which include *V. cholerae*, *Pseudomonas aeruginosa* and *Escherichia coli*. These organisms are adept at acquiring mobile DNA that impacts virulence and antibiotic resistance [46-48]. As a genus, *Shewanella* was first isolated from butter in 1931 as an agent of food spoilage and was originally identified as *Achromobacter putrefaciens* (*Ac. putrefaciens*) [49]. From 1930 to 1980 the taxonomic

status of *Shewanella* remained unclear (Figure 2-1). Biochemical and morphological analysis of *Ac. putrefaciens* led to the organism being renamed as *Pseudomonas putrefaciens* (*P. putrefaciens*) [50] in 1941 [51]. In 1977, *P. putrefaciens* was reclassified as *Alteromonas putrefaciens* (*Al. putrefaciens*) based on the deoxyribonucleic acid guanine + cytosine (mol% G+C) content and the ability of the species to reduce trimethylamine-N-oxide (TMAO) and produce deoxyribonucleases (DNAses) [52, 53]. In 1985 a new genus was described (*Shewanella*) on the basis of 5S rRNA gene sequences [54], and *Al. putrefaciens* was redescribed as *Shewanella putrefaciens* (*S. putrefaciens*) in honour of James Shewan and his work as a marine microbiologist.

The species *S. algae* was first isolated in 1990 as a new strain of tetrodotoxin-producing bacteria from the surface of *Jania* sp. red algae [55]. 16S rDNA sequence analysis showed that it was a member of the genus *Shewanella*. As it did not cluster closely with any other species in the *Shewanella* genus it was designated as a new species, *Shewanella alga* (*S. alga*). Further species characterisation found that *S. alga* had: a 52-54 mol% G+C content; grew on *Salmonella-Shigella* agar and in 6% NaCl; grew at 42°C but not at 4°C; and haemolysed blood agar (Table 2-1; Nozue, 1992 #158). Interestingly, clinical isolates previously identified as *S. putrefaciens* were found to be homologous with *S. alga* based on these characteristics [56]. This led to a clear distinction between *S. putrefaciens* and *S. alga*. In 1997, the genitive Latin species name meaning ‘of an alga’ was amended leading to the current species designation of *S. algae* [57].

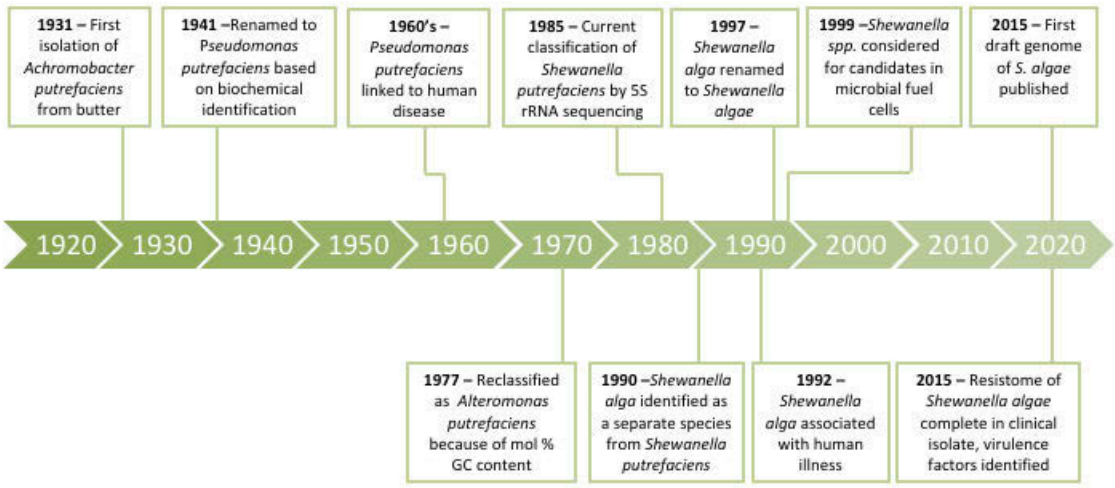

Figure 2-1 The history of *Shewanella algae*.

Currently, there are 63 described species within the genus *Shewanella* [7, 58], 49 of which have been discovered since the turn of the century. *Shewanella* species are extremely diverse at the genomic level. One study of ten *Shewanella* genomes belonging to six species showed they shared only 54% sequence identity [59] in the core genome. For many years, the 'gold standard' method for describing bacterial phylogeny was 16S rDNA gene sequencing [60] but low substitution values between closely related species in the *Shewanella* genus [37], and mounting evidence that the 16S rDNA gene within the *Shewanella* species is undergoing concerted evolution, suggests that this target is unsuitable for determining phylogeny [61]. Housekeeping genes, such as *gyrB* and *ropB*, have also been used as a phylogenetic target to distinguish between closely related species within the *Shewanella* genus [62] and are advised to be more accurate than 16S rDNA.

Previous phylogenetic studies based on 16S rDNA and *gyrB* sequences found that *Shewanella* species fell into two major branches: (i) the halotolerant species including *Shewanella baltica*, *S. putrefaciens* and *Shewanella oneidenesis* and (ii) the halophilic species including *Shewanella woodyi* and *Shewanella benthica* [51, 62, 63]. Notably, *S. algae* and *Shewanella amazonensis* formed their own branches, either clustering together [51] or separately [62] depending on the analysis performed (16S rDNA or *gyrB*). A phylogenetic tree of all *Shewanella* genomes constructed using PhyloSift [64] is depicted in Figure 2-2. PhyloSift, concatenates the alignments of 37 universally-conserved marker genes [64] and has been used widely to determine bacterial relatedness [65-67]. This analysis agrees with previous literature as it contains a halophilic branch, a halotolerant branch and a branch with *S. amazonensis* clustering separately from *S. algae* and *Shewanella haliotis*.

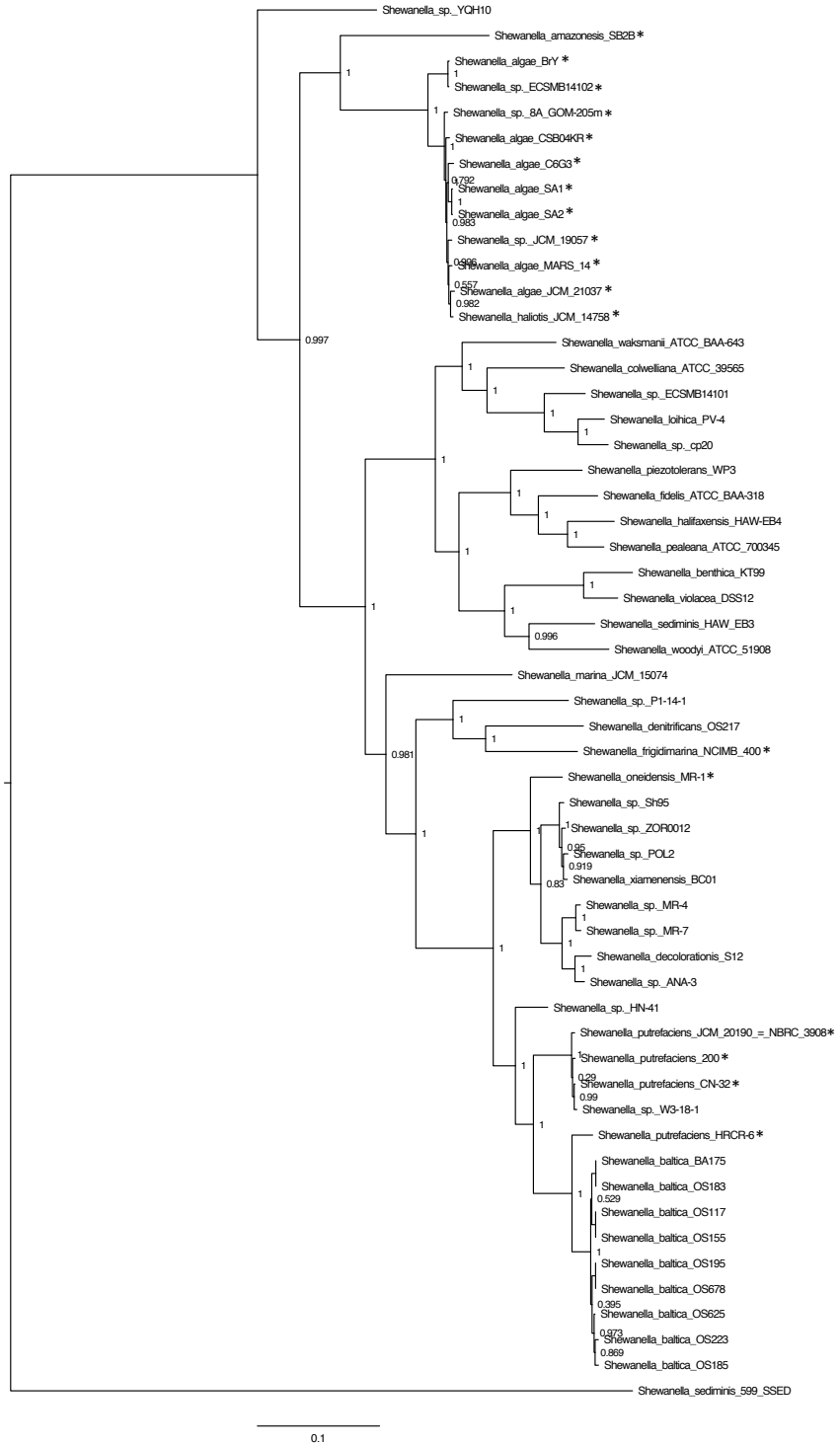

Figure 2-2 Phylogenetic tree of complete *Shewanella* spp. genomes and draft *S. algae* genomes.

The phylogenetic tree was constructed using PhyloSift [64] and all genomes were downloaded from the NCBI FTP GenBank server (ftp://ftp.ncbi.nlm.nih.gov/GenBank). The numbers located on each node are confidence values between 0 and 1, with values near 1 indicating that the branch at that node exists with high probability. The x-axis represents the substitutions per site and the numbers at each node give the clade confidence value. The phylogeny was inferred using FastTree [68] and the tree was visualized using FigTree version 1.4.0 (http://tree.bio.ed.ac.uk/software/ figtree/). * indicates a halotolerance.

Based on phylogenetic analysis (Figure 2-2), *S. algae* strains are most closely related to *S. haliotis* JCM 1475B (a draft genome) which was isolated from the gut microflora of abalone collected from the South Sea, Republic of Korea [69] and *S. amazonensis* S2B2, an environmental metal-reducing, facultative anaerobe isolated from Amazonian shelf muds [70] (a complete genome sequence). Of the five *S. algae* genomes available in the NCBI FTP GenBank database, four are environmental isolates (strains C6G3, CSZ04KR, BrY, and JCM 21037) and one is a clinical isolate (MARS 14, a multidrug resistant strain isolated from a patient suffering pneumoniae after plunging into the Mediterranean Sea). It should be noted that this isolate was determined to be *S. algae* via multiple methods, including matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) and sequence analysis of the *rpoB* gene, rather than 16S rDNA which can give misleading results.

2.2.3 Biological characteristics

Species in the genus *Shewanella* are widely distributed in freshwater and marine environments, including intertidal and benthic zones, sediments, terrestrial environments and oil-field wastelands throughout the world [1, 2]. Their physiological diversity means they are found in numerous locations throughout the world where the temperature, salinity and pH within these environments can fluctuate quite dramatically depending on the season. Examples of extreme locations from which they have been isolated include Antarctic coastal areas [71], the Amazonian shelf muds [70] and the orbital cavity of the mummified remains of St. Marcian [72]. *Shewanella* species are known to exist as free-living bacteria in marine environments and their sediments [73], in commensal relationships with marine life [2], in mutualistic

relationships with some organisms such as the *Loligo paelei* squid [74, 75] and in parasitic relationships with human and marine life, where they are known to cause disease [33].

Shewanella species also have robust sensory and regulatory systems that allow them to live at low temperatures (less than 4°C), high salt concentrations and at a wide range of barometric pressures [3, 4]. This is due to the ability of *Shewanella* species to metabolise an array of terminal electron acceptors including heavy metals and radio nucleotides (such as iron, magnesium and chromium) and even environmental pollutants such as arsenic and uranium [5]. This makes *Shewanella* species the most respiratory-diverse organisms known to date [37]. Furthermore, these metabolic attributes have led to increased interest in using *Shewanella* species for bioremediation and sustainable energy production. These characteristics may be the key to the ecological success of *Shewanella* species allowing them to exploit unique niches in extreme environments that seasonally fluctuate in temperature, pH and salinity and are inhospitable to other organisms. This may also explain the large genotypic and phenotypic diversity present within the genus.

Shewanella algae has been isolated from diverse environments including: sediments from the Great Bay, New Hampshire [76]; an oil field in Canada [77]; oil brine [78]; seawater [79]; marsh sediments in the USA [80]; muddy sediments from the Arcachon Bay [81]; and sea sediment from eastern China [82]. Other studies have isolated *S. algae* from a variety of organisms including: fish [83]; echinoderms [84]; mammals, such as sea lions [73]; wild birds [85, 86]; and poultry [78]. In the literature however, *S.*

algae has most commonly been isolated from human clinical material as an infective disease-causing organism.

The physiological characteristics of *Shewanella* species are as diverse as the niches from which they have been isolated. *Shewanella* species are Gram-negative rod-shaped bacteria that are 2-3µm in length and 0.4-0.7µm in width and are motile via a single polar flagellum (Figure 2-3). Common characteristics of *Shewanella* species include: growth at 25-35°C; the ability to grow on MacConkey agar; positive reactions for DNAse; protease, gelatinase, lipase and TMAO reduction; ornithine decarboxylase and hydrogen sulphide production; as well as negative reactions for indole, lysine decarboxylase and arginine dihydrolase [8, 87-89]. However, not all species are defined by these criteria [2].

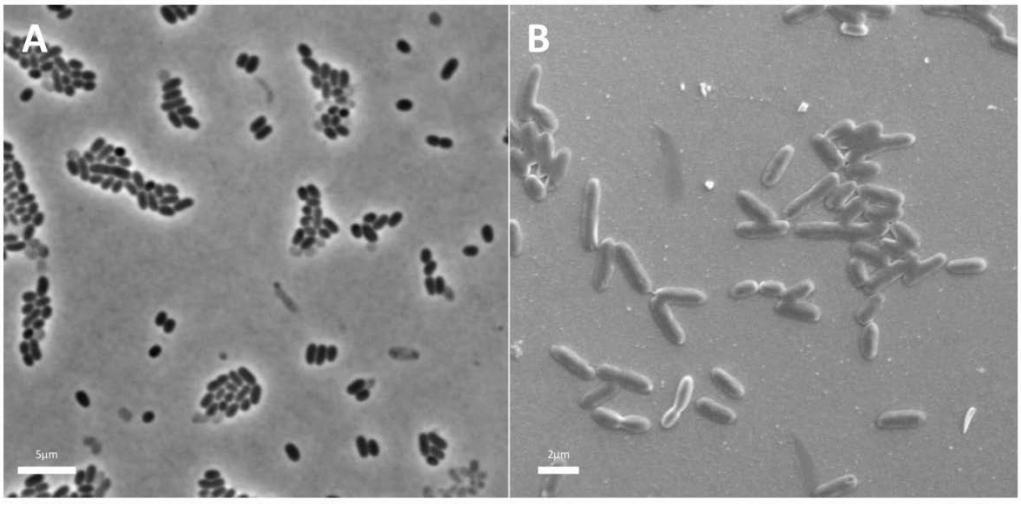

Figure 2-3 (A) Phase contrast images of *Shewanella algae* isolate SA1. (B) Scanning electron microscopy images of *S. algae*.

Despite *Shewanella* species being able to grow well on conventional media (e.g. Luria Bertani (LB) agar, marine agar and MacConkey agar) and for some species also on blood agar, *V. cholerae* selective agar and *Salmonella-Shigella* agar, in 2015 a selective and discriminatory media has been designed for the specific isolation of *Shewanella* species [90]. This media, *Shewanella* IRHLS agar, contains irgazan DP-300 and rifampicin which make it selective for growth of *Shewanella*, *Stenotrophomonas*, *Aeromonas*, *Serratia* and *Citrobacter* species. *Shewanella* IRHLS agar supports hydrogen sulphide and lipase activity and sorbitol fermentation allowing *Shewanella* species to be differentiated from species of *Stenotrophomonas*, *Aeromonas*, *Serratia* and *Citrobacter*. *Shewanella* IRHLS agar also prevents growth of species of *Salmonella*, *Escherichia*, *Enterobacter*, *Klebsiella*, *Shigella*, *Staphylococcus* and *Bacillus*.

Clinically, the ability to differentiate between different species of *Shewanella* using standard manual and automated identification systems such as the Vitek, MALDI-TOF and Biomeriux API 20NE/20E has been unsuccessful [1, 91] and has led to misidentification of *Shewanella* as other *Gammaproteobacteria*, such as *Pseudomonas,* and more recently the identification of different *Shewanella* species [1]. As such, automated identification systems are unreliable when attempting to identify the precise disease-causing agent in infections caused by a *Shewanella* species. *Shewanella putrefaciens* and *S. algae*, two of the most pathogenic *Shewanella* species, share similar phenotypic and biochemical characteristics and are often misidentified [56, 92]. The distinguishing genetic, biochemical and metabolic features between these two species have been clearly defined (Table 2-1; Nozue, 1992 #158). Examples of these differences include the ability of *S. algae* to grow on Salm*onella-Shigella* media, grow

in media containing 6% NaCl and at 42°C, form zones of haemolysis on sheep and bovine blood agar, mucoid colony growth, a mol% G+C content of 52-54%, while *S. putrefaciens* does not possess these characteristics [1, 8, 11, 35, 55, 56]. Furthermore, *S. algae* displays resistance to penicillin, ampicillin and tetracycline, while *S. putrefaciens* is susceptible [78]. Many of these features form the basis for distinguishing *S. putrefaciens* from *S. algae* by CDC biogrouping, Owens groupings and Gilardi biovars (Table 2-1). CDC biogroups are distinguished from one another by biochemical reaction patterns [93]. *S. algae* belongs in CDC biogroup Ib-2, whereas *S. putrefaciens* belongs in CDC biogroup Ib-1. Owens groups are differentiated by DNA reassociation kinetics that estimate genetic relatedness. Multiple studies have used the genome size, base composition and sequence similarity in conjunction with biochemical characteristics to group *S. algae* into Owens group IV and *S. putrefaciens* into Owens group I [94, 95]. Lastly, Gilardi biovars characterise bacterial species by differences in their physiological and biochemical properties [88]. Using these properties *S. algae* was allocated to Gilardi biovar group 2 and *S. putrefaciens* was allocated to Gilardi biovar group 3 [77]. It is essential that *Shewanella* species, but particularly *S. algae* and *S. putrefaciens*, are added to all the manual and automated clinical bacterial identification databases so that causative agents can be correctly identified to species level. This will also lead to a better understanding of *Shewanella* species and their role in the environment and as emerging agents of disease.

2.3 Role of *Shewanella algae* as an emerging human pathogen

Shewanella species are described as being rare, opportunistic or secondary community-acquired human pathogens commonly associated with skin and soft tissue infections. *Shewanella* species have not been intensively studied as pathogens and most descriptions of disease in the literature appear as case studies. The majority of case studies appeared after 2000 as a result of improved clinical diagnostics technology that allowed the genus to be identified [37]. Whilst *S. algae* and *S. putrefaciens* account for >85% of *Shewanella* infections recorded, *S. haliotis* [96-99] and *Shewanella xiamenesis* [100] have also been implicated in human disease. Furthermore, *S. oneidenesis* has been isolated from clinical material but its role in pathogenesis remains unknown [62].

Table 2-1 Physical and biochemical characteristics of *Shewanella algae* and *Shewanella putrefaciens.*

Property	*S. algae*	*S. putrefaciens*
Colony consistency	Mucoid	Non-mucoid
Colony size	2-4mm	2-4mm
Gram stain	Negative	Negative
Cell shape	Bacilli	Bacilli
Cell size	0.4µm by 2-4µm	0.4µm by 2-4µm
G + C content (mol %)*	52-54	45-48
Spore production	–	–
Motility	+	+
Growth at:		
- 4°C*	–	+
- 42°C*	+	–
Growth in 6-6.5% NaCl*	+	–
Growth on *Salmonella-Shigella* agar*	+	–
Haemolysis (ovine blood)*	+	–
Haemolysis (bovine blood)*	+	–
Nitrite reduction	+	–
Acid production from:	–	+
- D-ribose	+	–
- D-glucose	(+)	+
- Fructose	(+)	+
- Maltose*	–	+
- Sucrose	–	+
- L-arabinose*	–	+/V
Hydrogen sulphide production	+	+
Catalase reaction	+	+
Citrate reaction	–	–
Oxidase reaction	+	+
Indole	–	–
Hydrolysis of:		
- Urea	–	–
- Lechithin	+	–
- Gelatin		V
Caprate	+	–
Utilization of:		
- N-acetylgalactosamine	+	–
- α-hydroxybutyrate	+	–
Classification		
Gilardi's Biovar **[88]**	Biovar 2	Biovar 1
CDC biogroup [87]	Ib-2	Ib-1
Owen genomic group [94]	IV	I
Antibiotic resistance		
Penicillin	R	S
Ampicillin	R	S
Tetracycline	R	S

+ represents a positive reaction, - represents the absence of a reaction, V represents a varied reaction, () represents a delayed/weak positive reaction and an * represents a distinguishing feature between *Shewanella algae* and *S. putrefaciens*. Table collated from [1, 8, 11, 35, 51, 56, 78, 88, 89, 95].

The first case of *Shewanella* infection in humans was reported by King in 1967 [101] when the National Communicable Disease Centre linked an unknown oxidase-positive, H_2S producing, CD Group I-b strain (which conforms with the phenotypic classification of *P. putrefaciens*) as the causative agent of 22 different infections. However, it was not until 1970 that the pathogen was recognized as *P. putrefaciens* (now known to be *S. putrefaciens*) [102]. Soon after this, case studies were presented [103, 104] describing infected leg ulcers and septicaemia, that were both caused by *P. putrefaciens* (*S. putrefaciens*). These reports marked the first recognition that some *Shewanella* species were pathogenic for humans. *Shewanella algae* was first reported as a human pathogen in 1996 when it was isolated from two patients with bacteraemia linked to lower leg ulcers [35].

The age, sex, location, comorbidities, type of infection, co-infecting organisms, treatment, outcome and risk factors associated with infections caused by *S. algae* have been documented in 233 published case studies (Table 2-2). These case studies were identified via the PubMed and Google Scholar websites using the following key search term *Shewanella algae* or *Shewanella alga*. Notably, a large percentage of infections caused by *S. putrefaciens* were originally incorrectly assigned and were later recognised to be infections caused by *S. algae*. These case studies only describe infections caused by *S. algae* (and not *S. putrefaciens*) that have already been described in other reviews [33, 105]. One study reported that 83% of clinical isolates identified originally as *S. putrefaciens* were actually *S. algae* [56].

Table 2-2 Summary of case studies of *Shewanella algae* infections in humans.

*exposure to seawater/seafood 48 hours prior to infection

Abbreviations: qid = 4 times a day; PO = medication by mouth; q4h = every 4 hours; q8h = every 8 hours; q12h = every 12 hours.

Age (years)	Sex (M/F)	Location	Co-morbidities	Type of infection	Co-infecting organisms	Treatment	Outcome	Exposure to sea water/seafood*	Year	Reference
Unknown	35 cases	Japan	Unknown	Mixed infections	Unknown	Unknown	Unknown	Unknown	1992	[56]
69	M	Denmark	Lower leg ulcer, chronic congestive heart failure	Bacteraemia	Monomicrobial	IV penicillin, IV gentamicin, IV ampicillin and IV cefuroxime for 4 weeks followed by oral cefuroxime for 2 weeks	Cured	Yes	1996	[35]
80	F	Denmark	Lower leg ulcer, diabetes, rheumatoid arthritis, immunocompromised	Bacteraemia	Monomicrobial	IV penicillin followed by IV ampicillin and gentamicin for 7 days	Cured	Yes	1996	[35]
2-69	67 cases	Denmark	Previous ear disease, previous tympanic perforation, previous intubation	Acute otitis media, chronic otitis media, external otitis, non-specific aural discharge	Some pure (33), some co-isolated with *Staphylococcus aureus, Vibrio alginolyticus, Acinetobacter* spp., *Pseudomonas* spp., *Streptococcus Group A, Enterococcus faecialis,* corynebacteria, coagulase negative *Staphylococcus*	Topical antibiotics (tetracycline, polymixin B), systemic antibiotics and/or surgery	Cured	47/67 seawater	1997	[79]
64	F	Japan	Haemodialysis	Septicaemia		Cefazolin (2g/day), gentamicin (40mg/day) and levofloxacin (100mg/day) for 7 days followed by minomycin (100mg/day), cefpirome (1g/day) and levofloxacin (100mg/day)	Cured	No	1999	[106]

71	M	France	Appendicular peritonisis, hypertension, lower leg ulcer	Ruptured aortic aneurysm	Monomicrobial	Piperacillin	Cured	No	2001	[107]
66	M	Madrid, Spain	Multiple myeloma	Bacteraemia, cellulitis	Monomicrobial	Ceftazidime, amikacin	Cured	No	2002	[108]
58	M	Texas, USA	End stage renal disease, hypertensive nephrosclerosis, history of thrombosis, artherosclerosis	Necrotic ulcers and bellous lesions	Monomicrobial	Cefepime, clindamycin, amputation	Cured, amputation	Yes	2003	[109]
66	M	France	Immunocompromised	Cellulitis	Monomicrobial	IV Cefotaxime, ciprofloxacin and gentamicin followed by ciprofloxacin and erythromycin	Cured	Yes	2004	[110]
45	F	Turkey	Broken right tibia (open)	Osteomyelitis	Monomicrobial	Oral ciprofloxacin 1500mg/day, ceftazidime 3000mg/day and tobramycin 200mg/day for 2 weeks followed by oral ciprofloxacin 1500mg/day for 8 weeks	Cured	Yes	2005	[111]
14	M	Taiwan	None	Tonsillitis	Monomicrobial	Amoxacillin IV followed by oral cefuroxamine for 7 days	Cured	No	2006	[112]
65	M	Korea	Papillary mucinous tumour	Abdominal infection, spinal epidural abscess	Monomicrobial	Imipenem IV 500mg q6h followed by cefepime IV 2g q12hand meropenem IV 1000mg q8h	Cured	No	2006	[113]
41	M	India	None	Vomiting, abdominal pain	Monomicrobial	Cefoperazone-sulbactam IV 2g q12h	Cured	No	2007	[9]
75	M	India	None	Pneumonia	Monomicrobial	Ceftriaxone IV 2g once daily	Cured	No	2007	[9]
42	M	Taiwan	Liver cirrhosis, hepatitis B	Soft tissue infection	Monomicrobial	Ceftriaxone IV 2g/day and doxycycline orally 200mg/day followed by ciprofloxacin IV 1200mg/day	Cured	Yes	2008	[105]

Unknown	25 cases	Korea	Outbreak caused by a contaminated measuring cup reused in a hospital ward, hepatobiliary disease (58% cases)	Soft tissue infection, sepsis	Monomicrobial	Unknown	3 deaths	No	2008	[114]
76	F	Taiwan	Gastric cancer, gallbladder cancer	Pericarditis	Monomicrobial	Flomoxef IV 1g q8h followed by piperacillin-tazobactam 4.5g q6h for 10 days and then imipenem 500mg q6h	Cured	No	2008	[115]
58	M	Korea	Alcoholic liver disease	Necrotizing fasciitis	Monomicrobial	Ceftriaxone IV 2000mg/day and ciprfofloxacin IV 800mg/day	Death (gastrointestinal bleeding)	Seafood	2009	[116]
77	M	Costa Rica	None	Bloody diarrhoea (lasting 8 months)	Monomicrobial	Cefalexin 500mg qid for 7 days	Cured	Yes	2009	[10]
43	M	India	Ulcer left foot	Ulcer	Coagulase negative *Staphylococci*	Unknown	Cured	Unknown	2010	[11]
62	M	India	Diabetes mellitus, cellulitis (left foot), ulcers, peripheral neuropathy	Ulcer	Unknown	Unknown	Cured	Unknown	2010	[11]
34	M	India	None	Gastroenteritis	*Escherichia coli*	Ciprofloxacin, metronidazole, amlodipine	Cured	Unknown	2010	[11]
58	M	France	Back pain, obesity, hypertension, gout	Cellulitis left leg, septic spondylitis	Monomicrobial	Pristinamycin for 2 weeks followed by IV ceftriaxone and amikacinthen for 2 weeks and then oral ciprofloxacin for 12 weeks	Cured	Yes	2010	[92]
10	M	Croatia	Ventriculoperitoneal shunt (VPS)	Infected VPS	Monomicrobial	Amoxicillin and clavulanic acid, cefuroxamine, meropenem, vancomycin	Cured	Yes	2010	[117]
44	M	China	Diabetes mellitus, hypertension, Klinefelter syndrome	Infected foot ulcer	*Escherichia coli, Staphylococcus aureus*	Unknown	Cured	No	2010	[118]

48	F	China	None	Finger infection	*Klebsiella* spp., *Enterococcus* spp.	Unknown	Cured	Yes	2010	[118]
73	M	China	Lung carcinoma, asthma, colonic polyps	Wound infection	Monomicrobial	Unknown	Cured	No	2010	[118]
30	F	China	Aplastic anaemia	Neutropenic fever	Monomicrobial	Unknown	Cured	No	2010	[118]
64	M	China	Hepatitis C, cirrhosis, hepatocellular carcinoma, diverticulosis	Cholangitis	Monomicrobial	Unknown	Cured	No	2010	[118]
35	M	India	Non-healing ulcer	Ulcer	Monomicrobial	IV ampicillin 500mg qid and IV gentamicin 80mg twice daily followed by oral ampicillin 500mg 3 times daily for 10 days	Cured	No	2011	[119]
70	F	India	None	Bloody diarrhoea	Unknown	Ciprofloxacin oral 500mg q8h	Cured	Seafood	2011	[12]
20	M	India	None	Bloody diarrhoea	*Escherichia coli*	Ciprofloxacin oral 500mg q8h	Cured	Seafood	2011	[12]
71	M	India	Diabetes (type 2), hypertension	Soft tissue infection	Monomicrobial	Cefoperazone-sulbactam followed by colistin 1, vancomycin and amphotericin B	Death (cardiac arrest)	No	2012	[120]
<1	M	Puerto Rico	Preterm birth	Bacteraemia	Monomicrobial	Ampicillin, gentamycin, amikacin, imipenem	Cured	Yes	2012	[121]
76	F	Martinique, France	Heart failure	Cellulitis	Monomicrobial	Unknown	Cured	No	2013	[122]
79	M	Martinique, France	None	Cellulitis	Monomicrobial	Unknown	Cured	Yes	2013	[122]
63	M	Martinique, France	Chronic respiratory failure	Pneumonia	Monomicrobial	Unknown	Cured	No	2013	[122]

52	F	Germany	Autoimmune vasculitis, sensomotoric polyneuropathy, myasthenia gravis	Ulcers and haemorrhagic bullae	Monomicrobial	Piperacillin 0.5 g tid, ciprofloxacin 500mg bid for 20 days	Cured	No	2013	[123]
55	M	Taiwan	Hepatitis B	Cellulitis	Monomicrobial	Amoxicillin-clavulanic acid	Cured	Yes	2013	[99]
71	F	Taiwan	Lung cancer	Bacteraemia	Monomicrobial	Amoxicillin-clavulanic acid	Death	Yes	2013	[99]
68	M	Taiwan	Gall stones	Cholecystitis	Monomicrobial	Ampicillin	Cured	No	2013	[99]
Unknown	38 cases	China	Unknown	Food poisoning (symptoms including abdominal pain (n=35); diarrhoea (n= 35); vomiting (n = 24); nausea (n = 16); poor peripheral circulation and dizziness (n =20))	Monomicrobial	Unknown	Unknown	No	2013	[124]
24	M	Iran	None	Wound infection	Monomicrobial	Cloxacillin 500mg q6h for 7 days, cefixime 400mg q12h for 5 days and ciprofloxacin 500mg q12h for 7 days	Cured	Yes	2014	[125]
72	M	New York, USA	Ulcer right leg, hypertension, atrial fibrillation, chronic venous stasis, obesity	Necrotizing fasciitis	Monomicrobial	Piperacillin-tazobactam, wound dressings with sodium hypochlorite/boric acid	Cured	Yes	2014	[126]

25	M	New Haven, USA	Bullet wound in chest	Bacteraemia, empyema	Monomicrobial (in blood), sputum *Staphylococcus aureus* and *Hemophulius influenza*; *Vibrio alginolyticus* and *Vibrio parahaemolyticus* (corneal ulcer)	Gatifloxacin ophthalmic drops, vancomycin, trimethoprim/sulfamethoxazole, ceftazidime, oxacillin, piperacillin/tazobactam, levofloxacin, metronidazole	Cured	Yes	2014	[127]
57	M	Seoul, Korea	Alcoholic liver cirrhosis, diabetes	Peritonitis	*Streptococcus mitis, Escherichia coli*	Cefotaxime 6g/day and metronidazole 1500mg/day followed by gentamycin 160mg/day, amikacin 500mg/day, piperacillin/tazobactam 12/1.5g/day	Death (hepatic failure)	No	2014	[128]
62	M	Singapore	Cholecystectomy	Bacteraemia and cholangitis	*Escherichia coli,* alpha haemolytic *Streptococcus*	Ceftriaxone IV 2g/day for 2 weeks followed by metronidazole IV 500mg q8h	Cured	No	2014	[129]
27	F	Taiwan	Cobra bite	Wound infection	Monomicrobial	Ampicillin/sulbactam	Cured	No	2014	[130]
74	M	India	Chronic osteomyelitis, squamous cell carcinoma	Osteomyelitis	Monomicrobial	Amputation	Cured	No	2014	[131]
43	Male	**Denmark**	Haglund's heel and rupture of the Achilles tendon	Cellulitis	Unknown	Unknown	Cured	Unknown	2014	[132]
36	F	Thailand	Open lower limb fracture	Cellulitis	*Vagococcus fluvialis*	Azithromycin, ciprofloxacin	Cured	Unknown	2014	[133]
45	M	Karnataka, India	None	Acute gastroenteritis (bloody diarrhoea)	Monomicrobial	Oral ciprofloxacin 500mg q8h	Cured	Unknown	2015	[13]
67	M	Kochi, India	Hypertension, peripheral vascular occlusive disease	Superinfection of chronic ulcer	*Pseudomonas aeruginosa, MRSA*	Linezoid, gentamycin	Cured	No	2015	[34]
62	M	Kochi, India	Diabetes mellitus, hypertension, dyslipidemia	Gangrene and cellulitis of the left toes	*Proteus vulgaris, Escherichia coli*	Amputation	Death (cardiac arrest)	Yes	2015	[34]

63	M	Kochi, India	Diabetes mellitus, hypertension, dyslipidemia, chronic renal failure	Superinfection of ulcer on toe	*MRSA, Pseudomonas aeruginosa*	Cefepime 2g IV q12h for 10 days	Cured	Yes	2015	[34]
50	M	Kochi, India	Diabetes mellitus, hypertension, dyslipidemia	Pyoderma gangrenosum	*Escherichia coli, Pseudomonas aeruginosa*	Meropenem 1gm IV q8h for 14 days	Cured	Yes	2015	[34]
62	F	Kochi, India	Burns to hand, diabetes mellitus	Blisters/cellulitis of hand	*Escherichia coli*	Piperacillin/tavobactam 4.5g IV q8hr for 10 days	Cured	No	2015	[34]
64	M	Kochi, India	Hypertension	Superinfection of ulcer	*Escherichia coli*	Meropenem 500mg IV q8hr and clindamycin 600mg q8hr for 10 days	Died (sepsis)	No	2015	[34]
57	F	Kochi, India	Hypertension, peripheral vascular occlusive disease	Superinfection of ulcer	*Proteus vulgaris, Proteus mirabilis*	Moxifloxacin 400mg PO q24h and clindamycin 600mg q8h for 14 days, amputation	Cured amputation	No	2015	[34]
62	F	Kochi, India	Hypertension, diabetes mellitus	Wound infection	Monomicrobial	Cefepime 2g IV q12h for 10 days	Cured	No	2015	[34]
67	M	Kochi, India	Hypertension, peripheral vascular occlusive disease	Superinfection of ulcer	*Escherichia coli, Proteus mirabilis*	Ampicillin-cloxacillin 500mg q8h PO for 5 days	Death (sepsis)	Yes	2015	[34]
28	M	Kochi, India	None	Superinfection of ulcer	Monomicrobial	Ampicillin-cloxacillin 500mg q8h PO for 5 days	Cured	No	2015	[34]
52	M	Kochi, India	None	Superinfection of ulcer	Unknown	Levofloxicillin 750mg q24h PO for 14 days	Cured	Yes	2015	[34]
57	M	Kochi, India	Diabetes mellitus	Cellulitis	*Streptococcus spp. Klebsiella pneumoniae*	Ampicillin-cloxacillin 625mg q8h PO for 10 days	Cured	No	2015	[34]
<1	M	India	Newborn	Sepsis	Monomicrobial	IV ampicillin and gentamicin	Cured	No	2015	[134]
39	M	Marseille, France	None	Pneumonia	Monomicrobial	Unknown	Cured	Yes	2015	[36]
70	M	India	Diabetic, renal stones (3 years previous)	Urinary tract infection	Monomicrobial	Norfloxacin 400mg twice daily	Cured	No	2015	[135]
65	M	Toronto, Canada	Type 2 diabetes, chronic foot ulceration, hypertension, chronic kidney disease	Foot ulcer	Monomicrobial	Ceftriaxone IV 1g daily for 3 days followed by ciprofloxacin 250g q12h for 7 days	Cured	Seawater	2016	[136]

39	M	Texas, USA	None	Infection of finger (from fish hook injury)	*Proteus vulgaris*	IV doxycycline and vancomycin followed by oral levofloxacin for 14 days	Cured	Seawater	2016	[137]
65	M	China	Hypertension, diabetes, hyperlipidemia, nephrotic syndrome, chronic renal impairment, and congestive heart failure	Bacteremia and Fournier's gangrene, vomiting and diarrhoea	Bacteraemia momomicrobial Urine *Klebsiella* Scrotal tissue *S. algae, Staphylococcus haemolyticus, Enterococcis faecium* and *Candidia albicans*	IV amoxicillan and clavulanate, switched to ceftazidime with levofloxacilin	Death	None	2016	[14]
64	M	Malaysia	Weekly peritoneal dialysis	Abdominal pain and diarrhoea	Monomicrobial	Intraperitoneal cloxacillin 250mg qid and ceftazidime 250mg qid	Cured	None	2016	[15]
54	M	Malaysia	Continuous ambulatory peritoneal dialysis	Abdominal pain, cloudy peritoneal fluid	Peritoneal fluid monomicrobial Catheter site *S. aureus* and *Corynebacterium sp.*	Intraperitoneal cloxacillin 250mg qid and ceftazidime 250mg qid	Death	Possible mining water	2016	[15]
71	M	Japan	Chronic kidney disease, glomerulonephritis, arteriovenous fistula in left arm	Gastroenteritis	Monomicrobial	Cefmetazole (1g/day), followed by meropenem (2g/day) and ceftazidime (2g/day) and lastly levofloxacin.	Cured	Yes, seafood	2017	[138]

*exposure to seawater/seafood 48 hours prior to infection

Abbreviations: qid = 4 times a day; PO = medication by mouth; q4h = every 4 hours; q8h = every 8 hours; q12h = every 12 hours.

The demographics of those who have been infected with *S. algae* are as follows. Where the sex of the patient was identified, males were more likely to be infected than females: 22.7% of infected patients were male and 6.4% were female (70.8% unknown) giving a male to female ratio of 3.53:1. The average age of infected patients was 53.6 years with an age range from newborn to 89. *S. algae* infections have been reported from all over the world with 202 of the 227 cases coming collectively from Denmark (30.0%), China (18.9%), Japan (15.5%), Korea (12.0%) and India (10.7%). The distribution of *S. algae* infections is most likely under reported in many instances, as even with good identification systems there is no referral requirement and many clinicians would not write up case reports of *S. algae* infections.

While it was widely accepted that the role of *Shewanella* species as either human or animal pathogens has been difficult to determine, mainly because it was noted that infections occurred as poly-microbial infections. Case reports clearly show that *S. algae* infections, however, are predominantly mono-microbial infections (60.1% of cases) with poly-microbial infections totalling just 23.2% of cases. It is clear then that *S. algae* has the ability to cause disease as a primary pathogen and further studies are needed to understand the mechanisms of pathogenesis. Of the mono-microbial infections, most were associated with gastroenteritis, cellulitis, bacteraemia or pneumonia and occurred predominantly in males aged between [45-60].

2.3.1 Clinical manifestations

The clinical manifestations caused by *Shewanella* species, particularly *S. algae* and *S. putrefaciens*, include skin and soft tissue infections, sepsis, hepatobiliary disease and

otitis media [33] and are similar to conditions caused by marine halophilic bacteria such as *Vibrio* species and *Aeromonas* species [116]. The common infectious presentation associated with *S. algae*, in order of frequency of appearance are otitis media (29.2%), cellulitis (abscesses, ulcers, wound infections; 24.9%), gastroenteritis (17.6%), septicaemia (11.6%) and bacteraemia (4.3%), which represent 199 of the 239 cases (Figure 2-4). When comparing the clinical manifestations of *Shewanella* species in general, they share similar characteristics. Of the 227 patients presenting with an *S. algae* infection, 145 had underlying comorbidities (Figure 2-5) which included previously reported hepatobiliary disease, malignancy, diabetes mellitus and neutropenia. Whilst most of these are still associated with *S. algae* infection (hepatobiliary disease (1.8%), malignancy (3.0%) and diabetes (5.6%)), there are more prominent comorbidities such as previous ear disease (28.8%), abdominal problems (renal/liver; 13.7%), hypertension (6.0%), broken skin (4.7%) and a compromised immune system (1.3%).

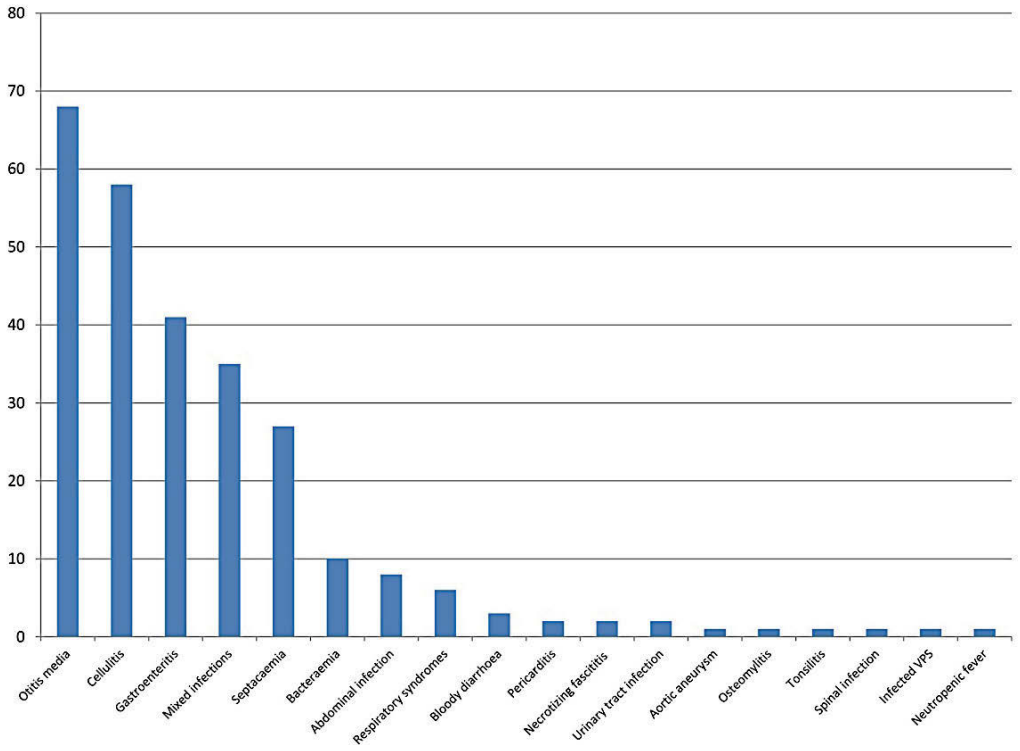

Figure 2-4 Frequency of different clinical presentations of infection caused by *Shewanella algae*.

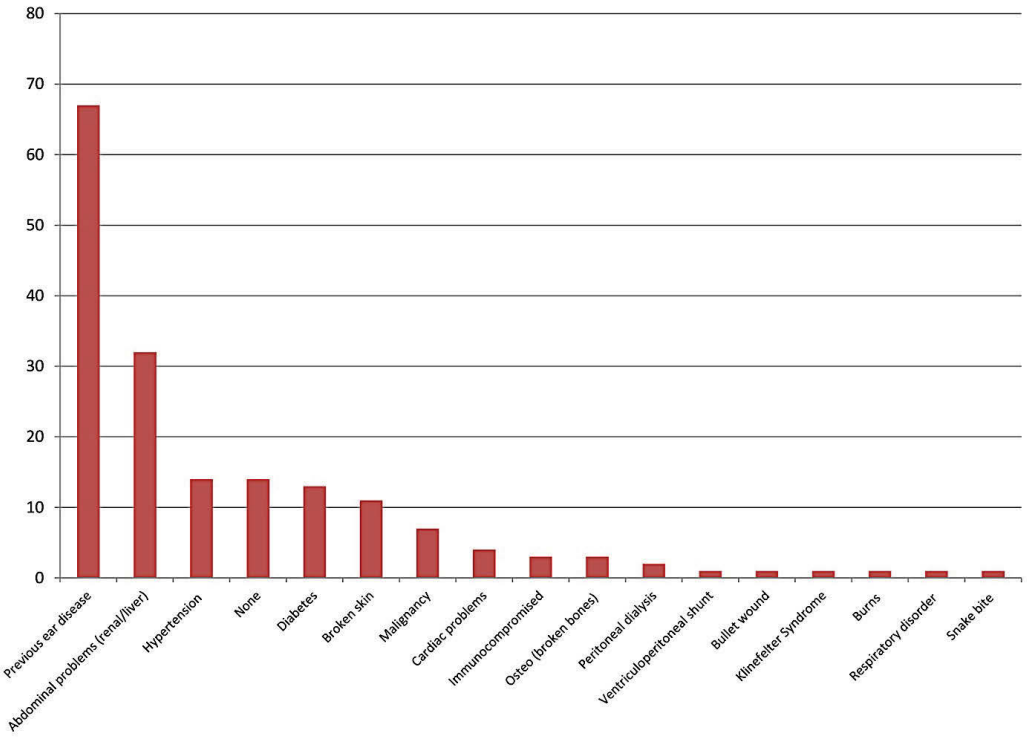

Figure 2-5 The prevalence of different co-morbidities associated with *Shewanella algae* infection of humans.

2.3.2 Outbreaks

S. algae has been implicated in only a single outbreak in the general surgery unit of a Korean hospital [114]. The outbreak was caused by contact transmission from an unwashed measuring cup over a 6-month period with an infection rate of 5%. *S. algae* was isolated from blood, bile, ascitic fluid and infections of the skin and soft tissue of patients. It caused sepsis and infection of surgical wounds leading to intra-abdominal, hepatobiliary and superficial and deep wound infections. This outbreak shows that *S. algae* can be transmitted in a contact-dependant manner and cause a range of clinical manifestations.

2.3.3 Risk factors

Risk factors associated with infection by *Shewanella* species are associated with geography, season, socioeconomic status, occupation, underlying conditions (which have previously been mentioned), food consumption and environmental exposure. This is also largely true for *S. algae* infections, with the particular exception of socioeconomic status. Typically, *S. algae* infections occur in warmer climates or warmer seasons as they are often associated with exposure to a marine environment, whether through the patient's occupation such as fishing, or through marine activities such as swimming and diving. Furthermore, exposure to marine environments with an open lesion or trauma increases the risk of *S. algae* infection dramatically, with 31.3% of patients having reported exposure to a body of water 24-48 hours prior to symptoms of infection.

With geography, season and climate being known risk factors, it should also then be noted that the impact of climate change could cause a higher incidence of infections caused by *S. algae*. This can be modelled for water-borne diseases by investigating the variation present in seasonal change [139]. For example, cholera outbreaks in Bangladesh occur most commonly in the monsoon season where precipitation is at its highest [140].

The ingestion of food contaminated with *Shewanella* species, particularly raw seafood, has also been implicated as a route for bacterial transmission, as has the ingestion of seawater containing *Shewanella* species. However, this was only relevant to *S. algae* in 1.3% of patients [12, 116]. One study did implicate *S. algae* as the cause of food poisoning in 38 individuals and was isolated from marinated beef; the age, storage conditions and cooking of this meat were not known [124].

Whilst socioeconomic status is known to play an important role in infection by some *Shewanella* species it does not seem to be implicated in disease caused by *S. algae* as first world countries report the highest incidence of these infections. However, this could be due to misidentification of *S. algae* or because individuals living in third world countries do not have access to health care centres and medical assistance to help diagnose and treat their infections, and therefore they are under reported.

2.3.4 Transmission

The natural presence of *S. algae* in marine environments and soils as an associated risk factor for *S. algae* infection, as well as the previously described outbreak, suggest that

transmission of *S. algae* is contact dependent. No studies have been done to investigate the spread of *S. algae* by other transmission routes. The ability of patients with pneumonia to spread *S. algae* via aerosol or droplet transmission or for patients with gastroenteritis to spread it via the faecal-oral route remain undetermined.

There is some evidence for transmission of *S. algae* through the environment [86]. *S. algae* has been isolated from 14 of 50 stranded *Somateria mollissim*, (common eiders) ducks with impacted cloacae and was present in 6 of 52 oral/nares/blow samples from live, stranded and caught marine vertebrates. The authors suggested that the presence of organisms like *S. algae* in diseased marine vertebrates (like these ducks) could be a mechanism for transmission of these pathogens to different aquatic reservoirs during migration and could also aid in the spread of antimicrobial resistance genes.

2.3.5 Treatment and antibiotic resistance

Current therapies for treating infections caused by *Shewanella* species include antibiotics (oral, IV and topical treatments), wound drainage and surgical therapies such as excision of tissue or amputation in extreme cases with 63.5% of *S. algae* infections being cured using these treatment strategies (Table 2-2). A poor outcome of treatment was usually associated with the presence of an underlying condition or pre-existing disease, as seen in Table 2-2.

Whilst it can be seen from these case studies that infections mediated by *S. algae* are commonly treated with IV antibiotics followed by oral antibiotics upon discharge, this is only because reported cases of *S. algae* infection are only documented in emergent

cases. It is highly likely, given that the common infections caused by this organism are otitis media, cellulitis and gastroenteritis, that cases of infection by this organism are highly under documented. This is because patients would simply seek treatment from a general practitioner who would not seek the identity of the organism and would prescribe a broad spectrum antibiotic to cover a variety or organisms. What can also be seen from the documented case studies is that IV antibiotics are generally commenced and then amended once the causative agent is identified and the antibiotic resistance profile is determined. Whilst his is standard healthcare practice which is linked to improved individual patient outcomes in serious Gram-negative infections, it should also be noted that these practices encourage oversubscription of antibiotics and favours development of multiple antibiotic resistance.

The *Shewanella* species are still susceptible to many common and readily available antibiotics, however there has been an increase in the occurrence of antibiotic resistance, with the identification of a multidrug resistance transposon Tn*1696* in *Shewanella xiamenensis* [141] and multidrug resistance in *S. algae* strains. Antibiotic resistance has been reported in clinical strains of *S. algae* since they were first linked to human disease in 1996 [35]. The most commonly reported antibiotic resistance is against beta-lactams, such as amoxicillin, ampicillin and penicillin; and against cephalosporins, such as cephalothin, cefazolin and cefotaxime. There are now reports of *S. algae* strains resistant to all classes of antibiotics and many strains are resistant to ≥ three antibiotics [12, 34-36] (Table 2-1). Notably, resistance to colistin has been reported for *S. algae* in many studies [130, 134]. In one instance, modification of the

bacterial outer membrane by the addition of phosphoethanolamine via ethanolamine phopshotransferase activity was shown to be the mechanism [91].

It has been suggested that genes encoding antibiotic resistance in *S. algae* can be transferred to, and between, other bacterial species *in vivo* [42]. For example, *S. algae* is considered to be the original source of the *qnrA* gene, which now has 8 known alleles [142], that encodes resistance to fluoroquinolones, variants of this gene are disseminated widely in the *Enterobacteriaceae* [143]. It has been demonstrated that the *qnrA3* gene from *S. algae* can be transferred between *Klebsiella pneumoniae* and *Kluyvera ascorbate in vivo* in an immunocompromised patient [42]. The *qnrA* gene increases bacterial fitness in the absence of fluoroquinolone resistance in *Escherichia coli* [144] and can be induced by cold shock in *S. algae* [145]. These data suggest that resistance to fluoroquinolones is not the primary function of the *qnrA* gene but that it may have a major role in regulating gene expression via DNA supercoiling [146, 147].

The resistome of *S. algae* strain MARS 14 encodes numerous genes implicated in resistance to antibiotics and heavy metals, including resistance to arsenic, copper, acriflavin, chromium, zinc, magnesium, nickel, chromium and molybdenum as well as bacteriocins and genes encoding beta-lactamases [36]. Furthermore, the gene encoding $bla_{OXA\text{-}55}$ was found in close proximity to a transposon, which suggests that it could have been acquired via lateral gene transfer [36]. Genes that encode multidrug efflux pumps such as *multidrug and toxic efflux* (MATE) genes which function in sodium/drug antiporters and *cmeA*, *cmeB* and *cmeC* which function in Resistance-Nodulation-Division (RND) efflux systems have been identified [36].

Table 2-3 Reports of antibiotic drug resistance in clinically-derived *Shewanella algae* isolates.

Antibiotic resistance	Reference(s)
Drugs targeting the cell wall	
Amoxicillin	[13, 36, 79, 112, 122, 125]
Penicillin	[78, 117, 148]
Meropenem	[36, 127]
Imipenem	[15, 34, 36, 113]
Carbepenam	[113]
Ampicillin	[79, 108, 125, 135]
Carbenicillin	[79]
Piperacillin	[113, 115]
Ticaricillin	[36]
Etrapenem	[36]
Cephalothin	[13, 92, 125, 135]
Cefotaxime	[12, 122]
Cefazolin	[99, 112, 117, 130]
First generation cephalosporins	[108]
Ceftazidimine	[12, 134]
Cephalexin	[125]
Ceftizoxime	[125]
Vancomycin	[125]
Cefoxitin	[36]
Drugs targeting the cell membrane	
Colistin	[130, 134]
Polymixin B	[79, 134]
Drugs targeting DNA synthesis	
Quinolones	
Nalidixic acid	[12]
Ciprofloxacin	[15, 34, 112, 115]
Drugs that modify protein synthesis	
Fosfomycin	[92, 122]
Gentamycin	[113, 122]
Doxycycline	[125]
Tetracycline	[125]
Drugs that target energy metabolism at the folate cycle	
Sulphonamides	[79]
Trimethoprim	[79]
Cotrimoxazole	[122]
Trimethoprim/sulfamethoxazole	[112]
Multidrug resistant	[12, 34-36]

Whilst the development of antibiotic resistance is infrequent and has not seriously impacted the treatment of *S. algae* infections, this organism shows potential for acquiring genes for antibiotic resistance and transferring them to other bacteria. As antibiotic resistance is a looming threat to today's society, it is crucial to determine the mechanisms by which *S. algae* is able to resist antibiotics, and the frequency at which this can occur.

2.3.6 Prevention

Strategies employed to avoid infection by *S. algae* include avoiding aquatic environments with open wounds or trauma to the body, or covering the wound with waterproof dressings before entering the water. It is recommended that all food (particularly seafood) is adequately cooked before consumption and that swallowing large quantities of water from marine or freshwater environments should be avoided to prevent *S. algae* entering the gastrointestinal tract. Lastly, cleaning down any equipment related to marine activities (e.g. fishing hooks and diving equipment) appropriately to ensure no living *S. algae* remain as a reservoir of infection.

2.3.7 Mechanisms of pathogenicity

The mechanisms for pathogenicity of *Shewanella* species to humans and animals remains largely unknown. Whilst it is known that some *S. algae* strains produce virulence factors such as haemolysins, tetrodotoxin (TTX), siderophores and exoenzymes, their exact role in pathogenesis is unclear.

Recent studies have uncovered some of the genes encoding potential virulence factors in *S. algae* strain MARS 14 [36]. In addition to multiple genes for chemotaxis, genes encoding a number of virulence factors were identified: six putative haemolysins; mannose-sensitive haemagglutinin (*msh*) proteins that are involved in bacterial adhesion; a bacteriocin that is an antimicrobial peptide known to have a role in bacterial interspecies competition; and genes for the types I, IV, VI and VII secretion systems that are all involved in the translocation of virulence factors from the bacterial cell [149-154]. The type VI secretion system has also been identified in some *S. algae* strains (SAI) [142], in *S. woodyi* and in *Shewanella frigidmarina* [155]. None of these studies, however, investigated the role it may play in pathogenicity.

Genes involved in quorum sensing and biofilm formation have also been described [36]. Biofilms aid bacterial pathogenesis by: protecting the bacteria from the external environment and the host's immune system; decreasing susceptibility to antibiotics; and allowing the dispersion and growth of bacteria in hostile environments [31, 156-161]. Furthermore, biofilms provide bacteria with an environment that facilitates the colonisation of wounds, which could be essential in *S. algae* infection. Studies describing the role of biofilms in *S. algae* pathogenesis have, to our knowledge, not been published, but the role of biofilms of *S. algae* strain CECT 5071 in bacterial fouling have been reported [162]. The biomass of *S. algae* biofilm in these experiments was dependent on the temperature and the constituents of the culture medium, which should be noted in any future studies investigating the role of biofilms in *S. algae* pathogenicity. Investigations into biofilm formation, persistence and breakdown could help fill present gaps in our knowledge about the pathogenicity of *Shewanella* species,

particularly wound colonization and the establishment of poly-microbial infections. They also provide insight into potential therapies for controlling persistent infections by *Shewanella* species. *Shewanella algae* isolated from patients suffering food poisoning produced tetrotoxin TTX [124]. Male mice injected intraperitoneally with extracts derived from 7 d old culture supernatants suffered 100% mortality within 2.5 minutes of injection and demonstrated limb-weakness and convulsions, whereas injection of the overnight supernatant caused no infection or mortality. These symptoms are typical of TTX poisoning [163].

While the full repertoire of virulence factors produced by *S. algae* are yet to be determined, there is sufficient evidence to suggest that TTX and haemolysins may play an important role. For *S. algae* to cause skin and soft tissue infections, blood-borne infections and gastroenteritis, it must have mechanism(s) that allow it to adhere and colonise (adhesins, biofilm promoting factors) and cause tissue damage (proteases, toxin, haemolysins etc.) Protein secretion systems are hallmarks of pathogenesis [164] for a wide range of pathogens that have an environmental niche (e.g. *Vibrio* species [165] and *Legionella* species [166]). The secretion of putative virulence factors may be a response by *S. algae* to competition for limited resources in poly-microbial infections and as a mechanism to avoid predation [37]. Further studies are needed to determine how *S. algae* causes disease.

2.3.8 Pathogenicity in human cells lines and animal models

Studies investigating the pathogenicity of *S. algae* in human cell lines and animal models are extremely limited. Only one study has investigated cell invasion and

adherence of *S. algae* using human epithelial (HEp-2) cells and compared the differences in pathogenicity between *S. algae* and *S. putrefaciens* [8]. HEp-2 cells were grown as a monolayer in chamber slides in antibiotic-free medium and 1 x 10^6 *S. algae* or *S. putrefaciens* cells were added. After 90 minutes of incubation, extracellular bacteria were removed by washing the cells with PBS and adding gentamicin to the culture medium. After a further 3 hours of incubation, cells were either lysed with Triton X-100 and colonies plated to determine the number of viable bacterial cells, or cells were fixed for microscopy of invaded cells. Whilst *S. putrefaciens* showed some adherence to Hep-2 cells, it did not invade or result in cytotoxic effects on the cells. *S. algae* also failed to adhere or invade Hep-2 cells, but did result in a cytotoxic effect. The mechanism for this toxicity remains unknown.

The lethal dose to kill 50% (LD_{50}) was also determined for *S. algae* and *S. putrefaciens* against female Swiss Webster mice [8]. The bacterial strains were grown overnight and 10^9 viable bacterial cells were serially diluted to give a range of concentrations and injected intraperitoneally into 8-10 week old mice. Mice were observed for 1-week post-inoculation, deaths recorded and the LD_{50} calculated. The authors concluded that *S. algae* was more virulent than *S. putrefaciens*, with an LD_{50} of 1.9 x 10^8 viable bacterial cells for *S. algae* compared with 8.4 x 10^8 viable bacterial cells for *S. putrefaciens* [8]. Another study also determined the LD_{50} for *S. algae* using similar methods and found it to be $10^{-5.5}$/0.1 mL [167]. These authors also compared the pathogenicity of *S. algae* to mice and Sanhuang cockerels injected abdominally with 1.2 x 10^8 viable bacteria cells/ml. They found that 90% of mice and 100% of cockerels died within 24 hours due to infection by *S. algae* [167]. Autopsies showed that the

animals had died from sepsis; that their livers, spleens, kidneys, lungs and hearts displayed symptoms of congestive bleeding and swelling; and that their stomach and mucosal linings had been bleeding. No other studies have investigated the role that *S. algae* plays in the disease of livestock, however given the mortality shown here in chicks this warrants further investigation, especially in coastal areas where contamination of livestock water supplies is more likely.

A handful of studies have also investigated the role of *S. algae* in infection of marine life. Studies determined an LD_{50} for *S. algae* against 20-day-old post-larval abalone (*Haliotis diversicolor supertexta*) to be 1.8 x 10^4 viable bacterial cells mL^{-1} [168] after incubation in bacterial suspensions for 72 hours. Additional studies have characterised the pathogenicity of *S. algae* to the sea cucumber *Apostichopus japonicas* (*A. japonicas*) and the sea urchin *Strongylocentrotus nudus* (*S. nudus*) [84]. Following injection of *S. algae* into their coelomic fluid, 100% of *A. japonicas* and *S. nudus* died within 3-8 days. *Shewanella algae* survived and replicated within the amoebocytes (phagocytes) of these animals. However, it was also demonstrated that *S. algae* was not invasive unless the epithelium of either animal species had been previously broken.

While only a handful of invasion and pathogenicity assays have been performed on human cells lines and in animal models, they clearly show that *S. algae* is pathogenic (and more pathogenic than *S. putrefaciens*) to humans and marine life. Further studies are needed to investigate the mechanisms of pathogenicity in this species.

2.3.9 Genomic and proteomic studies of *Shewanella algae*

Genomic, proteomic and transcriptomic studies provide insights into the potential of pathogens to cause disease [169]. Only a handful of genomic, proteomic and transcriptomic studies of *S. algae* have been reported (Table 2-4).

Table 2-4 Genomic, proteomic, and transcriptomic studies of *Shewanella algae.*

Study	Year	Reference
Genomics		
Ribotyping and 16SrRNA analysis to differentiate *S. putrefaciens* and *S. algae*	1997	[78]
RAPD analysis to determine *S. algae* homogeneity	2000	[170]
A plasmid-borne Shewanella algae gene, *qnrA3*, and its possible transfer *in vivo* between *Kluyvera ascorbata* and *Klebsiella pneumoniae*	2008	[42]
Detection of a new *qnrA7* genotype in *S. algae*	2010	[171]
Cold shock induces *qnrA* expression in *S. algae*	2011	[145]
Investigating chlorate reduction in *S. algae* ACDC	2014	[172]
Draft genome sequence of *S. algae* C6G3	2015	[81]
Draft genome sequence of *S. algae* MARS 14	2015	[36]
Functional genomics to discover antibiotic resistance genes of *S. algae* MARS 14	2015	[91]
Identification of the novel *qnrA* allele, *qnrA8*, in environmental *S. algae*	2017	[142]
Proteomics		
Whole cell protein profiling to differentiate *S. putrefaciens* and *S. algae*	1997	[78]
Homogeneity of Danish environmental and clinical isolates of *S. algae*	2000	[170]
Investigating the production of TTX from *S. algae*	2013	[124]
Investigating chlorate reduction in *S. algae* ACDC	2014	[172]
Studies investigating biolfilm formation in antifouling *S. algae*	2014	[162]
Resistome		
Resistome of *S. algae* MARS 14, identification of virulence factors	2015	[36]
Transcriptomics		
Time course transcriptome changes in *S. algae* in response to salt stress	2014	[173]

The first *Shewanella* species genome was sequenced in 2002 [5] and there are currently 54 genomes publically available on the National Centre for Biotechnology Information website. To date, 5 draft genomes sequences of *S. algae* are currently available, of which four are environmental isolates (strains JCM 20137, C6G3, BrY, and CSB04KR) and one is a clinical isolate (MARS 14). The properties of these genomes are varied, as exemplified by the size of the genomes which ranged from 4.8 to 5.0 Mb and encode for 4,484 to 5,792 genes (Table 2-5).

Table 2-5 Genomic features of *Shewanella algae* strains

S. algae strain	Assembly accession number	Status	Size (Mb)	Predicted ORF's	Predicted proteins	Predicted RNA genes
JCM 21037	GCA_000615045.1	Incomplete	4.82	5,257	5,188	69
MARS 14	GCA_000947195.1	Incomplete	5.00	4,484	4,363	121
C6G3	GCA_000956365.1	Incomplete	4.87	5,792	5,660	132
BrY	GCA_001870495.1	Incomplete	4.55	4,047	3,994	53
CSB04KR	GCA_001858195.1	Incomplete	4.80	4,362	4,259	103

2.4 Role of *Shewanella algae* as an animal pathogen

S. algae is known to cause: i) ulcer disease [174] and kidney swelling [175] in the fish S*ciaenops ocellata*; ii) mortality in post-larval stages of the abalone *H. diversicolor* [168]; iii) death of the sea cucumber *A. japonicas* and the sea urchin *S. nudus* [84]; iv) impacted cloacae in *S. mollissim* ducks [86]; and v) peritonitis in sea lions [73].

2.5 Role as a food spoilage agent

Shewanella species, particularly *S. putrefaciens*, are major food spoilage bacteria. Whilst *S. putrefaciens* is most commonly associated with spoilage of iced fish, there have been reports of it causing spoilage in chicken carcasses, beef and pork, resulting

in the production of foul sulphurous odours, changes in food flavour, slime formation and discolouration [176]. In the food industry, *S. putrefaciens* is a spoilage bacterium of marine fish, some vacuum-packed meats, and chicken due to its ability to produce volatile sulfides, amines, and trimethylamine [177]. *S. algae* has caused multiple cases of gastroenteritis, but it is not specifically associated with food spoilage. However, *S. algae* has also been isolated from fresh oysters in the Delaware Bay [178], and if consumption of raw oysters occurs it has the potential to cause gastroenteritis in humans.

2.6 Future directions and concluding remarks

The genus *Shewanella* is an extremely diverse group of organisms. The main interest in this genus is for its use in bioremediation and microbial fuel cells. *S. algae* is recognised as an emerging human pathogen but there is little research investigating mechanisms of pathogenicity or the role it plays in disease. The taxonomic status of *S. algae* and its physical and biochemical properties are now well defined and it is imperative that this organism be added to all manual and automated clinical identification systems so that the true incidence of the diseases that it causes can be determined.

We have summarised all published case studies on *S. algae* associated with human infection, highlighting that *S. algae* is capable of causing mono-microbial disease in humans. *S. algae* is increasingly resistant to multiple antibiotics and has been shown to accept and transfer antimicrobial resistance genes by lateral gene transfer. With the reported incidence of *S. algae* infections on the rise and the increasing levels of

resistance it displays to antimicrobials, the pathogenic potential of this organism is underreported and should be thoroughly investigated before it is adopted widely in the biotechnology industry.

Environmental screening for *S. algae* in freshwater and marine environments is needed to understand the breadth of niche it occupies. Such studies should encompass efforts to investigate the potential role of *S. algae* infection in livestock, as we know that it has the ability to cause disease in poultry.

Chapter Three

The draft genome sequence of

Shewanella algae SA2

Chapter 3 – The draft genome sequence of *Shewanella algae* SA2

3.1 Compound abstract

Rationale: *S. algae* are predominantly marine, Gram-negative facultative anaerobes that are increasingly isolated from human infections from different body sites, raising concerns that the species is an emerging human pathogen. With very few *S. algae* genome sequences currently available and none from Australia, we present the draft genome sequence of *S. algae* strain SA2 isolated from the Georges River in Sydney and identify potential virulence factors in this organism.

Methods: The *S. algae* SA2 genome was sequenced using Illumina paired-end technology and the genome annotated via RAST. Phylogenetic analysis was performed using Phylosfit. Antibiotic susceptibility testing was performed using the CDS method and the Vikek 2 automatic system and potential virulence factors were identified using the online server MP3.

Results: The genome of *S. algae* SA2 is 5.07 Mbp in size with a G + C content of 52.8%. Phylogenetic analysis using PhyloSift showed that the SA2 isolate clustered with other environmental *S. algae* genomes. MP3 identified multiple secretion systems, haemolysins, an RTX toxin, heamagglutinins and proteins encoding a flagellar system which could contribute to the ability of this organism to adhere to, colonise and infect multiple sites in humans. Antimicrobial resistance genes were also identified, including genes resistant to

beta-lactamases, fluoroquinolones and multidrug-efflux pumps and SA2 was found to be resistant to cefazolin, sulfafurazole and apramycin. MP3 identified 1,361 protein to be potentially pathogenic, including the identification of haemolysins, cytotoxins, adhesins, proteases and genes involved in bacterial motility/chemotaxis and the resistance to antimicrobial and toxic compounds. Harbouring this suite of genes is consistent with the hypothesis that *S. algae* may be an emerging opportunistic pathogen of humans.

3.2 Introduction

The genus *Shewanella* comprises an extremely diverse group of facultative anaerobes that are widely distributed in freshwater and marine environments, including intertidal and benthic zones, their sediments and oil field wastes throughout the world [1, 2]. They are Gram-negative bacilli that are 1 - 2 µm in length and 0.4 - 0.7 µm in width which are motile via a single polar flagellum, exhibit un-paralleled respiratory diversity, and have robust sensing and regulatory systems which allow them to survive environments with low temperatures (less than 4°C), high salt concentrations and an extensive range of barometric pressures [3, 4]. These features lend themselves to phenotypic and physiological differences within the genus, but also have elicited interest in their use for bioremediation and microbial fuel cells [5, 6].

There are 63 species that comprise the *Shewanella* genus [7], and a handful of these are known to cause disease in humans and animals. The main species associated with human infection is *S. algae* [1, 8], which naturally resides in aquatic environments and has been

isolated from marine and freshwater sediments, oil fields, animals, marine life (including fish, sea lions, echinoderms, birds and poultry), and largely from human clinical material as the causative organism of diseases such as otitis media, cellulitis, septicemia and increasingly gastroenteritis [9-15]. There are more than 230 clinical studies, mostly case reports that describe *S. algae* as the causative agent of a range of human afflictions, with approximately 20% associated with gastroenteritis. Patients with gastrointestinal symptoms include nausea, vomiting, (bloody) diarrhoea and abdominal cramps. In addition, the incidence of antibacterial drug resistance within *S. algae* is on the rise, with several reports of multiple drug resistance [12]. To date, there are five draft genomes of *S. algae*, and limited studies investigating the mechanisms of pathogenesis and antimicrobial resistance. Furthermore, there are no genome sequences from isolates recovered in Australia. There is a need to characterise the carriage of putative virulence and antimicrobial resistance genes and determine how they are acquired. Here we report the draft genome of *S. algae* SA2 isolated from the Georges River that flows through a suburban area in Sydney, Australia. We undertook an analysis of the genome to identify potential virulence factors and genes that are predicted to encode resistance to antimicrobial agents.

3.3 Methods

3.3.1 Strain isolation and genomic DNA preparation

S. algae SA2 was coincidently co-isolated alongside environmental *V. cholerae* as a part of a project investigating the presence of non-toxigenic *V. cholerae* in the Sydney area [46].

Water samples (50 mL) were collected from the Georges River, transported to the University of Technology Sydney and enriched as described previously [179, 180]. Samples were plated on TCBS agar [181], a *V. cholerae* selective medium, and incubated at 37°C for 18-24 hours. Whilst *V. cholerae* generate large yellow colonies, small, green colonies 1-2 µm in diameter were consistently co-isolated. A PCR targeting the 16S rDNA was characterised by Sanger sequencing and tentatively identified the organisms that generated green colonies as *Shewanella*. To characterise several isolates further, whole genome sequencing was undertaken.

3.3.2 Genome sequencing, assembly and annotation

SA2 was grown aerobically in LB broth at 37°C with shaking at 180 rpm. SA2 genomic DNA was extracted from an overnight culture using the Promega Wizard genomic DNA purification kit. Isolated DNA was prepared using the Nextra DNA Library Prep kit and sequenced at the Beijing Genomics Institute on the Illumina HiSeq using paired-end sequencing technology. Raw reads from the paired end library were assembled using the A5 pipeline [65, 182, 183]. The latest version of A5 has been modified to improve assembly contiguity by accepting reads up to 500 nt long and is able to construct *de Bruijn* graphs with *k*-mers up to 500 nt. Genome annotation was performed using the RAST online server and FigFAM release 70 [184].

3.3.3 Bacterial phylogeny

Phylogeny was inferred using PhyloSift, which identifies closely related organisms based on similarities and differences from a set of 37 universally conserved housekeeping proteins. The genes that encode these 37 proteins represent approximately 1% of the core *E. coli* genome [64]. All complete *Shewanella* genomes and draft *S. algae* genome sequences were obtained from the NCBI FTP GenBank website (ftp://ftp.ncbi.nlm.nih.gov/genomes) for phylogeny analysis. The assemblies were checked by identifying clusters of read pairs that map to disjoint locations in the assembled genome as described previously [65].

3.3.4 Identification of potential virulence factors

Proteins believed to be involved in bacterial pathogenesis were identified by genome annotation via RAST and by using the online server MP3. RAST assigns proteins to a specified subsystem based on abstract functional roles, whilst MP3 uses a combination of Support Vector Machine (SVM) and Hidden Markov Model (HMM) to predict pathogenic proteins based on dipeptide composition (the fractions of amino acids and their local order) and Pfam domains (protein functional domains), respectively [185].

3.3.5 Identification of antibiotic resistance genes and antibiotic susceptibility testing

The *S. algae* SA2 genome was screened against ResFinder to identify genes involved in the resistance to antibiotics [186]. Antibiotic susceptibility was carried out using two methodologies; the calibrated dichotomous sensitivity (CDS) test and the Vitek 2 Compact

system (Version 05.04 at Concord Hospital). All methods for CDS testing were followed as per the CDS Manual (6th edition). The two control strains used for CDS testing were *E. coli* ACM 5185 and *E. coli* ACM 5186. Isolates are determined to be resistant to antibiotics if the diameter of the zone of clearing is <6mm (or <4mm for neomycin and tetracycline).

For the Vitek 2 sensitivity testing, SA2 was prepared following the Biomerieux guidelines. The Concord hospital version of GLOBAL, 2003 MIC interpretation guideline, based on the CLSI *Enterobactericae* model with *Escherichia coli,* was selected as standards since *Shewanella* was not in their system. Antibiotic resistance was determined based on the optical density of inoculated wells compared to the standards.

3.4 Results and Discussion

Once assembled via A5, the draft genome of *S. algae* SA2 assembled into 185 scaffolds to produce a 5,072,041 bp genome that had a G+C content of 52.8%, a 116-fold median depth coverage and median scaffold length (N50) of 147,209 bp (Table 3-1). Sequencing generated 6,833,334 raw reads with a total number of 615,000,060 nucleotides. The annotation by RAST revealed there to be 4,489 DNA coding sequences and 92 RNA coding sequences (all RAST annotations can be seen in Table S-1). The overall functional profile of the genome as determined by RAST is shown in Figure 3-1, where 51% of the coding sequence (CDS) was assigned to a subsystem. The high percentage of genes unallocated to a subsystem by RAST is not uncommon, as the subsystems are proteins grouped by a relationship in function and are therefore not comprehensive [187]. Furthermore, is it also

not uncommon to have a large percentage of genes encode hypothetical proteins, which remain hypothetical due to a lack of experimental studies, with the number of hypothetical genes in genomic databases at almost 4 million [188]. The main functional categories determined by RAST include amino acids and derivatives, carbohydrates and cofactors, vitamins, prosthetic groups and pigments.

Table 3-1- Genome properties of *S. algae* SA2.

Gene feature	Value	% of Total
Genome size (bp)	5,072,041	100.00
DNA coding (bp)	4,348,478	85.73
DNA G+C (bp)	2,351,604	54.08
DNA scaffolds	185	-
Total genes	4,640	100.00
Protein coding genes	4,489	96.75
RNA genes	92	1.98
Pseudo genes	59	1.27
Genes with function prediction	2,248	48.45

Phylogenetically, all *S. algae* cluster in their own clade, with the closest relative identified as *Shewanella amazonensis* SB2B, an environmental metal-reducing, facultative anaerobe isolated from Amazonian shelf muds [70]. SA2 clusters with other environmental *S. algae* strains C6G3, CSZ04KR, and JCM 21037 (Figure 3-2), which were isolated from the upper 2cm of muddy sediment in the Arcachon Bay on the southwest coast of France [81], the gut of a sea cucumber *Apostichopus japonicus* in South Korea, and the surface of red alga (*Jania* species) [55], respectively, suggesting they have a common ancestor. The clinical *S. algae* strain MARS 14, isolated from a patient with pneumonia [36], clustered separately

from the environmental *S. algae* strains. Interestingly, the environmental strain BrY, isolated from sediment in the Great Bay estuary in New Hampshire [76], is phylogenetically distinct from all other *S. algae* strains.

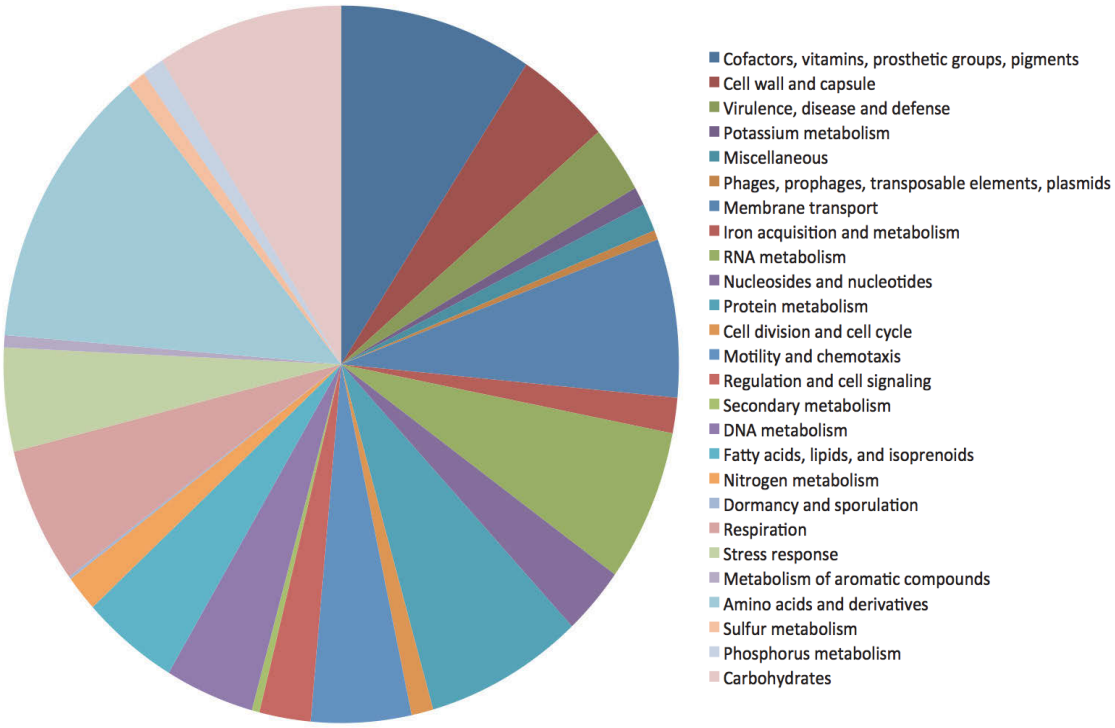

Figure 3-1 RAST subsystems and their relative abundance in the *S. algae* SA2 genome, where 51% of the CDS have been assigned to the subsystems in RAST.

To identify potential virulence factors in the *S. algae* SA2 genome, we utilised MP3, which determines the pathogenic proteins by dipeptide composition (the fractions of amino acids and their local order) and Pfam (protein functional domains) by HMM and SVM, respectively. Proteins are then identified as either HMM, SVM or a hybrid, denoting it was detected by both HMM and SVM analysis. The analysis via MPS determined 1,361 proteins

to be potential pathogenic proteins (Table S-2). This included the prediction of 209 to be hybrid, 118 by HMM and 1,034 by SVM analysis.

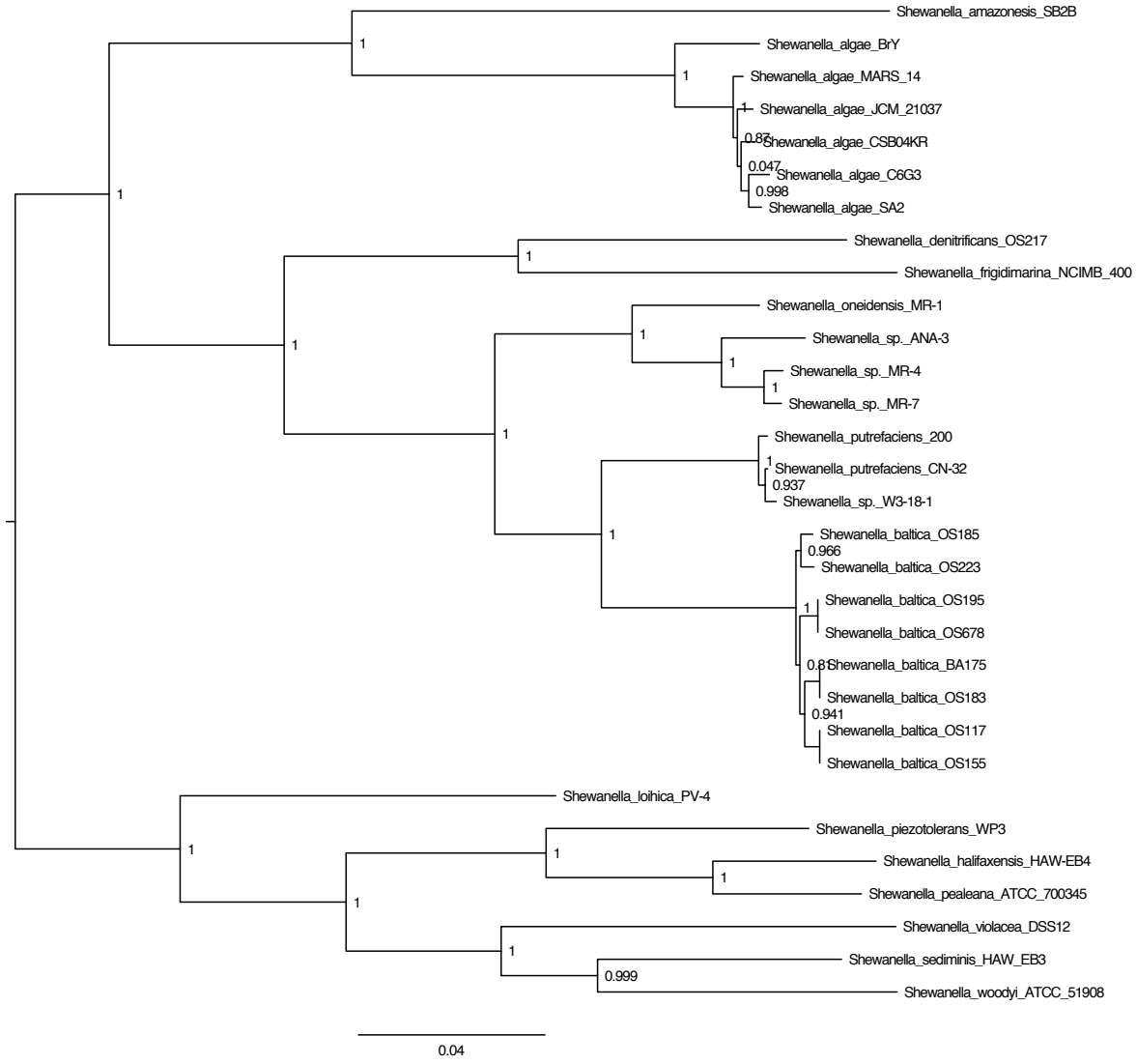

Figure 3-2 Phylogeny tree of complete *Shewanella* genomes and draft *S. algae* genomes based on PhyloSift.

The x-axis represents the substitutions per site and the numbers at each node give the clade confidence value. The phylogeny was inferred with FastTree [68], and the tree visualized with FigTree version 1.4.0 (http://tree.bio.ed.ac.uk/software/ figtree/).

The pathogenic proteins identified include virulence factors involved in bacterial adhesion, cytotoxicity, haemolysis, motility, chemotaxis and biofilm formation. Multiple secretion systems known to be involved in bacterial pathogenicity and biofilm formation were identified, including the Type I, II, IV, VI, and VIII secretion systems [189, 190]. There are numerous genes encoding haemolysins, an RTX toxin [191-193] and mannose sensitive heamagglutinins (*Msh* genes) [149, 150, 194], which may contribute to adhesion of the bacteria to the gastrointestinal lining and bloody diarrhoea that is often reported with gastroenteritis caused by *S. algae*.

Protein function predictions by RAST also identified various genes involved in pathogenicity, bacterial fitness and resistance to antibiotics. These included proteins involved in the resistance to antibiotics, such as fluoroquinolones (*gyrA*, *gyrB*, *parC*, and *parE*), quinolones (*qnr*), and beta-lactamases (*blaOXA-55*). There were also multiple genes encoding multidrug resistance efflux pumps (*MATE*, *arcB*, *RND*, *ydhE/norM*, *mtrF*, *cmeA*, *cmeB*, *cmeC* and *tolC*).

RAST also predicted that SA2 genome encodes genes involved in the resistance to toxic compounds such as arsenic (*arsA, arsC*, *arsR*, *CymA* and *ARC3*), bile hydrolysis (*bsh* and *damX*), cobalt-zinc-cadmium (*cusA*, *czcB*, and *czcD*), copper (*CIA/CSA*, *CRTR*, *ccmI*, *ccmH*, *clfA*, *cutA*, *cutE*, *corC*, *scsA*, *scsB*, and *scsD*), chromium (*chrA*), zinc (*znrA*), and nickel (*nikB2* and *nikR*) (Table 3-2), which all increase bacterial fitness and the ability to colonise a variety of environmental niches. In addition, genes with known roles in cell invasion and

intracellular resistance (Mycobacterium virulence operons involved in DNA transcription, protein synthesis and quinolinate biosynthesis), and resistance to bacteriocins and ribosomally synthesized antibacterial peptides (*creA, creD*-like protein), were also identified. Many of these genes are similar to those found in the genome and resistome of the clinical isolate, *S. algae* MARS 14 [36].

To determine the presence of genes involved in antibiotic resistance, the *S. algae* SA2 genome was scanned by ARGD. This database identified *blaOXA-55* and a *qnrA* allele (later determined to be a novel allele *qnrA8* in Chapter 6). Antibiotic susceptibility testing was carried out by the Vitek 2 and CDS methods. Results from the Vitek 2 revealed SA2 to be resistant to cefazolin, a cephalosporin (Table 3-3). Results from the CDS method showed resistance to sulfafurazole, a sulphonamide, and apramycin, an aminoglycoside (Table 3-4).

Table 3-2 List of genes associated with resistance toxic compounds in the *S. algae* SA2 genome.

Resistance agent	Gene	Location in genome	Function
Arsenic	*arsA*	fig|1236544.6.peg.2995	Arsenate reductase (EC 1.20.4.1)
	arsC	fig|1236544.6.peg.4305	Arsenical pump-driving ATPase (EC 3.6.3.16)
	arsR	fig|1236544.6.peg.2398	Arsenical resistance operon repressor
	ARC3	fig|1236544.6.peg.2397	Arsenical-resistance protein ACR3
	CymA	fig|1236544.6.peg.1137	Membrane anchored tetraheme cytochrome c, CymA
Bile	*bsh*	fig|1236544.6.peg.981	Choloylglycine hydrolase (EC 3.5.1.24)
	DamX	fig|1236544.6.peg.3713	DamX, an inner membrane protein involved in bile resistance
Coblat-zinc-cadmium	*CusA*	fig|1236544.6.peg.1143 fig|1236544.6.peg.2445 fig|1236544.6.peg.2770 fig|1236544.6.peg.4129 fig|1236544.6.peg.493	Cation efflux system protein CusA
	CzcB	fig|1236544.6.peg.1142 fig|1236544.6.peg.1692 fig|1236544.6.peg.2004 fig|1236544.6.peg.2446 fig|1236544.6.peg.2985 fig|1236544.6.peg.3193 fig|1236544.6.peg.3305 fig|1236544.6.peg.4273	Probable Co/Zn/Cd efflux system membrane fusion protein
	CzcD	fig|1236544.6.peg.1143 fig|1236544.6.peg.2445 fig|1236544.6.peg.2770 fig|1236544.6.peg.4129	Cobalt-zinc-cadmium resistance protein CzcA
Copper	*CIA/CSA*	fig|1236544.6.peg.2923 fig|1236544.6.peg.3780 fig|1236544.6.peg.4301	Copper-translocating P-type ATPase (EC 3.6.3.4)
	CRTR	fig|1236544.6.peg.4300	Cu(I)-responsive transcriptional regulator
	CcmI, CcmF	fig|1236544.6.peg.2418 fig|1236544.6.peg.3359	Cytochrome c heme lyase subunit CcmF
	CcmH	fig|1236544.6.peg.3358	Cytochrome c heme lyase subunit CcmH
	ClfA	fig|1236544.6.peg.1533 fig|1236544.6.peg.2947	Multidrug resistance transporter, Bcr/CflA family
	CutE	fig|1236544.6.peg.3120	Copper homeostasis protein CutE
	CorC	fig|1236544.6.peg.1813 fig|1236544.6.peg.3119	Magnesium and cobalt efflux protein CorC
	ScsA	fig|1236544.6.peg.693 fig|1236544.6.peg.1894	Membrane protein, suppressor for copper-sensitivity ScsB
	ScsD	fig|1236544.6.peg.1895	Membrane protein, suppressor for copper-sensitivity ScsD
	CutA	fig|1236544.6.peg.3569	Periplasmic divalent cation tolerance protein CutA
	ScsB	fig|1236544.6.peg.1896	Secreted protein, suppressor for copper-sensitivity ScsC
Zinc	*ZraR*	fig|1236544.6.peg.3971	Response regulator of zinc sigma-54-dependent

			two-component system
Nickel	*nikB2*	fig|1236544.6.peg.374	Nickel transport system permease protein nikB2 (TC 3.A.1.5.3)
	nikR	fig|1236544.6.peg.377	Nickel responsive regulator NikR
Chromium	*ChrA*	fig|1236544.6.peg.2107	Chromate transport protein ChrA

Table 3-3 Antibiotic susceptibility of *S. algae* SA2 determined by the Vitek 2.

The Gram-negative Identification Card was used and the CLSI* *Enterobactericae* model with *Escherichia coli* selected as standards (as *Shewanella* was not in their system). *CLSI – Clinical and Laboratory Standards Institute; MIC = Minimum inhibitory concentration.

Antibiotic	*S. algae* SA2	
	MIC (μg/mL)	Interpretation
Ampicillin	≤ 2	Susceptible
Amoxicillin/clavulanic acid	≤ 2	Susceptible
Ticarcillin/clavulanic acid	≤ 8	Susceptible
Piperacillin/tazobactam	≤ 4	Susceptible
Cefazolin	≥ 64	Resistant
Cefoxitin	≤ 4	Susceptible
Ceftazidime	≤ 1	Susceptible
Ceftriaxone	≤ 1	Susceptible
Cefepime	≤ 1	Susceptible
Meropenem	≤ 0.25	Susceptible
Amikacin	≤ 2	Susceptible
Gentamicin	≤ 1	Susceptible
Tobramycin	≤ 1	Susceptible
Ciprofloxacin	≤ 0.25	Susceptible
Norfloxacin	≤ 0.5	Susceptible
Nitrofurantoin	≤ 16	Susceptible
Trimethoprim	4	Susceptible
Trimethoprim/sulfamethoxazole	≤ 20	Susceptible

Table 3-4 Antibiotic susceptibility of *S. algae* SA2 determined by the CDS method.

Resistance was determined if the diameter was <6mm. Control strains used were *Escherichia coli* ACM 5185 and *Escherichia coli* ACM 5186. *Resistance determined if the diameter was <4mm.

Antibiotic concentration	*S. algae* SA2	
	Diameter	Resistant/Susceptible
Ampicillin (25µg)	12	Susceptible
Apramycin (15µg)	5	Resistant
Augmentin (60µg)	14	Susceptible
Azithromycin (15µg)	8, 14	Susceptible
Cefotaxime (5µg)	11.5	Susceptible
Cefoxatin (µg)	8, 14	Susceptible
Cephalexin (100µg)	8	Susceptible
Chloramphenicol (30µg)	8	Susceptible
Ciprofloxacin (2.5µg)	8, 13	Susceptible
Gentamicin* (10µg)	10	Susceptible
Imipenem (10µg)	12	Susceptible
Kanamycin (50)	9	Susceptible
Nalidixic acid (30µg)	12	Susceptible
Neomycin* (30µg)	9	Susceptible
Streptomycin (25)	10	Susceptible
Sulphafurazole (300µg)	5	Resistant
Tetracycline* (10µg)	8.5	Susceptible
Tircillin/clauvulanic acid (7.5:1) (85µg)	14	Susceptible
Trimethoprim (5µg)	8	Susceptible

3.5 Conclusion

Whole genome sequencing revealed that the *S. algae* SA2 genome contains genes encoding proteins involved in antibacterial drug resistance and bacterial pathogenicity. These insights have identified potential virulence factors that may allow *S. algae* to colonise and proliferate in a variety of niches in humans, including the gastrointestinal tract.

Chapter Four

Differential expression of virulence factors in *Shewanella algae* SA1, an emerging human pathogen

Chapter 4 - Differential expression of virulence factors in *S. algae* SA1

4.1 Compound abstract

Rationale: *Shewanella algae* are Gram-negative facultative anaerobes that are being recognised as emerging human pathogens. To date, there are limited genome sequences of *S. algae* and there have been limited studies investigating the presence and expression of virulence factors in *S. algae*. Here we applied a proteogenomic approach to screen for the presence and expression of potential virulence determinants in *Shewanella algae* strain SA1, an environmental isolate from Sydney, Australia.

Methods: Whole genome sequencing analysis was combined with differential proteomics following the growth of *S. algae* SA1 on traditional and infection relevant media (complex media containing either bile salts, blood, brains and heart). Antibiotic resistance genes were identified by screening the genome through the Antibiotic Resistance Genes Database (ARGD) and antibiotic susceptibility testing was performed using the CDS method and the Vikek 2 automatic system. Lastly, potential virulence factors were identified using the online server MP3.

Results: Comparative whole-cell proteomic analysis carried out on a variety of media confirmed the differential regulation of a number of proteins that have the potential to contribute to the pathogenicity of this organism, including the type VI secretion system (T6SS). These findings demonstrate that whole genome sequencing combined

with comparative proteomics offers insights into the characterisation of factors that may contribute to virulence in emerging pathogens.

4.2 Introduction

Infectious diseases are a major threat to public health and one quarter of deaths worldwide are a result of an infectious pathology incurred by an infectious agent [17]. Bacteria cause disease by expressing distinct sets of virulence factors, which allow them to adhere, colonise and invade target host cells, and evade the host immune system [18]. The rapid speed at which bacteria can evolve gives them an adaptive advantage, allowing them to acquire new virulence traits (often via horizontal gene transfer mechanisms), colonise new environmental and host niches, and withstand selection pressures imposed by antibiotics and various arms of the immune response [195, 196]. Emerging bacterial infectious diseases are defined as an infectious disease that has newly appeared in a population, or are infectious agents that have re-emerged and is increasing in incidence, geographic location or host range [32]. The study of emerging human bacterial pathogens and their mechanisms of pathogenesis is essential to further develop our knowledge and understanding of basic cellular biological principles and mechanisms, host-pathogen interactions and host immunity, and the pursuit of potential therapeutic agents [197].

In the current age, there are many different approaches to studying bacterial pathogens. These include genomics [198-201], proteomics [202, 203], transcriptomics [204], computational biology [205, 206] and other technologies. These allow an integrative and cyclical systems biology approach, which is an interdisciplinary

approach combining different methods and techniques to investigate biological areas in order to gain a more holistic understanding of the problem area, with the notion that 'the sum is greater than the individual parts' [19]. In infectious disease, this would see the integration of multiple 'omics in a high throughput manner to produce data that could be used to construct predictive models of the networks and interactions between the biological components of the host-pathogen interaction [19, 207, 208].

Members of the genus *Shewanella* are Gram-negative facultative rods that have largely been studied because of their ability to demonstrate an electrochemical-active phenotype important for bioelectric catalysis, bioremediation and applications in microbial fuel cells [37]. However, members of this genus are known to be emerging as human pathogens, particularly *S. algae* which has been isolated from numerous soft tissue and blood borne infections [1, 33]. Since the isolation and biochemical characterisation of the species in 1990 [55, 56], approximately 230 case studies have been published. This number is also likely to be under represented because the ability to differentiate between different species of *Shewanella* using standard manual and automated identification systems such as the Vitek 2, MALDI-TOF and Biomeriux API 20NE/20E has been unsuccessful [1, 91] and has led to misidentification of *Shewanella* as other Gammaproteobacteria, such as *Pseudomonas* and *Vibrio*. More recently the identification of different *Shewanella* species [1] has added to this problem. The gradual increase in the temperature of coastal waters provides conditions that are favourable for *Shewanella* spp., which could significantly impact the locations *S. algae* can inhabit. It is important that the virulence potential of *S. algae* is understood and managed before the large-scale industrial use of this organism proliferates. More work

is needed to identify potential virulence factors and the extent to which it causes disease.

Only limited studies have been performed to investigate the pathogenicity of *S. algae*. It is known that the *qnrA* antibiotic resistance gene possibly originated in this species and appears to have been transferred from this organism to others via horizontal gene transfer [143]. To date, there are only 5 draft genome sequences of *S. algae*, of which four are environmental isolates (strains JCM 21037, C6G3, CSB04KR and BrY). Only one draft genome belongs to a clinical isolate (MARS 14) [36]. The genome announcement of MARS 14 investigated potential virulence factors and the resistome, or collection of all antimicrobial resistance genes [209], of *S. algae* MARS 14, however, there have been no proteomic studies focused on investigating the expression of potential virulence factors in this organism.

S. algae SA1 was isolated from the Georges River in Sydney in 2010 alongside *V. cholerae* as a part of a project surveying Sydney waterways for environmental *V. cholerae*. In this study, we present the genome sequence of *S. algae* SA1 and identify potential virulence factors in this emerging human pathogen. Furthermore, we took a 'differential' proteomic approach and investigated the expression of potential virulence factors via whole cell proteomics with SA1 grown in a variety of media, including LB broth, LB agar, blood agar, TCBS agar and brain heart infusion (BHI) broth. The use of both liquid and solid media aims to explore differences that may occur in different bacterial lifestyles, as bacteria grow in communities, or biofilms, in the environment and within the host [210] and this lifestyle differs dramatically from free-

living cells in broth culture. As it is well documented that bacteria are sensitive to environmental changes, such as pH, temperature and stressors [211], this study uses media containing components of eukaryotic cells aimed to represent infection relevant conditions, with bile, blood and brain/heart mimicking enteric and systemic infections.

4.3 Materials and Methods

4.3.1 Strain isolation and storage

S. algae SA1 was isolated from the George's River, Sydney, by enrichment on TCBS agar, known commonly as '*V. cholerae* selective media' as previously described [46]. A diagnostic PCR for *V. cholerae* was carried out with primers designed to target *ompW* [212]. Isolates that were negative for *ompW* were further evaluated by 16S rDNA PCR [213]. SA1 was stored long-term in LB broth supplemented with 20% glycerol at -80°C. Sub-culturing of the isolate was performed on LB agar at 37^{o}C.

4.3.2 Genomic sequencing and annotation

Genomic DNA was extracted from an overnight culture (in 2 mL LB broth at 37°C and 180 rpm) using the Promega Wizard DNA extraction kit following manufacturer's instructions. Ten µg of DNA was prepared via the Nextra DNA Library Preparation Kit and sent to Beijing Genomics Institute for genome sequencing via the Illumina HiSeq platform using paired end sequencing technology. Raw reads from the paired end library were assembled using the A5 pipeline [65, 182, 183]. Genome annotation was performed using the RAST online server and FigFAM release 70 [184].

4.3.3 Phylogenetic analysis

Phylogeny was inferred using PhyloSift, which identifies closely related organisms based on similarities and differences from a set of 37 universally conserved housekeeping proteins. The genes that encode these 37 proteins represent approximately 1% of the core *E. coli* genome [64]. The phylogeny was inferred with FastTree [68], and the tree visualized with FigTree version 1.4.0 (http://tree.bio.ed.ac.uk/software/ figtree/). All complete *Shewanella* and draft *S. algae* genome sequences were obtained from the GenBank ftp website (ftp://ftp.ncbi.nlm.nih.gov/genomes) for phylogeny analysis.

4.3.4 'All vs. all' genome comparison

The N-way BLAST was performed to determine the core genome of the *Shewanella* species. To do this, the nucleotides sequence of all complete *Shewanella* genomes and all draft *S. algae* genomes were downloaded from the NCBI FTP website. These sequences were uploaded to RAST for genome annotation. The amino acid sequences were downloaded, and an 'all vs. all' homology search protocol, deposited in the github repository as cRBLH (https://github.com/cerebis/crblh/ tree/v0.1), was carried out as previously described [67]. A heat map was then generated from the resulting analysis to depict the presence and absence of genes across all genomes.

4.3.5 Antibiotic sensitivity and haemolytic activity testing

Antibiotic susceptibility was carried out using two methodologies; the calibrated dichotomous sensitivity (CDS) test and the Vitek 2 Compact system (Version 05.04 at Concord Hospital). All methods for CDS testing were followed as per the CDS Manual

(6th edition). The two control strains used for CDS testing were *E. coli* ACM 5185 and *E. coli* ACM 5186. Isolates are determined to be resistant to antibiotics if the diameter of the zone of clearing is <6mm (or <4mm for neomycin and tetracycline).

For the Vitek 2 sensitivity testing, SA1 was prepared following the Biomerieux guidelines. The Concord hospital version of GLOBAL, 2003 MIC interpretation guideline, based on the CLSI *Enterobactericae* model with *Escherichia coli,* was selected as standards since *Shewanella* was not in their system. Antibiotic resistance was determined based on the optical density of inoculated wells compared to the standards.

For haemolytic activity testing, SA1 was streaked to single colonies on Columbian horse blood agar and incubated over night at 37°C. Control strains indicative of alpha, beta and gamma haemolysis *(Streptococcus viridans, Streptococcus pyogenes* and *Enterococcus facealis respectively)* were grown alongside and the associated growth characteristics of each on the plate were compared to SA1.

4.3.6 Proteomic analysis

To harvest cells from broth culture, SA1 was grown to early stationary phase in 50 mL LB broth or BHI broth (OD_{595}= 5) in a shaking incubator (170-180 rpm) at 37°C). Protease inhibitors were added to the culture and the cells harvested by centrifuging at 10,000 rpm for 10 minutes. The cell pellets were re-suspended in 1% SDS and 50 mM Tris-hydrochloride (HCl) pH 8.8 buffer and probe sonicated on ice for 3 × 30 second intervals. To harvest cultures from agar plates, 100 µL of stationary phase LB

broth culture of SA1 cells grown overnight in a shaking incubator (170-180 rpm) at 37°C was spread onto an agar plate (LB agar, Columbian horse blood agar or TCBS agar) and incubated overnight at 37°C. The agar plates were flooded with 5 mL PBS and protease inhibitors and the colonies gently scraped from the agar plate. The cells were then pelleted by centrifugation at 10,000 rpm for 10 minutes. The SA1 cell pellets were re-suspended in 1% SDS and 50 mM Tris-HCl buffer and lysed by 3 × 30 second intervals of sonication.

The whole cell protein extracts were reduced with 5 mM Tributylphosphine (TBP), alkylated with 20 mM acrylamide monomers for 90 minutes at room temperature, and precipitated with acetone overnight at -20^{o}C. Proteins were then pelleted and re-suspended in a minimum volume of 1% SDS and 50 mM Tris buffer. Bio-Rad MicroBiospin column equilibrated with 1% SDS and 50 mM Tris buffer was used to desalt the samples.

One hundred and twenty µg of protein was added to 2 × sample buffer and loaded onto 1D-SDS-PAGE and run at 160 V for approximately 1 hr. The gel was fixed with 40% methanol and 10% acetic acid, stained and imaged initially with Flamingo Fluorescent Gel Stain and a PharosFX Plus imager (Bio-Rad) followed by colloidal Coomassie Blue G-250 staining and imaging with an Epsom 4800 Perfection scanner. Gel lanes containing the resolved proteins were sliced into 14 equal pieces and proteins were subjected to in-gel trypsin digestion followed by LC-MS/MS on a QSTAR Elite to idnetify the peptides as previously described [214].

The SA1 genome was uploaded onto PEAKS Studio v.7.0 to use as the search database. Mascot generic format (mgf) files of the data from the LC-MS/MS were created using the data import filter on Mascot Daemon v.2.4. The following parameters were permitted for the SA1 database search: peptide score of 10 logP > 20; parent mass tolerance 100 ppm; fragment mass error tolerance 0.2 Da; monoisotopic precursor mass search type; 3 maximum missed cleavage sites; a maximum of 3 missed cleavage sites per peptide; a false discovery rate of <1%; and variable modifications including deamidation (NQ), oxidation (M) and propionamide (C). For a protein to be identified, it had to be present in 2 or more of the biological replicates and have more than 1 peptide identifying the protein. Lastly, any protein identified to be 'hypothetical' underwent BLASTp analysis to identify potential protein function.

4.3.7 Identification of potential virulence factors

Proteins believed to be involved in bacterial pathogenesis were identified by genome annotation via RAST and by using the online server MP3. RAST assigns proteins to a specified subsystem based on abstract functional roles, whilst MP3 uses a combination of Support Vector Machine (SVM) and Hidden Markov Model (HMM) to predict pathogenic proteins based on dipeptide composition (the fractions of amino acids and their local order) and Pfam domains (protein functional domains), respectively [185].

4.4 Results

4.4.1 Genome sequencing and assembly

Once assembled via A5, the draft genome of *S. algae* SA1 was assembled into 142 scaffolds to produce a 5,112,347 bp genome that had a G+C content of 52.7% (Table

4-1). Assembly generated a sequence with median scaffold length (N50) of 212,306 bp, an N90 of 44,372 bp and an average scaffold size of 55,013 bp. The annotation by RAST revealed there to be 4,556 DNA coding sequences and 119 RNA coding sequences. Genome annotations are available in Table S-3. The overall functional profile of the genome as determined by RAST is shown in Figure 4-1, where 48% of the coding sequence (CDS) was assigned to a subsystem.

Table 4-1 Genome properties of *S. algae* SA1.

Gene feature	Value	% of Total
Genome size	5,112,347 bp	100
DNA G+C	2,6942,07 bp	52.7
DNA Scaffolds	142	-
Total genes	4,675	100
Protein coding genes	4,556	97.5
RNA genes	119	2.5
Genes with function prediction	3,092	66.1

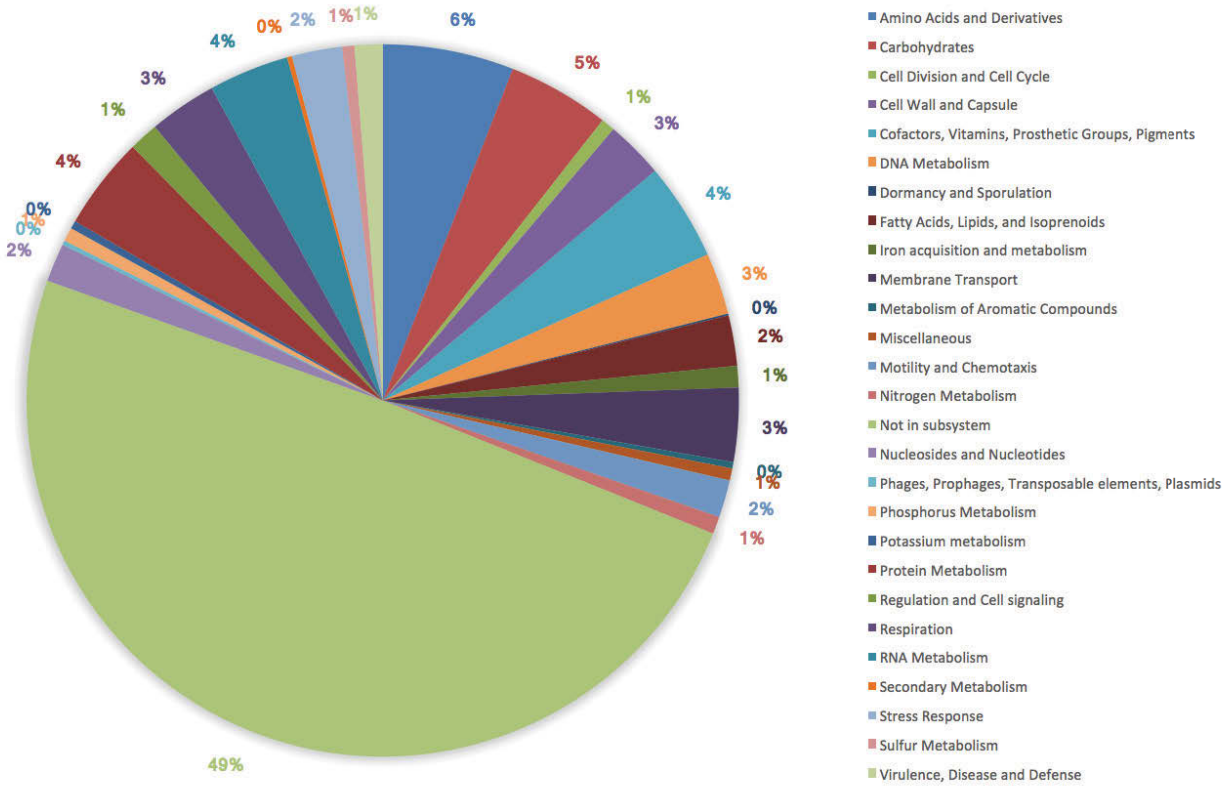

Figure 4-1 RAST subsystem analysis of *S. algae* SA1.

4.4.2 Phylogenetic analysis

Phylogenetically, all *S. algae* cluster in their own clade, with the closest relative identified as *Shewanella amazonensis* SB2B, isolated from Amazonian shelf muds [70]. SA1 clusters with other environmental *S. algae* strains C6G3, CSZ04KR, and JCM 21037 (Figure 4-2), which were isolated from the upper 2cm of muddy sediment in the Arcachon Bay on the southwest coast of France [81], the gut of a sea cucumber *Apostichopus japonicus* in South Korea, and the surface of red alga (*Jania* species) [55], respectively, suggesting they have a common ancestor. The clinical *S. algae* strain MARS 14, from a patient with pneumonia [36], clustered separately from the environmental *S. algae* strains. Interestingly, the environmental strain BrY, isolated

from sediment in the Great Bay estuary in New Hampshire [76], is phylogenetically distinct from all other *S. algae* strains.

4.4.3 'All vs. all' genome comparison

Results of the 'all vs. all' protein comparison between all complete *Shewanella* genomes and draft *S. algae* genomes are depicted in the heat map in Figure 4-3. In this analysis, we included 26 *Shewanella* genomes (with 15 different species). Members determined to be of a similar species by this analysis have been grouped together and this determined the order of genomes on the right hand side. The pangenome contained 8,527 proteins (Table S-4); of this 20% make up the core *Shewanella* genome. Within the defined parameters, there appeared to be 82 protein families exclusively conserved within the *S. algae* genomes, the majority of which are annotated as 'hypothetical' proteins and have no known function.

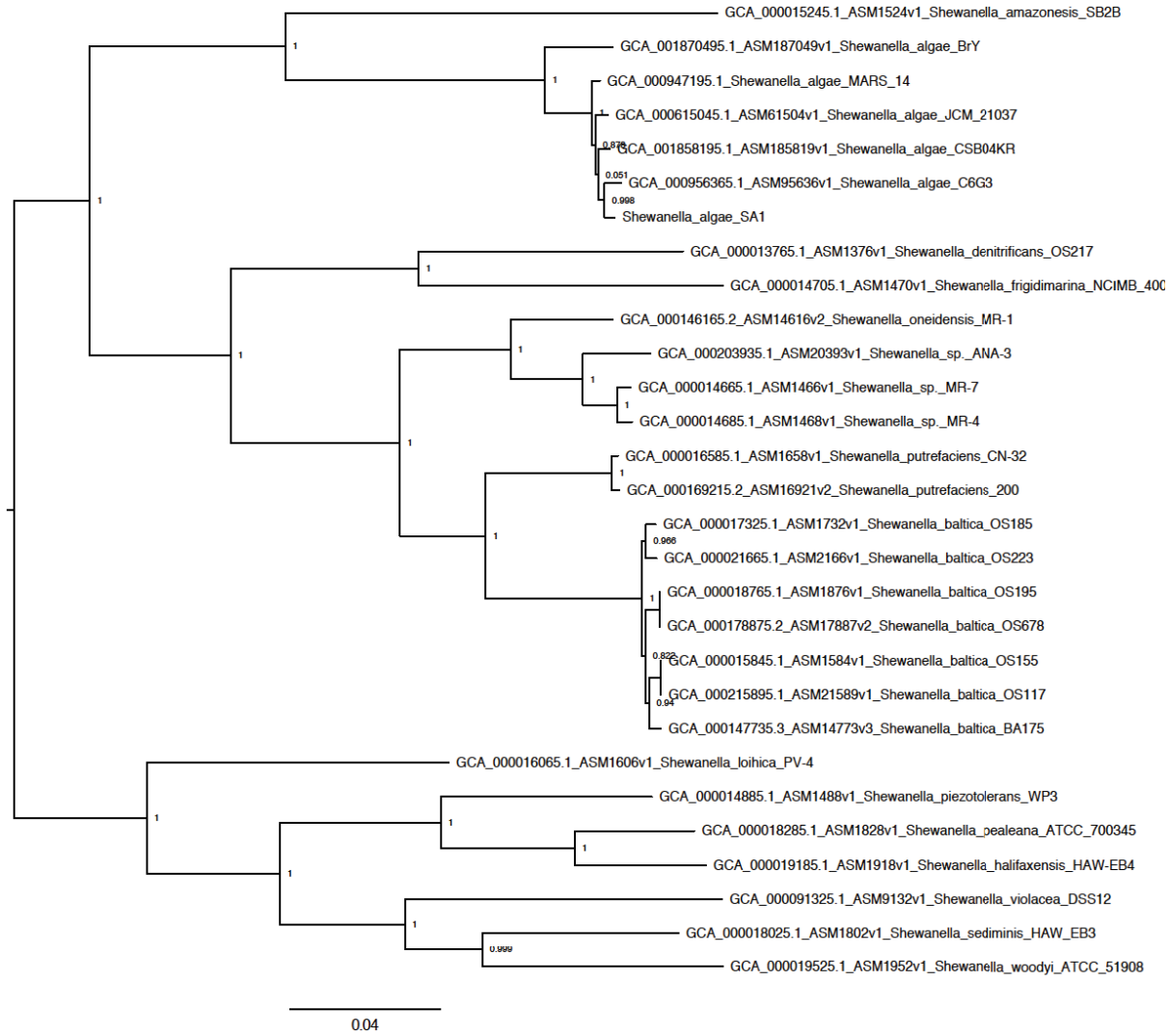

Figure 4-2 Phylogeny of the complete *Shewanella* and draft *S. algae* genomes, as determined by PhyloSift.

The x-axis represents the substitutions per site and the numbers at each node give the clade confidence value.

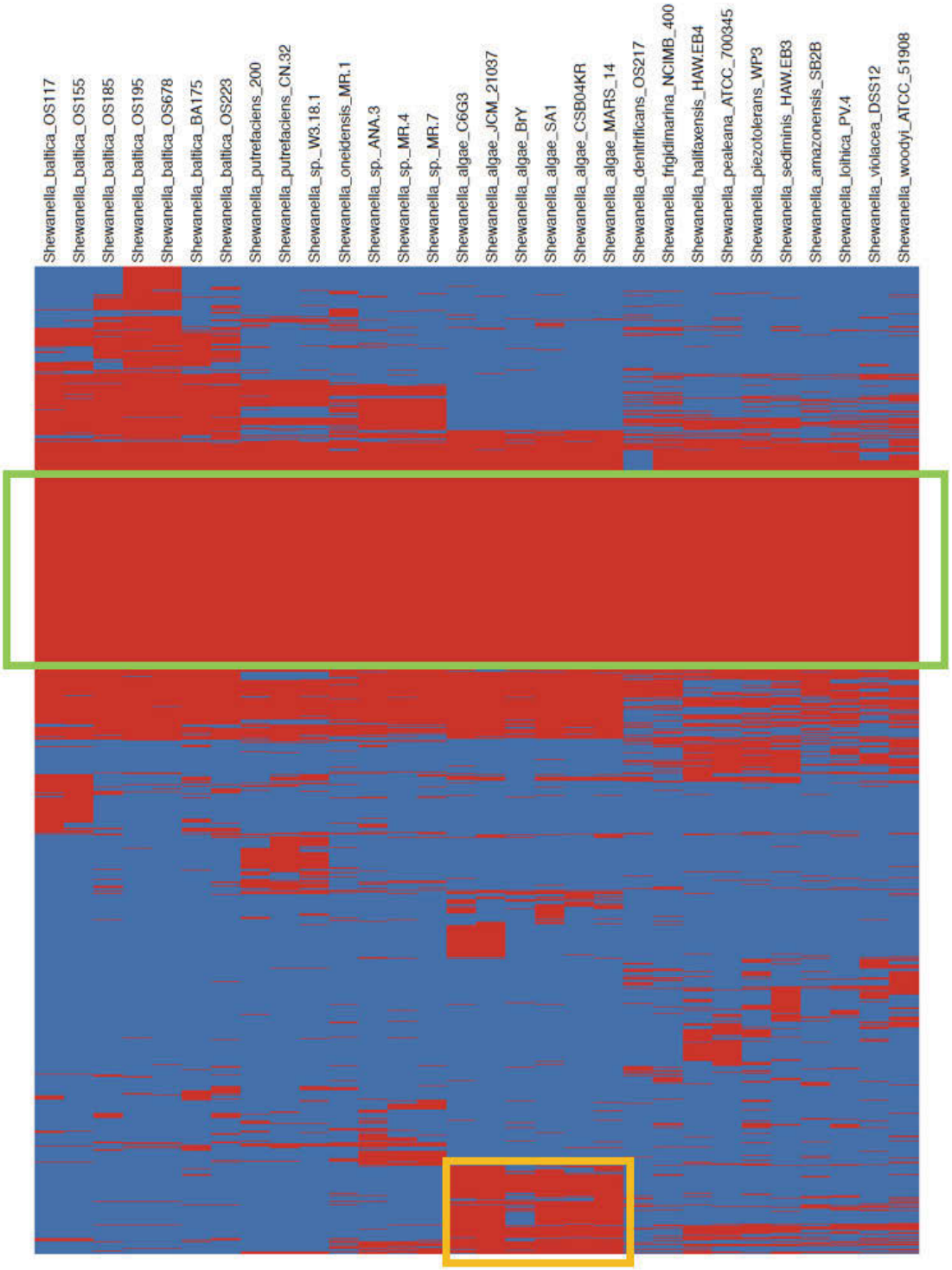

Figure 4-3 A heat map depicting the results of the 'all vs. all' protein clustering genome comparison. Each pixel represents the occupancy for individual protein clusters (of which there are 8,527), where red designates the presence of that cluster and blue denotes the absence of that cluster. The green box represents the core genome of the Shewanella genus (approximately 1705 genes), and the orange box represents 82 genes unique to *S. algae* species.

4.4.4 Antibiotic susceptibility and haemolytic activity

Antibiotic susceptibility carried out by the Vitek revealed SA1 to be resistant to cefazolin, a cephalosporin (Table 4-2), and to sulfafurazole, a sulphonamide, and apramycin, an aminoglycoside, using the CDS disc-diffusion method (Table 4-3). The growth of SA1 on Columbian horse blood agar plates revealed it to be α-haemolytic, compared to the control strains, indicating it causes the incomplete lysis of red blood cells (Figure 4-4).

Table 4-2 Antibiotic susceptibility of *S. algae* SA1 determined by the Vitek 2.

The Gram-negative Identification Card and the CLSI* *Enterobactericae* model with *Escherichia coli* selected as standards (as *Shewanella* was not in their system) were used. *CLSI – Clinical and Laboratory Standards Institute; MIC = Minimum inhibitory concentration.

Antibiotic	*S. algae* SA1	
	MIC (μg/mL)	Interpretation
Ampicillin	≤ 2	Susceptible
Amoxicillin/clavulanic acid	≤ 2	Susceptible
Ticarcillin/clavulanic acid	≤ 8	Susceptible
Piperacillin/tazobactam	≤ 4	Susceptible
Cefazolin	≥ 64	Resistant
Cefoxitin	≤ 4	Susceptible
Ceftazidime	≤ 1	Susceptible
Ceftriaxone	≤ 1	Susceptible
Cefepime	≤ 1	Susceptible
Meropenem	≤ 0.25	Susceptible
Amikacin	≤ 2	Susceptible
Gentamicin	≤ 1	Susceptible
Tobramycin	≤ 1	Susceptible
Ciprofloxacin	≤ 0.25	Susceptible
Norfloxacin	≤ 0.5	Susceptible
Nitrofurantoin	≤ 16	Susceptible
Trimethoprim	2	Susceptible
Trimethoprim/sulfamethoxazole	≤ 20	Susceptible

Table 4-3 Antibiotic susceptibility of *S. algae* SA1 determined by the CDS method.

Resistance was determined if the diameter was <6mm. Control strains used were *Escherichia coli* ACM 5185 and *Escherichia coli* ACM 5186. *Resistance determined if the diameter was <4mm.

Antibiotic concentration	*S. algae* SA1	
	Diameter	Resistant/Susceptible
Ampicillin (25µg)	14.5	Susceptible
Apramycin (15µg)	3.5	Resistant
Augmentin (60µg)	15	Susceptible
Azithromycin (15µg)	11, 17	Susceptible
Cefotaxime (5µg)	14	Susceptible
Cefoxitin (µg)	13	Susceptible
Cephalexin (100µg)	9	Susceptible
Chloramphenicol (30µg)	10.5	Susceptible
Ciprofloxacin (2.5µg)	12	Susceptible
Gentamicin* (10µg)	8	Susceptible
Imipenem (10µg)	9.5, 11	Susceptible
Kanamycin (50)	7.5	Susceptible
Nalidixic acid (30µg)	10	Susceptible
Neomycin* (30µg)	8	Susceptible
Streptomycin (25)	9	Susceptible
Sulphafurazole (300µg)	4	Resistant
Tetracycline* (10µg)	5.5	Susceptible
Tircillin/clauvulanic acid (7.5:1) (85µg)	15.5	Susceptible
Trimethoprim (5µg)	9, 12	Susceptible

Figure 4-4 Alpha haemolytic activity of *S. algae* SA1.

Alpha haemolysis is shown by the green tinge around the SA1 colonies, caused by the partial haemolysis of red blood cells.

4.4.5 Proteomic analysis

The 1D-SDS-PAGE of the SA1 differential proteomes can be seen in Figure 4-5, in which each lane contains 120 μg of protein and represents a biological replicate of the respective growth media. Proteins were identified if they were present in two or more of the biological replicates and had more than one peptide identifying the protein (Table S-5 contains the SA1 proteome Peaks output, with all PEAKS protein scores, the number of peptides identifying the protein, the number of unique peptides, and the percentage of protein coverage). Proteomic analysis of SA1 from growth in LB and BHI broth and on LB, blood and TCBS agar identified a total of 1,192 proteins, with 602 in LB broth, 521 on LB agar media, 438 on TCBS agar, 410 on blood agar, and 1,028 in BHI broth (Figure 4-6, Table S-6). In total, only 195 proteins were found in common between all growth conditions.

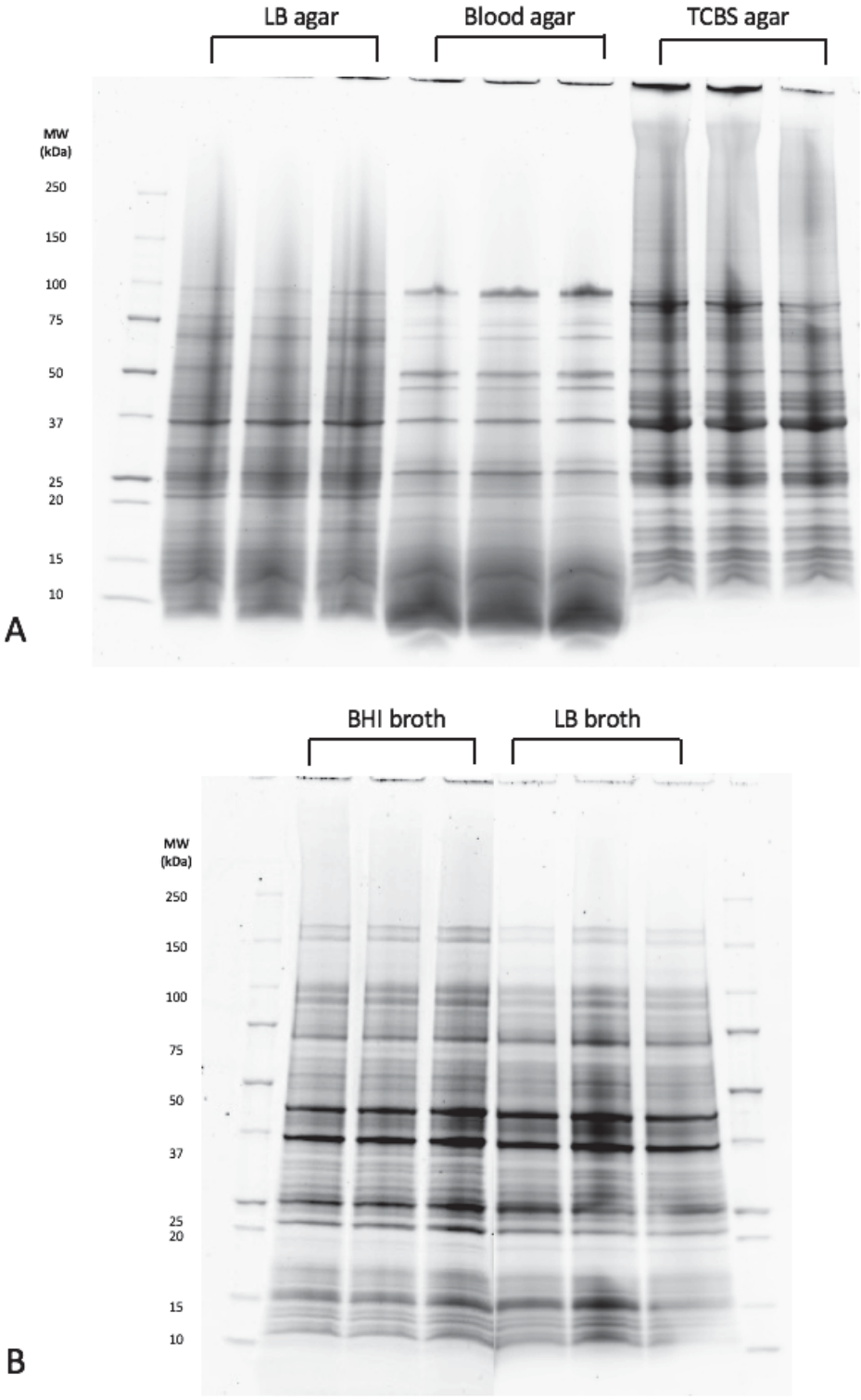

Figure 4-5 1D-SDS-PAGE of the SA1 differential proteome.

(A) depicts the proteome from LB agar, blood agar and TCBS agar, whilst (B) depicts the proteome grown in LB and BHI broth. Each lane contains 120μg of protein and represents a biological replicate of the respective growth media.

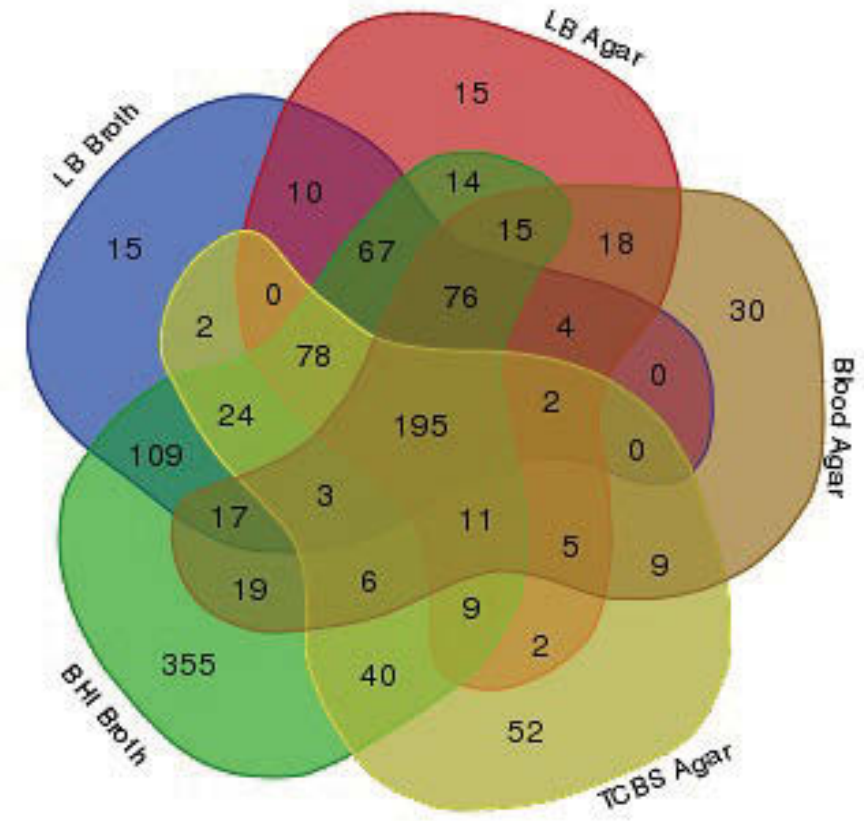

Figure 4-6 Venn diagram of the differentially expressed proteins identified in the *S. algae* SA1 proteome in LB broth, LB agar, blood agar, TCBS agar and BHI broth.

4.4.6 Identification of potential virulence factors

The RAST server annotates genomes by predicting the most closely related protein family for each open reading frame in the genome and categorises them into different subsystems, including those involved in 'virulence, defence and disease', which incorporate genes involved in pathogenicity, and the resistance to antibiotics and toxic compounds. RAST showed the SA1 genome has multiple genes encoding resistance to antibacterial and toxic compounds (Table S-3). SA1 encoded genes for multidrug efflux pumps (*arcB, RNA, MATE, ydhE/norM, mtrF, cmeB, cmeA, cmeC* and *tolC*) and proteins involved in the resistance to toxic compounds include arsenic (*arsA, arsB*, *arsC*, *arsR*, *cymA* and *ARC3*), bile hydrolysis (*bsh* and *damX*), cobalt-zinc-cadmium (*cusA*, *czcB*,

czcD, and *czcR*), copper (*CIA/CSA*, *CRTR*, *ccmI*, *ccmH*, *clfA*, *cutA*, *cutE*, *corC*, *scsB*, *scsC*, and *scsD*), chromium (*chrA*), zinc (*zraR*), and nickel (*nikR*) as well as genes encoding resistance to colicin (*creA, creD*).

MP3, which uses a combined approach of SVM and HMM to predict pathogenic proteins, was also used to identify potential virulence factors in the *S. algae* SA1 genome. The output from this server gives either HMM, SVM and hybrid determination of protein pathogenicity, based on the positive results for protein identification for HMM, SVM, and both HMM and SVM (hybrid) respectively. In total, MP3 identified 1,393 proteins to be involved in pathogenicity, of which 120, 1,063, and 210 were determined by HMM, SVM, and hybrid (Table S-7). These genes encode numerous categories of virulence factors, including those involved in motility and chemotaxis, protein degradation, production of bacteriocins, toxins, haemolysins, antibiotic resistance, and the resistance to toxic compounds.

To determine the expression of these putative virulence factors, we looked at the SA1 whole cell proteome grown under different conditions to check whether the predicted genes were constitutively expressed or differentially expressed. In total, 231 proteins were identified (Table 4-4, Figure 4-7). BHI medium induced the expression of the highest number of virulence factors (165 putative virulence proteins identified) of which 55 were unique to this medium. This was followed by LB agar, which expressed 112 virulence factors and 5 uniquely, then TCBS agar, LB broth and blood agar, with 107, 96 and 82 virulence factors expressed and 2, 20 and 10 unique to the respective medium.

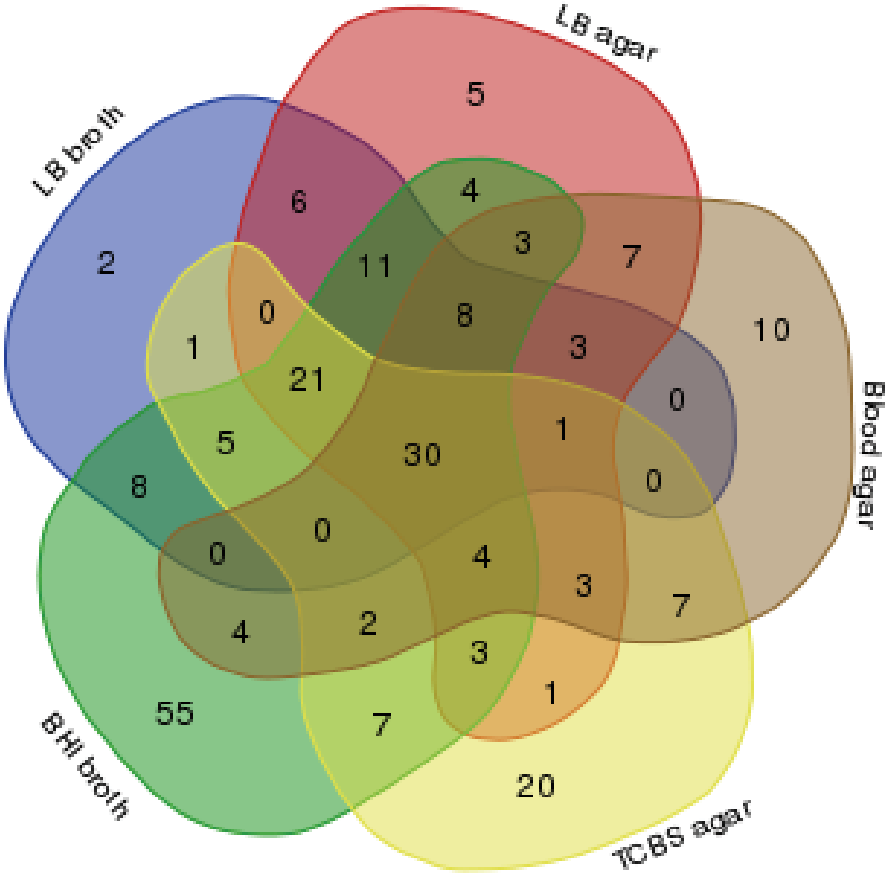

Figure 4-7 A Venn diagram of the differentially expressed virulence factors identified in the *S. algae* SA1 proteome in LB broth, LB agar, blood agar, TCBS agar, and BHI broth.

Table 4-4 Potential virulence factors expressed in the *S. algae* SA1 differential proteome.

Protein name	Protein Location	Proteins identified by MP3			Proteins present in the *S. algae* core genome	LB broth	LB agar	Blood agar	TCBS agar	BHI broth
		Hybrid	HMM	SVM						
21 kDa hemolysin precursor	Scaffold 9.1|size154538 31548 30979		✓							✓
3-oxoacyl-[acyl-carrier protein] reductase (EC 1.1.1.100)	Scaffold 34.1|size41106 35054 34308			✓		✓	✓	✓	✓	✓
4-hydroxyphenylpyruvate dioxygenase (EC 1.13.11.27)	Scaffold 16.1|size90721 29856 30932		✓			✓	✓	✓		✓
ABC transporter ATP-binding protein	Scaffold 1.1|size386180 23768 22878			✓			✓	✓		✓
ABC transporter ATP-binding protein uup	Scaffold 6.1|size257651 254901 252988	✓								✓
ABC transporter protein	Scaffold 7.1|size227575 78690 80342			✓				✓		
ABC transporter, ATP-binding protein	Scaffold 13.1|size109835 57813 59480		✓			✓	✓		✓	✓
ABC transporter, ATP-binding/permease protein	Scaffold 7.1|size227575 80342 82441			✓				✓		
Acetyltransferase, GNAT family	Scaffold 12.1|size131859 77999 77550			✓						✓
Aculeacin A acylase	Scaffold 12.1|size131859 74872 77433	✓						✓		
Aerobactin siderophore receptor IutA	Scaffold 3.1|size313968 158112 155875			✓		✓	✓	✓		✓
Agglutination protein	Scaffold 14.1|size254705 124076 125479		✓			✓	✓	✓	✓	✓
Alkyl hydroperoxide reductase protein C (EC 1.6.4.-)	Scaffold 25.1|size57286 54467 53898		✓							✓
Alkyl hydroperoxide reductase subunit C-like protein	Scaffold 3.1|size313968 232338 232943		✓			✓	✓	✓	✓	✓
Alpha-aspartyl dipeptidase Peptidase E (EC 3.4.13.21)	Scaffold 2.1|size354851 73356 74066			✓		✓	✓			✓
Amidophosphoribosyltransferase (EC 2.4.2.14)	Scaffold 3.1|size313968 113009 114523									✓
Aminopeptidase	Scaffold 5.1|size304996 170216 168813		✓						✓	✓
Ankyrin repeats containing protein	Scaffold 3.1|size313968 78928 79647			✓						✓
Antioxidant, AhpC/Tsa family	Scaffold 18.1|size91380 23300 23773		✓			✓	✓			✓
Arsenate reductase (EC 1.20.4.1)	Scaffold 6.1|size257651 117701 118051					✓	✓			✓
Arylsulfatase (EC 3.1.6.1)	Scaffold 20.1|size72974 39598 39047		✓			✓	✓		✓	✓
ATP-dependent Clp protease ATP-binding subunit ClpX	Scaffold 3.1|size313968 7128 8408		✓			✓				✓
ATPase involved in DNA repair	Scaffold 23.1|size66068 65018 65566			✓						✓
Azurin	Scaffold 17.1|size87895 23012 23479			✓		✓	✓			
Bacterioferritin	Scaffold 5.1|size304996 59981 60454			✓				✓		✓
BatA (Bacteroides aerotolerance operon)	Scaffold 16.1|size90721 45960 46949	✓								✓
Biphenyl-2,3-diol 1,2-dioxygenase III-related protein	Scaffold 1.1|size386180 37432 37815		✓							✓
Chitin binding protein	Scaffold 22.1|size67676 39726 41168			✓			✓			✓

Cobalt-zinc-cadmium resistance protein CzcA; Cation efflux system protein CusA	Scaffold 1.1|size386180 313887 310681			✓		✓			
Colicin I receptor precursor	Scaffold 3.1|size313968 20266 22179			✓	✓	✓	✓		
Copper metallochaperone, bacterial analog of Cox17 protein	Scaffold 1.1|size386180 185040 184573			✓	✓	✓			
Cytochrome c heme lyase subunit CcmH	Scaffold 40.1|size25507 4328 5581				✓			✓	✓
Cytochrome c-type biogenesis protein CcmG/DsbE, thiol:disulfide oxidoreductase	Scaffold 40.1|size25507 1881 1327		✓		✓	✓		✓	✓
D-alanyl-D-alanine carboxypeptidase (EC 3.4.16.4)	Scaffold 35.1|size35122 8676 7495	✓			✓	✓		✓	✓
DNA gyrase subunit A (EC 5.99.1.3)	Scaffold 10.1|size148235 10143 7504				✓	✓	✓	✓	✓
DNA gyrase subunit B (EC 5.99.1.3)	Scaffold 30.1|size46769 8596 10938				✓	✓	✓	✓	✓
DNA-directed RNA polymerase beta subunit (EC 2.7.7.6)	Scaffold 45.1|size16040 11913 7882				✓	✓	✓	✓	✓
DNA-directed RNA polymerase beta' subunit (EC 2.7.7.6)	Scaffold 45.1|size16040 7797 3586				✓	✓	✓	✓	✓
Esterase ybfF (EC 3.1.-.-)	Scaffold 6.1|size257651 18036 17293		✓						✓
FAD/FMN-containing dehydrogenases	Scaffold 7.1|size227575 200888 200397			✓	✓				✓
Ferrichrome-iron receptor	Scaffold 14.1|size254705 11754 9328			✓	✓	✓			✓
Ferrichrome-iron receptor	Scaffold 37.1|size26884 18211 20295			✓					✓
FIG001592: Phosphocarrier protein kinase/phosphorylase, nitrogen regulation associated	Scaffold 5.1|size304996 109401 111635			✓					✓
FIG002095: hypothetical protein	Scaffold 35.1|size35122 19462 19962			✓	✓	✓	✓		✓
FIG002903: a protein of unknown function perhaps involved in purine metabolism	Scaffold 11.1|size212306 177399 178016			✓	✓				✓
FIG00950053: hypothetical protein	Scaffold 7.1|size227575 183784 184287			✓					✓
FIG01056714: hypothetical protein	Scaffold 61.1|size3298 1664 3142			✓	✓	✓	✓	✓	✓
FIG01056995: hypothetical protein	Scaffold 4.1|size281651 26025 26480			✓	✓			✓	✓
FIG01057098: hypothetical protein	Scaffold 6.1|size257651 122618 121473			✓					✓
FIG01057138: hypothetical protein	Scaffold 12.1|size131859 42099 41773			✓	✓				
FIG01057229: hypothetical protein	Scaffold 19.1|size145815 130030 130560			✓					✓
FIG01057367: hypothetical protein	Scaffold 6.1|size257651 192047 192364			✓					✓
FIG01057974: hypothetical protein	Scaffold 9.1|size154538 66270 65692			✓	✓	✓		✓	✓
FIG01057997: hypothetical protein	Scaffold 14.1|size254705 135887 134922			✓				✓	
FIG01058172: hypothetical protein	Scaffold 5.1|size304996 35292 34552			✓		✓		✓	✓
FIG01058394: hypothetical protein	Scaffold 6.1|size257651 33455 34084			✓					✓
FIG01058520: hypothetical protein	Scaffold 3.1|size313968 294719 293781			✓					✓
FIG01059796: hypothetical protein	Scaffold 13.1|size109835 97076 95967			✓			✓	✓	
FIG014328: hypothetical protein	Scaffold 1.1|size386180 184560 183589			✓	✓	✓	✓	✓	✓
FIG067310: hypothetical protein	Scaffold 11.1|size212306 141691 139637			✓		✓	✓	✓	
FIG139976: hypothetical protein	Scaffold 6.1|size257651 142052 143557			✓	✓			✓	
Flagellar biosynthesis protein FliL	Scaffold 14.1|size254705 191196 191603		✓			✓			✓
Flagellar biosynthesis protein FliL	Scaffold 15.1|size91351 55754 55230		✓		✓	✓		✓	✓
Flagellar biosynthesis protein FliS	Scaffold 15.1|size91351 68177 67851		✓		✓	✓	✓	✓	✓

Flagellar hook protein FlgE	Scaffold 15.1|size91351 81507 80146	✓				✓			✓	✓
Flagellar protein FlgO	Scaffold 15.1|size91351 88249 87641			✓		✓	✓		✓	✓
Flagellar protein FlgP	Scaffold 15.1|size91351 87641 87174	✓							✓	
Flagellar regulatory protein FleQ	Scaffold 15.1|size91351 67572 66142		✓							✓
Flagellin protein FlaA	Scaffold 15.1|size91351 71315 70497			✓		✓	✓	✓	✓	✓
Flagellin protein FlaA	Scaffold 15.1|size91351 72352 71531			✓		✓	✓		✓	✓
Formate hydrogenlyase transcriptional activator	Scaffold 3.1|size313968 166658 165135			✓						✓
General secretion pathway protein D	Scaffold 14.1|size254705 229368 231491	✓						✓	✓	✓
General secretion pathway protein E	Scaffold 14.1|size254705 231484 233049		✓					✓		✓
General secretion pathway protein G	Scaffold 14.1|size254705 234326 234760	✓				✓				✓
GGDEF domain family protein	Scaffold 2.1|size354851 340111 341667			✓						✓
Glyoxalase family protein	Scaffold 5.1|size304996 34442 34092		✓				✓	✓		✓
Hcp (*TssD*)	Scaffold 36.1|size31486 31468 31163			✓						✓
Hcp (*TssD*)	Scaffold 53.1|size10419 8937 8533			✓			✓	✓	✓	✓
High-affinity choline uptake protein BetT	Scaffold 1.1|size386180 36296 34311			✓					✓	
HlyD family secretion protein	Scaffold 8.1|size194190 86980 86117			✓					✓	
Hydrogen peroxide-inducible genes activator	Scaffold 5.1|size304996 105312 106220			✓					✓	✓
Hypothetical protein	Scaffold 10.1|size148235 45811 44927			✓	✓				✓	
Hypothetical protein	Scaffold 11.1|size212306 21920 23209			✓	✓				✓	
Hypothetical protein	Scaffold 14.1|size254705 166879 167247			✓	✓	✓	✓		✓	✓
Hypothetical protein	Scaffold 17.1|size87895 22803 22282			✓	✓		✓			
Hypothetical protein	Scaffold 17.1|size87895 24784 24209			✓	✓		✓			✓
Hypothetical protein	Scaffold 18.1|size91380 75682 76137			✓	✓				✓	
Hypothetical protein	Scaffold 21.1|size70319 21684 22100			✓	✓					✓
Hypothetical protein	Scaffold 26.1|size55846 19617 19264			✓	✓		✓	✓		
Hypothetical protein	Scaffold 28.1|size53367 31924 32451			✓	✓	✓	✓			✓
Hypothetical protein	Scaffold 4.1|size281651 206559 205909			✓	✓		✓			
Hypothetical protein	Scaffold 9.1|size154538 126523 127023			✓	✓	✓	✓	✓	✓	✓
IcmF-related protein	Scaffold 36.1|size31486 5290 1730			✓				✓	✓	
IncF plasmid conjugative transfer pilus assembly protein TraE	Scaffold 8.1|size194190 151585 152211			✓						✓
IncF plasmid conjugative transfer pilus assembly protein TraK	Scaffold 8.1|size194190 152195 153091	✓								✓
Inter-alpha-trypsin inhibitor domain protein	Scaffold 3.1|size313968 260527 262839	✓					✓			
L-asparaginase (EC 3.5.1.1)	Scaffold 14.1|size254705 180839 181264			✓						✓
L-aspartate oxidase (EC 1.4.3.16)	Scaffold 5.1|size304996 119110 117500					✓	✓			✓
Lactoylglutathione lyase (EC 4.4.1.5)	Scaffold 11.1|size212306 74660 75070		✓							✓
Large extracellular alpha-helical protein	Scaffold 14.1|size254705 52169 55003,			✓						✓

Late competence protein ComEA, DNA receptor	Scaffold 2.1|size354851 24292 23984		✓				✓	✓		
Lipase-like protein	Scaffold 3.1|size313968 70967 73435			✓				✓		
Lipid A export ATP-binding/permease protein MsbA (EC 3.6.3.25)	Scaffold 2.1|size354851 109415 107661			✓						✓
Lipoprotein	Scaffold 15.1|size91351 975 1448			✓		✓	✓		✓	✓
Lipoprotein-related protein	Scaffold 1.1|size386180 81935 81522			✓		✓	✓		✓	✓
Lipoprotein, putative	Scaffold 1.1|size386180 291797 291090			✓		✓				✓
Lipoprotein, putative	Scaffold 13.1|size109835 8668 9246			✓						✓
Lipoprotein, putative	Scaffold 13.1|size109835 90157 89552			✓		✓	✓	✓	✓	✓
Long-chain fatty acid transport protein	Scaffold 16.1|size90721 54117 55403	✓				✓	✓	✓	✓	✓
Long-chain fatty acid transport protein	Scaffold 9.1|size154538 125026 126372	✓					✓	✓	✓	✓
LppC putative lipoprotein	Scaffold 9.1|size154538 34399 32570	✓							✓	✓
LPS-assembly lipoprotein RlpB precursor (Rare lipoprotein B)	Scaffold 35.1|size35122 16742 16233			✓		✓	✓		✓	✓
LptA, protein essential for LPS transport across the periplasm	Scaffold 23.1|size66068 53097 53630			✓		✓	✓		✓	✓
LysM domain protein	Scaffold 20.1|size72974 62250 61336			✓					✓	✓
Magnesium and cobalt efflux protein CorC	Scaffold 35.1|size35122 27149 26268		✓				✓			✓
Major outer membrane lipoprotein, putative	Scaffold 5.1|size304996 75713 75447	✓					✓	✓	✓	✓
Membrane protein	Scaffold 1.1|size386180 203436 204209			✓				✓	✓	
Membrane protein, putative	Scaffold 53.1|size10419 5110 1544			✓	✓				✓	
Membrane protein, suppressor for copper-sensitivity ScsB	Scaffold 12.1|size131859 39867 39325								✓	
Membrane protein, suppressor for copper-sensitivity ScsD	Scaffold 26.1|size55846 4791 4264	✓							✓	
Membrane-bound lytic murein transglycosylase B precursor (EC 3.2.1.-)	Scaffold 35.1|size35122 10578 9583			✓						✓
Membrane-fusion protein	Scaffold 16.1|size90721 56566 57591			✓					✓	
Methyl-accepting chemotaxis protein	Scaffold 4.1|size281651 79498 81408			✓				✓		✓
Methyl-accepting chemotaxis protein	Scaffold 21.1|size70319 36774 35140			✓						✓
Methyl-accepting chemotaxis protein	Scaffold 33.1|size44372 29299 31305			✓						✓
Methyl-accepting chemotaxis protein I (serine chemoreceptor protein)	Scaffold 4.1|size281651 279148 280737			✓				✓		
Methyl-accepting chemotaxis protein, homolog 13	Scaffold 25.1|size57286 38544 36886			✓		✓	✓		✓	✓
Methyl-accepting chemotaxis protein, homolog 4	Scaffold 7.1|size227575 63529 65439			✓						✓
MltA-interacting protein	Scaffold 4.1|size281651 25221 26009			✓		✓	✓	✓	✓	✓
MoxR-like ATPase in aerotolerance operon	Scaffold 16.1|size90721 43566 44522		✓			✓	✓		✓	✓
MSHA biogenesis protein MshE	Scaffold 7.1|size227575 159651 157897		✓							✓
MSHA biogenesis protein MshI	Scaffold 7.1|size227575 165272 164673			✓					✓	✓
MSHA biogenesis protein MshJ	Scaffold 7.1|size227575 164676 164017	✓							✓	
MSHA biogenesis protein MshL	Scaffold 7.1|size227575 163710 162022	✓					✓	✓	✓	
MSHA pilin protein MshA	Scaffold 7.1|size227575 154955 154440	✓								✓
MSHA pilin protein MshA	Scaffold 7.1|size227575 155477 154971		✓							✓

MSHA pilin protein MshB	Scaffold 7.1|size227575 156114 155512	✓			✓					✓
NAD-dependent malic enzyme (EC 1.1.1.38)	Scaffold 17.1|size87895 3799 5487			✓	✓	✓	✓	✓	✓	✓
Nucleoprotein/polynucleotide-associated enzyme	Scaffold 6.1|size257651 3267 3812			✓						✓
Nucleoside-binding outer membrane protein	Scaffold 7.1|size227575 195847 196599			✓	✓	✓	✓	✓	✓	
OmpA-like transmembrane domain protein	Scaffold 51.1|size8289 4621 4037	✓			✓	✓		✓	✓	✓
Outer membrane lipoprotein omp16 precursor	Scaffold 23.1|size66068 61548 60886	✓			✓	✓		✓	✓	✓
Outer membrane porin, putative	Scaffold 2.1|size354851 10159 8960	✓						✓	✓	✓
Outer membrane porin, putative	Scaffold 4.1|size281651 273463 272375	✓			✓	✓	✓	✓	✓	✓
Outer membrane protein	Scaffold 5.1|size304996 163284 162169	✓			✓	✓	✓	✓	✓	✓
Outer membrane protein	Scaffold 5.1|size304996 279478 281961	✓			✓	✓	✓	✓	✓	✓
Outer membrane protein A precursor	Scaffold 2.1|size354851 242583 243776	✓					✓			
Outer membrane protein assembly factor YaeT precursor	Scaffold 9.1|size154538 77954 77400			✓	✓	✓	✓	✓	✓	✓
Outer membrane protein C precursor	Scaffold 28.1|size53367 40170 42482	✓						✓		
Outer membrane protein H1	Scaffold 29.1|size47000 18189 19031			✓	✓	✓	✓	✓	✓	✓
Outer membrane protein Imp, required for envelope biogenesis / Organic solvent tolerance protein precursor	Scaffold 1.1|size386180 321527 323521			✓						✓
Outer membrane protein OmpK	Scaffold 7.1|size227575 132707 131307	✓						✓		
Outer membrane protein W precursor	Scaffold 1.1|size386180 85809 85129	✓			✓	✓	✓	✓	✓	✓
Outer membrane protein, MtrB	Scaffold 32.1|size45110 24718 26805			✓	✓	✓	✓			✓
Outer membrane protein, MtrE	Scaffold 32.1|size45110 12568 14679			✓			✓			
Outer membrane protein, probably efflux family	Scaffold 1.1|size386180 316311 315037	✓			✓	✓				
Outer membrane receptor for ferric coprogen and ferric-rhodotorulic acid	Scaffold 8.1|size194190 4906 2804			✓	✓	✓	✓			
Outer membrane vitamin B12 receptor BtuB	Scaffold 10.1|size148235 125174 127123	✓								✓
Oxidoreductase, short-chain dehydrogenase/reductase family	Scaffold 6.1|size257651 2430 3248			✓	✓	✓				✓
Paraquat-inducible protein B	Scaffold 11.1|size212306 150167 152788	✓						✓		✓
Phage peptidoglycan binding peptidase	Scaffold 17.1|size87895 30792 29518	✓				✓	✓			
Phage shock protein A	Scaffold 2.1|size354851 31382 30699		✓		✓					✓
Phosphoenolpyruvate carboxykinase [ATP] (EC 4.1.1.49)	Scaffold 14.1|size254705 226721 225180			✓	✓	✓		✓		✓
Phospholipase/carboxylesterase family protein	Scaffold 3.1|size313968 226845 227516	✓								✓
Polysaccharide biosynthesis/export protein	Scaffold 15.1|size91351 29366 26547	✓			✓			✓		✓
Probable Co/Zn/Cd efflux system membrane fusion protein	Scaffold 1.1|size386180 315007 313898			✓	✓	✓				
Protein of unknown function YceH	Scaffold 34.1|size41106 22274 22978			✓						✓
Putative exported protein	Scaffold 2.1|size354851 16367 17065			✓	✓	✓				✓
Putative lipoprotein	Scaffold 20.1|size72974 33394 32096			✓				✓		
Putative membrane protein	Scaffold 14.1|size254705 177833 179122			✓	✓					
Putative membrane protein	Scaffold 32.1|size45110 1027 2793	✓						✓		

Putative nucleotide sugar-1-phosphate transferase	Scaffold 15.1|size91351 24980 24597			✓		✓		✓	✓
Putative periplasmic protein	Scaffold 16.1|size90721 55624 56340			✓	✓	✓		✓	✓
putative secreted protein	Scaffold 24.1|size61336 30637 31785			✓	✓	✓			✓
Putative SpoOM-related protein	Scaffold 4.1|size281651 209703 210443			✓					✓
Putative TonB dependent outer membrane receptor	Scaffold 12.1|size131859 49211 51214			✓	✓	✓			
Quinolinate phosphoribosyltransferase [decarboxylating] (EC 2.4.2.19)	Scaffold 1.1|size386180 376945 377805				✓			✓	✓
Quinolinate synthetase (EC 2.5.1.72)	Scaffold 6.1|size257651 26582 27649								✓
Rare lipoprotein A precursor	Scaffold 35.1|size35122 9578 8805			✓				✓	
Ribonuclease E inhibitor RraA	Scaffold 6.1|size257651 191836 191342			✓		✓	✓		
Ribonuclease E inhibitor RraA	Scaffold 7.1|size227575 177329 177814			✓	✓	✓	✓		✓
RNA-binding protein	Scaffold 14.1|size254705 252081 251632			✓			✓		✓
RND efflux system, inner membrane transporter CmeB	Scaffold 4.1|size281651 107179 110310			✓	✓	✓	✓	✓	✓
RND efflux system, membrane fusion protein CmeA	Scaffold 4.1|size281651 106128 107162			✓	✓	✓		✓	✓
Serine protease, subtilase family	Scaffold 22.1|size67676 29837 32338	✓				✓	✓		
Sigma factor RpoE negative regulatory protein RseB precursor	Scaffold 5.1|size304996 120542 121474			✓					✓
Surface lipoprotein VacJ	Scaffold 15.1|size91351 34764 33892			✓				✓	
T1SS secreted agglutinin (RTX)	Scaffold 14.1|size254705 114865 120384	✓							✓
Thiol peroxidase, Bcp-type (EC 1.11.1.15)	Scaffold 6.1|size257651 112273 112740		✓						✓
Thiol peroxidase, Tpx-type (EC 1.11.1.15)	Scaffold 13.1|size109835 86340 85750		✓			✓		✓	✓
TolA protein	Scaffold 5.1|size304996 19089 18130		✓						✓
TolB protein precursor, periplasmic protein involved in the tonb-independent uptake of group A colicins	Scaffold 5.1|size304996 18120 16795			✓	✓	✓			✓
TonB-dependent hemin , ferrichrome receptor	Scaffold 23.1|size66068 13271 11109			✓	✓	✓	✓		
TonB-dependent receptor	Scaffold 11.1|size212306 77734 75128			✓				✓	
TonB-dependent receptor	Scaffold 18.1|size91380 71877 74027			✓	✓	✓			
TonB-dependent receptor	Scaffold 2.1|size354851 157735 155084			✓			✓	✓	
TonB-dependent receptor	Scaffold 2.1|size354851 65514 63358			✓		✓		✓	
TonB-dependent receptor	Scaffold 2.1|size354851 8540 5787			✓			✓		
TonB-dependent receptor	Scaffold 3.1|size313968 195074 192180			✓	✓	✓	✓	✓	✓
TonB-dependent receptor	Scaffold 3.1|size313968 284542 287130	✓					✓		
TonB-dependent receptor	Scaffold 6.1|size257651 151269 148438			✓		✓	✓	✓	
TonB-dependent receptor	Scaffold 7.1|size227575 99629 101674	✓			✓	✓	✓	✓	✓
TonB-dependent receptor; Outer membrane receptor for ferrienterochelin and colicins	Scaffold 1.1|size386180 287962 290355			✓	✓	✓	✓	✓	✓
TonB-dependent receptor; Outer membrane receptor for ferrienterochelin and colicins	Scaffold 17.1|size87895 17916 15967			✓			✓	✓	
TonB-dependent receptor; Outer membrane receptor for ferrienterochelin and colicins	Scaffold 19.1|size145815 18615 16447			✓	✓	✓	✓		✓
TonB-dependent receptor; Outer membrane receptor for ferrienterochelin and colicins	Scaffold 2.1|size354851 160777 158183			✓	✓	✓	✓	✓	✓

TonB-dependent receptor; Outer membrane receptor for ferrienterochelin and colicins	Scaffold 3.1|size313968 191874 189232	✓					✓	✓		
TonB-dependent receptor; Outer membrane receptor for ferrienterochelin and colicins	Scaffold 4.1|size281651 99745 97559			✓		✓	✓		✓	✓
TonB-dependent siderophore receptor	Scaffold 3.1|size313968 29447 27291			✓		✓	✓	✓		✓
Topoisomerase IV subunit B (EC 5.99.1.-)	Scaffold 1.1|size386180 178634 176748									✓
TPR domain protein, putative component of TonB system	Scaffold 2.1|size354851 1404 154			✓	✓	✓	✓	✓	✓	✓
Translation elongation factor G	Scaffold 24.1|size61336 21590 19608					✓	✓	✓	✓	✓
Translation elongation factor G paralog	Scaffold 45.1|size16040 1984 317					✓	✓	✓	✓	✓
Translation initiation factor 3	Scaffold 6.1|size257651 80468 80016					✓	✓			✓
TRAP-type C4-dicarboxylate transport system, periplasmic component	Scaffold 2.1|size354851 203634 204653			✓			✓	✓		
Twin-arginine translocation protein TatA	Scaffold 7.1|size227575 182310 182555			✓						✓
Two-component system response regulator OmpR	Scaffold 18.1|size91380 27825 27100			✓						✓
Type I secretion outer membrane protein, TolC precursor	Scaffold 1.1|size386180 181432 182748	✓				✓	✓	✓	✓	✓
Type IV pilus biogenesis protein PilE	Scaffold 5.1|size304996 184389 184784	✓					✓			
Type IV pilus biogenesis protein PilQ	Scaffold 9.1|size154538 19429 21483	✓						✓	✓	✓
UDP-sugar hydrolase (EC 3.6.1.45); 5'-nucleotidase (EC 3.1.3.5)	Scaffold 3.1|size313968 228180 229874	✓					✓	✓		✓
Uncharacterized ABC transporter, periplasmic component YrbD	Scaffold 23.1|size66068 48103 47630	✓								✓
Uncharacterized ABC transporter, permease component YrbE	Scaffold 23.1|size66068 48932 48147			✓						✓
Uncharacterized protein ImpA	Scaffold 36.1|size31486 6707 5307			✓				✓	✓	
Uncharacterized protein ImpJ/VasE	Scaffold 36.1|size31486 13645 12314			✓				✓		
UPF0246 protein YaaA	Scaffold 5.1|size304996 167961 168737			✓						✓
Uroporphyrinogen-III methyltransferase (EC 2.1.1.107)	Scaffold 14.1|size254705 103185 104294			✓		✓				✓
VgrG protein (*tssI)*	Scaffold 12.1|size131859 656 1519			✓				✓	✓	
YcfL protein: an outer membrane lipoprotein that is part of a salvage cluster	Scaffold 2.1|size354851 66216 65827			✓			✓	✓	✓	✓
Zinc carboxypeptidase domain protein	Scaffold 10.1|size148235 28615 27488			✓						✓
Zinc-regulated TonB-dependent outer membrane receptor	Scaffold 1.1|size386180 220153 218948		✓						✓	
Total number of proteins identified in the SA1 proteome		**46**	**31**	**155**	**13**	**96**	**112**	**82**	**107**	**165**
Number of unique proteins in the SA1 proteome		**-**	**-**	**-**	**-**	**2**	**5**	**10**	**20**	**55**

4.5 Discussion

Of the 63 species within the genus Shewanella, eight are regarded as emerging human pathogens. *S. algae*, however, is the most clinically prominent, mediating more than 80% of Shewanella infections in reported case studies [1]. *S. algae* has the ability to survive and replicate in diverse habitats, including fresh and salt water, spoilt food, sewage, and in diverse compartments of the human body where it causes disease. At least one outbreak in a hospital is known to have occurred, infecting 24 patients [114] and the incidence of antibiotic resistance within the species is increasing, with reports of multidrug resistant strains isolated from clinical isolates [12, 34-36]. *Shewanella* as a genus is rapidly gaining notoriety for harbouring drug resistance genes, with reports of multidrug resistance *Shewanella* spp., the identification of *S. algae* as the origin of the *qnrA* resistant determinant [143], and the recent identification of a novel variant of the mercury resistant transposon Tn1696 that was housed on a novel plasmid, identified as pSx1. The plasmid encoded a large number of antibiotic resistance genes including *blaOXA-416*, a beta-lactamase [141]. Together, this leads to the recommendation that this bacterium be further investigated for its mechanisms of pathogenicity, ability to cause disease and ability to resist antibiotics, and that it be included in hospital surveillance programs.

SA1 was isolated from the Georges River, Sydney, as part of a study surveying Sydney waterways for the presence of *V. cholerae*. *S. algae* SA1, having the ability to grow on *Vibrio* selective media, was of interest as this media is selective based the ability of organisms to grow in pH 8.5-9.5 and in the presence of bile salts, and the selective ability to utilise sucrose as a carbohydrate source [215]. All isolates underwent

screening of the *ompW* gene by PCR, as *ompW* is a gene that is indicative of *V. cholerae*. The 16S rDNA gene was amplified by PCR and the amplicon was sequenced using Sanger technology to investigate their phylogeny. SA1 was negative for the *ompW* gene, and was determined to be *S. algae* on the basis of 16S rDNA gene sequencing. The similarity of *S. algae* infections to other marine pathogens, including members of the genus *Vibrio* and *Aeromonas* [116], is such that in numerous cases, soft tissue infection or food poisoning by *S. algae* have been misidentified, clearly suggesting diagnostic challenges. It also suggests that *S. algae* may contain virulence factors similar to those found in *Vibrio* species. In order to gain further insights into the pathogenicity of *S. algae* we carried out whole genome sequencing of *S. algae* SA1.

Whole genome sequencing determined the SA1 genome to be 5.11 Mbp, with a G+C content 52.7% and 4,556 DNA coding sequences. In comparison to the other *S. algae* draft genomes sequenced, this is the largest genome with the highest G+C content (Table 2-5). Genome annotation via RAST predicted the function of 2,187 genes, which represent 48% of the genome. The main subsystems to which these genes belonged include amino acid and derivatives, carbohydrates, protein metabolism, cofactors, vitamins, prosthetic groups and pigments, membrane transport and respiration (Figure 4-1). The high percentage of genes unallocated to a subsystem by RAST is not uncommon, as the subsystems are proteins grouped by a relationship in function and are therefore not comprehensive [187]. Furthermore, is it also not uncommon to have a large percentage of genes encode hypothetical proteins, which remain hypothetical due to a lack of experimental studies, with the number of hypothetical genes in genomic databases at almost 4 million [188].

Phylogenetically, *S. algae* cluster together with the closest *Shewanella* relative being *S. amazonensis* S2B2 (Figure 4-2). However, *S. algae* BrY, an environmental isolate, clusters separately from all other *S. algae* strains. The 'all vs. all' genome comparison of all Shewanella genomes, depicted as a heat map in Figure 4-3, identifies the core genome of the Shewanella genus (boxed in green), in which the protein sequences are conserved across members within this genus [216]. This core genome represents approximately 20% of the *Shewanella* pangeome, and consist of typical essential genes that encode proteins involved in protein synthesis, energy metabolism, DNA metabolism, regulatory functions, cellular processes, transcription and protein fate. To be noted, however, is that *S. algae* also appear to have their own set of unique accessory genes (boxed in orange). Accessory genomes in bacterial pathogens are of interest as they can encode genes involved in unique aspects of the pathogenicity as they contain customised genomic repertories, many of which can move laterally between bacterial species [217, 218].

The subset of proteins found unique to *S. algae*, which were determined to be any gene encoding a protein found solely in the six *S. algae* genomes, comprises 82 genes (Table S-4). The majority of these genes (73%) are hypothetical proteins with no assigned function, which again is not uncommon when it is known that 25% of the core genomes of the well known bacteria *Campylobacter jejuni* and *Neisseria meningitidis* are hypothetical proteins [216]. As hypothetical genes can encode unknown virulence factors, the SA1 genome was screened though MP3, an online server used to predict pathogenic proteins based on dipeptide composition via SVM and Pfam domains via HMM. This analysis revealed that 49 of the 82 proteins encode

potential pathogenic proteins (Table S-7), the majority of which are hypothetical proteins. Other identified virulence factors in the *S. algae* core genome include Haemagglutinin 2, which is known to be involved in the adhesion and colonisation of host cells [219], spermidine export protein MdtI which is a component of a multidrug resistance export pump, and a TRP domain protein known to be involved in the translocation of virulence factors [220, 221].

The presence of a subset of proteins unique to *S. algae* could help explain why *S. algae* has the ability to elicit disease and other species within the genus cannot, and further investigations should be conducted on these proteins to characterise their function. *S. algae* BrY, appears to have a significantly different genetic make-up to the other *S. algae* strains, as it does not contain many other genes unique to the other *S. algae* genomes, as seen in Figure 4-3 by the lack of red pixels in the orange box on the *S. algae* BrY line. This, along with the phylogenetic analysis (Figure 4-2), suggests that *S. algae* BrY has had a different evolutionary path compared to other *S. algae* strains.

Analysis of the annotated genome and MP3 results indicated the presence of numerous genes encoding resistance to antibiotic drugs, including fluoroquinolones, quinolones, beta-lactams, and tetracycline, as well as encoding genes for multidrug efflux pumps, and the resistance to toxins and heavy metals. As the incidence of antibiotic resistant *S. algae* strains rise along with the reports of multidrug resistant *S. algae* isolates [12, 34-36] and treatment failure leading to mortality [113], we investigated the antibiotic resistance of *S. algae* SA1. To investigate the expression of the antibiotic resistance genes, we tested the antibiotic resistance profile of SA1 using

two different methodologies and found it to be resistant to cefazolin (cephalosporin) by Vitek 2 analysis and to sulfafurazole (sulfonamide) and apramycin (aminoglycoside) using the CDS disc-diffusion method. We have also reported the identification of the novel *qnrA8* allele in SA1, which we hypothesise to play a role in bacterial fitness and thermotolerance, as it does not confer resistance to quinolones or fluoroquinolones [142].

Whilst SA1 was isolated from an environmental source, the genomic analysis indicated it was equipped with a repertoire of genes that were potentially important for the colonisation, survival and replication in human hosts. In this study, we used proteomics to investigate the differential expression of putative virulence factors in *S. algae* SA1 that might mimic growth conditions encountered in a mammalian host. To do this, we used a variety of complex media containing components of eukaryotic cells, including blood agar, BHI broth, and TCBS agar as well as standard laboratory growth medium at stationary time points. While it is well known that environmental conditions, cell density and growth phase can influence the expression of virulence factors, the early stationary time point was specifically chosen as previous studies have determined the expression of virulence factors to be maximal at late exponential and early stationary growth phases [222, 223] as this is when bacteria are more physiologically active. Future studies could investigate other time points, including mid-exponential and late stationary growth phases to further examine the differential expression of virulence factors. A previous study utilising infection relevant media not only used different growth medium, but also included environmental shocks (growth of media in media with a sudden change in environment e.g. temperature, presence of salt, etc.) to mimic

the exposure of bacteria to the a new 'host' environment to uncover the induction of pathogenicity islands in *Salmonella enterica* serovar Typhimurium [211]. In addition, the use of solid agar media allows for SA1 to grow in a colony biofilm, which is how bacteria grow *in vivo*, as biofilms offer protection from the host environment, host immune system and antibiotics. Previous studies investigating the growth of *S. algae* biofilm in antifouling utilised different growth media to determine the biofilm morphology, thickness, roughness, surface coverage, elasticity and adhesion forces, and found that the biofilm dramatically changes based on the growth media [162], thus showing the importance of simulating real-life conditions to study novel, emerging bacteria.

As seen in the 1D-SDS-PAGE in Figure 5-4, the protein profiles between each biological replicate are identical, however between each of the different media the protein profiles change dramatically, suggesting differential expression of proteins under the different conditions of each growth medium. A total of 1,192 proteins were identified in the *S. algae* SA1 differential proteome (Figure 4-6), with proteins being identified in 2 or more biological replicates with more than 1 peptide identifying the protein. This included 602 in LB broth, 521 on LB agar media, 438 on TCBS agar, 410 on blood agar, and 1,028 in BHI broth. Of interest is the expression of proteins found uniquely in each media, which includes 15 proteins unique to LB agar, 15 to LB broth, 30 to blood agar, 55 to TCBS agar, and 355 in BHI broth. These, along with proteins identified in complex media and not traditional lab culture media, highlight the importance of investigating the differential proteome. These pilot studies provide insight into the identification of growth parameters required for the expression of certain proteins. Previous studies

investigating differential proteomes have not only showed the expression of proteins under certain conditions, but also allowed for the speculation/determination of their function [224-226].

The proteomic data was also interrogated to investigate the expression of putative virulence factors found in the SA1 genome. In total, we identified 231 potential virulence factors, based on the identification of virulence factors from RAST and MP3. Many of the putative virulence factors were differentially expressed depending on growth medium (Table 4-4). MP3 analysis gives three different outputs, which convey if the virulence factors were determined via dipeptide composition in the SVM, and miniPfam domains in HMM. From this analysis, SVM identified 155 putative virulence factors in the SA1 genome and 30 in HMM. Only 46 of the 231 proteins were identified by both SVM and HMM MP3 analysis.

BHI broth expressed the highest number of proteins in total and uniquely, with 165 and 55 respectively. This was followed by LB agar, which expressed 112 virulence factors and 5 uniquely, then TCBS agar, LB broth and blood agar, with 107, 96 and 82 virulence factors expressed and 2, 20 and 10 unique to the respective medium. The virulence factors had identified functions in adhesion, invasion, colonisation, toxins, haemolysins, aiding in the transport of virulence factors and protein degradation and are listed in Table 4. Putative virulence factors that stood out in this analysis include the T1SS secreted agglutinin (RTX), a cytotoxin involved in virulence and adhesion [191-193, 227-229] which was found expressed in BHI broth; mannose-sensitive haemagglutinin (MSHA) pilin protein MshA plays a role in adhesion and biofilm

colonisation [194, 230] expressed in BHI broth; chaperone protein DnaK which plays a role in bacterial colonisation, adhesion and the expression of virulence factors [231-236], which was expressed in all media; and flagellar biosynthesis protein FliL and Flagellin protein FlaA, which are both involved in bacterial motility, adhesion and invasion [237-239]. As some of these proteins play a role in haemolysis, we tested the haemolytic ability of SA1 by growth on blood agar. Strain SA1 possesses α-haemolytic activity (Figure 4-4).

The main virulence factor of interest to us was the identification of proteins involved in the type VI secretion system (T6SS). The type T6SS system is a recently described bacterial export pathway that has been characterised in a range of Gram-negative organisms. It was first characterised in *V. cholerae* where it was correlated with an ability to be produce toxic effects on *Dictyostelium discoideum* and mammalian macrophages [240]. The system is now known to be involved in the export of a variety of effector molecules that can target both prokaryotic and eukaryotic cells, functioning in host cell invasion and virulence [241-243], bacterial competition [244-246], self-recognition [247], horizontal gene transfer [248, 249] and biofilm formation [155]. Although the repertoire of secreted proteins or 'effector molecules' have not been exhaustively catalogued, the genes and proteins forming the structural and functional components of the system have been well characterised [250, 251]. Structurally the T6SS apparatus has been suggested to resemble the canonical form of other secretory systems that utilise a core-element comprising a bacteriophage-like element spanning the bacterial membrane [252]. It is now reported that 25% of Gram-negative genomes encode at least one copy of the T6SS [152].

SA1 was found to possess a complete complement of genes encoding the T6SS, along with two copies of the gene encoding the T6SS effector molecule valine–glycine-repeat-containing protein (VgrG) and three copies of the gene encoding the T6SS effector molecule hemolysin co-regulated protein (Hcp). A BLASTpsi analysis (Figure 4-8) shows the SA1 T6SS is closely related to the T6SS of *V. cholerae* N16919, an O1 serotype *V. cholerae* responsible for causing the disease cholera in endemic areas. Phylogenetically, this T6SS gene cluster is family D, where the effector molecules VgrG and hcp lie upstream of the core components [152]. The presence of the T6SS gene cluster in SA1 suggests that this organism may be adapted to a pathogenic lifestyle which is consistent with its description as an emerging pathogen.

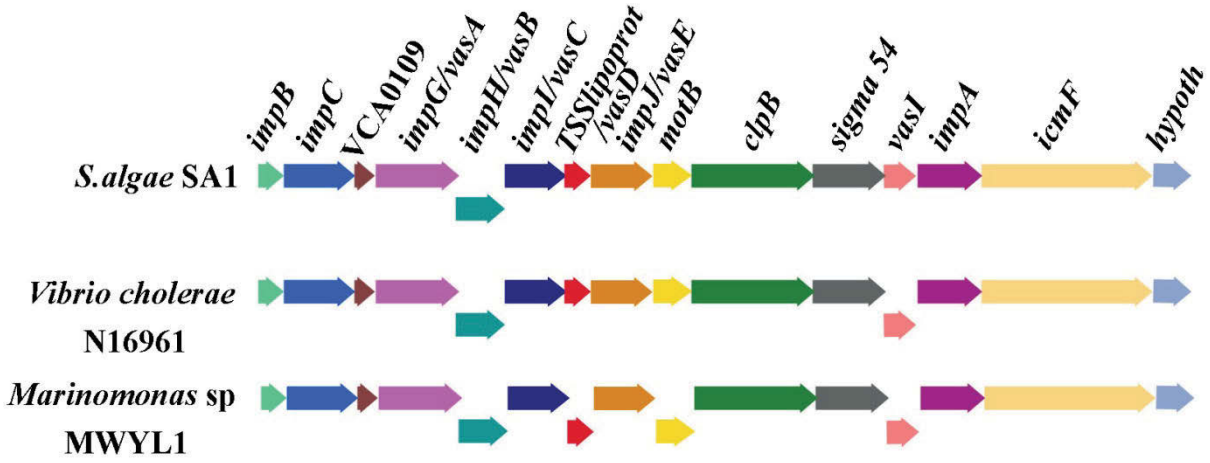

Figure 4-8 The T6SS gene cluster found in SA1 and its closest relatives identified by Psi-BLAST analysis. Sets of genes with similar amino acid sequences are colour coded. E –value cutoffs for colouring CDS sets was 1e-20.

The proteomic analysis of SA1 showed the expression of seven proteins involved in the T6SS, as well as the effector molecules VgrG and hcp. The expression of the proteins in SA1 was dependant on the media, with the structural components of the T6SS TssJ

(expressed in TCBS agar) and TSSM (expressed in blood agar and TCBS agar) responsible for anchoring the T6SS to the cell membrane. TssB (expressed in all media) and TssC (expressed in all media bar LB broth) proteins are homologous to T4 phage contractile tail sheath proteins. TssA (expressed in blood agar and TCBS agar) and TssK (expressed in blood agar) have an unknown function. We would not expect to see all components of the T6SS in this analysis, as some components lie within the bacterial cell membrane and this proteomic analysis was not selective for detecting components of the cell membrane.

VgrG (TssI) is involved in puncturing host cell membranes and also in virulence [253], binding host actin (adhesion) [254], and in the induction of apoptosis [255]. Of the two copies of VgrG, both were expressed, one in blood agar and TCBS agar, and the other in TCBS agar only. Hcp (TssD) is known to be involved in cellular adhesion and invasion [154, 256, 257] as well as the activation of apoptosis and for paralysing host macrophages [258]. Of the three copies of Hcp, all were expressed, with two copies being expressed in LB agar, blood agar, TCBS agar and BHI broth, and one copy expressed in BHI broth. The expression of the T6SS is known to be upregulated in a contact dependant manner with both prokaryotic and eukaryotic cells, and the expression of Hcp and VgrG are indicative of a functional T6SS. As most of the media expressing these proteins are the complex media, it is possible that these conditions are favourable for inducing components of the T6SS in SA1. Furthermore, TCBS supported the expression of most T6SS proteins. It has been noted that bile upregulates T6SS activation in *V. cholerae [259]* and *Salmonella* Typhimurium [260].

While T6SS elements are widespread in Gram-negative bacteria, only two other members of the Shewanellae, *Shewanella woodyi* and *Shewanella frigidimarina*, have identifiable T6SS components. In *Shewanella frigdimarina,* the T6SS was found to be upregulated in the early phases of biofilm formation [155].

4.6 Conclusion

While whole genome sequencing can generate rapid information on the identification and taxonomy of emerging pathogens, the role of virulence factors that may contribute to disease requires further analysis at the protein level. We have utilised a novel approach that combines genome sequence information with comparative differential proteomic data to identify virulence factors in an emerging human pathogen. We suggest that this type of analysis may be particularly significant for the study of emerging pathogens, as delving into the gene expression using infection relevant media gives a more realistic portrayal of bacterial pathogenicity. Our approach has enabled us to identify a panel of virulence genes in *S. algae* SA1, which are upregulated only in the presence of components of eukaryotic cells (including blood, brain, heart, and bile) in the media. This approach allows us to present a comprehensive multi-genome analysis of genetic loci harbouring gene clusters constituting the key differentially regulated T6SS elements.

Chapter Five

Analysis of the *Shewanella algae* SA1 secretome identifies novel virulence factors

Chapter 5 - Analysis of the *Shewanella algae* SA1 secretome identifies novel virulence factors

5.1 Compound abstract

Rationale: The secretion of proteins by bacteria underpins mechanisms to colonise and invade host cells, and as such investigations into the secretome of emerging pathogens are valuable to aid in the identification of potential virulence factors. This is the first study to investigate the secretome of *S. algae* and identify secreted virulence factors.

Methods: *S. algae* SA1 secretomes were harvested at mid-exponential and early stationary growth phases from growth in differential media (LB and BHI broth). Label-free quantitative analysis (spectral counting, emPAI and NSAF) was implemented to determine the true secretion of proteins with a comparison on the protein abundance in the secretome compared to the proteome. Virulence factors were determined by screening the SA1 secretome against MP3, a server which predicts pathogenic proteins.

Results: Analysis of the SA1 secretome with the implemented label-free quantitative analysis scoring system identified 210 proteins in the *S. algae* SA1 secretome, with 50, 125, 25 and 94 proteins present in the LB mid-exponential, LB stationary, BHI mid-exponential and BHI stationary time points respectively. We identified an array of potential virulence factors secreted by *S. algae* SA1 under different growth conditions at different growth phases utilising mass spectrometry and label-free quantitative analysis. Many of these identified virulence factors were moonlighting proteins.

5.2 Introduction

The secretome, or exoproteome, was originally defined as the proteins secreted by cells and the components of machineries used for protein secretion [261]. The secretion of proteins by bacteria underpins mechanisms to colonise and invade host cells, supply nutrients for growth, form biofilms, execute cell communication, and evade immune detection. Several secretion pathways are universally conserved in bacteria and secrete a diverse array of protein cargo while others are restricted to select genera, appear to have been acquired horizontally, and transport a limited repertoire of cargo. Over the past 30 years multiple protein secretion mechanisms have been described and these are distinguished from one another based on structural and functional specificity [190]. Many transported proteins are mobilised across the bacterial inner cell membrane via dedicated protein secretion pathways (Sec and TAT pathways). These secreted proteins carry secretion signal sequences that allow them to be recognised and targeted by the secretion apparatus.

In Gram-negative bacteria, a subset of translocated proteins remain in the periplasmic space only to be shunted across the outer cell membrane by other dedicated secretion machinery while others are promptly shunted across the outer membrane by Sec A and SecYEG machinery and promoted extracellularly. A subset of dedicated virulence factors that are secreted in Gram-positive bacteria contain C-terminal, cell wall anchoring motifs, while a subset of protein in Gram-negative bacteria display C-terminal secretion signals that are recognised by the Type I secretion system. Proteins that are transported across membranes include many enzymes (lipases, proteases, phosphatases), adhesins, toxins, lipoproteins, heme-binding proteins, and

antimicrobial peptides. The requirement for signal sequences and cell wall anchoring sequences led to the development of algorithms that were used to interrogate genome sequence data in an attempt to predict the identity of proteins that are ultimately destined for secretion. These tools have been used extensively in the efforts to identify pathogenicity factors and new vaccine antigens (reverse vaccinology). The secretion of proteins is an energy demanding, complex, and multifaceted process essential to all bacteria. Protein secretion determines how bacteria interact with and colonise their surrounding environment, survive as a community, and cause disease.

The secretion of proteins in Gram-negative bacteria is a tightly regulated process, which occurs through numerous specialised secretion systems, including the Sec pathway, the Tat pathway, and the types I to VI secretions systems (T1SS-T6SS), all of which are known to contribute to bacterial pathogenesis [190]. Many studies investigating pathogenic Gram-negative bacterial secretomes have uncovered novel mechanisms of virulence. The bacterial secretome has been predicted to represent between 10-30% of the genome [262, 263]. Bioinformatic tools, such as PSORTb [264], SecretomeP [265], SignalP [266], and predTAT [267], can be utilised to predict protein secretion. These tools identify secreted proteins based on the presence of signal peptides (PSORTb, SignalP and predTAT), or non-classical protein secretion (proteins secreted that do not contain a signal peptide). While the signal peptide sequences for different pathways are not homologous, they share the same structural composition, facilitating for their identification [268]. The prediction of a bacterial secretome using bioinformatics tools presents a number of difficulties, including that the prediction of

proteins depends entirely on the presence of a well annotated genome, and that not all proteins will be secreted under certain conditions [269].

Whilst genomic studies and bioinformatics tools can predict secreted proteins, proteomic studies have the advantage of investigating the differential expression of proteins in addition to protein abundance [270]. Significant developments in mass spectrometry over the past 15-year has led to the characterisation of proteins present in growth medium. These studies have generated controversy because many report proteins that have canonical functions in the cytosol and that lack signal sequence motifs. The main theories behind the presence of cytosolic proteins in the secretome include: (i) cell lysis within the bacterial population, which releases cytosolic proteins into the extracellular milieu; (ii) the formation of outer membrane vesicles which carry a diverse array of protein cargo; and (iii) the prediction that as yet unknown mechanisms of protein secretion remain to be discovered. The acceptance of proteins in bacterial secretomes that have essential functions in the cytosol, also known as geographic moonlighting proteins [271-274], is rapidly gaining momentum.

The genus *Shewanella* comprises an extremely diverse group of facultative anaerobes that are widely distributed in freshwater and marine environments, including intertidal and benthic zones, their sediments and oil field wastes throughout the world [1, 2]. Members of the *Shewanella* species, particularly *S. algae*, are now being recognised as emerging human pathogens which are isolated from numerous infection sites including blood borne and soft tissue infections. The mechanisms by which *S. algae* can cause disease remain largely unknown. A recently published genome sequence of *S. algae*

MARS 14 identified a number of putative virulence factors including a haemolysin, flagellum and biofilm associated proteins [36]. *S. algae* strains have been reported to carry diverse and novel resistance genes including beta-lactamases, multidrug-efflux pumps [91] an ethanolamine phosphotransferase that encodes resistance to colistin [82, 91] and genes encoding enzymes that alter DNA supercoiling [142].

The number of reported *S. algae* infections is rising. Here we sought to investigate the secretome of *S. algae* SA1 with the aim of identifying potential virulence factors. Secretomes were characterised at mid-exponential and early stationary phases of growth, in 'differential' growth media, namely LB broth and BHI broth. This is the first study to investigate the secretome of *S. algae*.

5.3 Methods

5.3.1 Determination of the growth phases of *S. algae* SA1

SA1 was streaked to single colonies on LB agar and incubated overnight at 37°C. A single colony was transferred to 2 mL LB broth and incubated at 37°C with shaking at 180 rpm. The optical density at 595 nm (OD_{595}) of a 1/10 dilution of the overnight culture was determined and used to dilute the overnight culture to OD_{595} = 0.05 in 50 mL LB broth or BHI broth. The cultures were incubated at 37°C with shaking at 180 rpm and OD_{595} was determined from a 1 mL aliquot sampled every 30 minutes during growth and plotted using GraphPad Prism v.5.0. for Windows, GraphPad Software, La Jolla California USA (www.graphpad.com). These analyses were undertaken as biological triplicates.

5.3.2 SA1 secretome and proteome harvest

Triplicate cultures of SA1 grown in LB broth and BHI broth to either mid exponential (OD_{595} = 0.6) or early stationary growth phase (OD_{595} = 5) were used for secretome studies. Complete ultra protease inhibitors (Roche) were added to the culture upon harvest to stop enzymatic protein degradation and the cells pelleted by centrifuging at 10,000rpm for 10 minutes at 4°C. The cell pellet was washed 3 times with ice cold PBS containing protease inhibitors and frozen at -20°C. Culture supernatants were filtered through a 0.2 µm filter, to remove remaining cellular membrane components and large outer membrane vesicles, and concentrated using a 3 kDa viva spin column until a final volume of 5 mL remained.

5.3.4 Determination of bacterial cell viability

Bacterial cells were strained with Syto 9 and prodidium iodide (PI) to assess cell lysis. At each harvest point, 1 mL of culture was centrifuged (10,000 rpm for 30 seconds), washed 3 times with 0.9% sodium chloride and the pellet was re-suspended in 1 mL 0.9% sodium chlorine containing prodidium iodide and Syto 9 at final concentrations of 15 µM and 5 nM respectively for mid-exponential time points, and 30 µM and 10 nM respectively for stationary phase time points. Cells were incubated in the dark for 15 minutes before viewing on the Zeiss Axioplan 2 microscope.

Cells were imaged using phase-contrast and fluorescence microscopy with a Zeiss Axioplan 2 fluorescence microscope as published [275] with the following modifications. The cells were excited at 488 nm and 561 nm, and the emissions were collected at 500–550 nm and 570–620 nm, respectively, with a 100 W high pressure

mercury lamp passed through filter set 02 (Carl Zeiss) as a light source. Image processing was then performed using AxioVision software version 4.5 (Carl Zeiss). Live cells appeared green and dead cells appeared red.

5.3.4 Protein extraction, fractionation and identification

The SA1 secretome samples were reduced and alkylated with 5 mM TBP and 20 mM acrylamide respectively, then precipitated with acetone overnight at -20°C. Proteins were pelleted and re-suspended in a minimum volume of 1% SDS and 50 mM Tris-HCl pH 8.8. Samples were then de-salted by running through a Bio-Rad Micro Biospin column that was equilibrated with 1% SDS and 50 mM Tris buffer. Remaining nucleic acid was removed via the addition of benzonaze (Sigma).

The SA1 cell pellet from stationary time points were re-suspended in 1% SDS and 50 mM Tris buffer and lysed by 3 x 30 second intervals of sonication. The samples were reduced and alkylated with 5 mM TBP and 20 mM acrylamide respectively, then precipitated with acetone overnight at -20°C. Proteins were pelleted and re-suspended in a minimum volume of 1% SDS and 50 mM Tris buffer and de-salted using a Bio-Rad Micro Biospin column that was equilibrated with 1% SDS and 50 mM Tris buffer.

Bacterial lysate (120 µg) was mixed with 2x sample buffer, loaded onto a 1D-SDS-PAGE and run at 160 V. The gel was fixed with 40% methanol and 10% acetic acid, stained and imaged initially with Flamingo Fluorescent Gel Stain and a PharosFX Plus imager followed by colloidal Coomassie Blue G-250 and an Epsom 4800 Perfection scanner. Gel lanes containing the resolved proteins were sliced into 14 equal pieces and

proteins were subjected to in-gel trypsin digestion followed by LC-MS/MS on a QSTAR Elite of the tryptic peptides as previously described [214].

Peptides were identified on an AB-Sciex QSTAR as described previously [276]. Wiff files were loaded onto Peaks Studio v.7.0 along with the SA1 translated amino acid fasta file. Known contaminants were detected computationally using amino acid fasta file containing known contaminants. The following parameters were permitted for the SA1 database search: parent mass tolerance 100 ppm; fragment mass error tolerance 0.2 Da; monoisotopic precursor mass search type; 3 maximum missed cleavage sites; a maximum of 3 missed cleavage sites per peptide; a false discovery rate of <1%; and variable modifications including deamidation (NQ), oxidation (M) and propionamide (C). For a protein to be identified, peptides from that protein had to be identified in two or more of the biological replicates. For all proteins whose Peaks protein score (-10lgP) was less than 80 or proteins only identified by 1 peptide, the spectra were manually interrogated to ensure confidence in the identification.

5.3.5 Determination of protein subcellular location

The SA1 genome was interrogated (Table 5-2) including PSORTb, SecretomeP, SignalP, and predTAT, for the prediction of subcellular location or to predict the presence of signal peptides. Classical protein secretion, or the secretion of proteins through known secretion systems requiring a signal peptide, was determined if the SignalP score was > 0.5 or predTAT identified the protein to be secreted through the Sec or Tat pathways (regardless of the SecretomeP score). Non-classical protein secretion, or the secretion

of proteins that do not contain signal peptides, was determined if the SecretomeP score was > 0.5 and there was no predicted secretion by SignalP or predTAT.

Table 5-1 Bioinformatic tools utilised in the secretome analysis.

Bioinformatic tool	Version	Bioinformatic prediction	Reference
PSORTb	3.0.2	Prediction of protein subcellular location utilizing a combination of homology-based, transmembrane domain, signal peptide and motif- and profile-matching domain prediction tools.	[264]
SecretomeP	2.0	Prediction of non-classical protein secretion.	[265]
SignalP	4.1	Prediction of protein secretion based on the presence or absence of signal peptides.	[266]
predTAT	-	Prediction of signal peptides for protein secretion through the twin-arginine translocation pathway or general secretion pathway.	[277]

Label-free quantitative analysis was performed to compare the SA1 mid-exponential secretomes and the SA1 stationary secretomes to the SA1 proteome utilising Scaffold (v.4.0). Peaks Studio output was exported (.mzid) into Scaffold v.4.0. and analysed using the following parameters: protein threshold 95%; minimum number of peptides 2; a peptide threshold of 95%; and a decoy false discovery rate of 0.1%. Three methods of label-free quantitative analysis were used: the normalised total spectra, the normalised exponentially modified Protein Abundance Index (emPAI) and the Normalised Spectral Abundance Factor (NSAF). The normalised total spectra count is the number of MS/MS spectra per peptide [278]. The emPAI offers quantification

based on protein coverage by the peptide matches in a database search result [279]. The emPAI is calculated as below, where N is the number of peptides:

$$emPAI = 10^{\frac{N\ obsered}{N\ observable}} - 1$$

The NSAF is the number of spectral counts (the total number of MS/MS spectra) identifying a protein, divided by the protein's length, divided by the sum of spectral counts divided by the protein length for all proteins in the experiment, as seen below [280]:

$$NSAF = \frac{\frac{Number\ of\ spectral\ counts}{Protein\ length}}{\sum \frac{Number\ of\ spectral\ counts}{Protein\ length} for\ all\ proteins\ in\ the\ experiment}$$

The normalised total spectra, the emPAI and NSAF values were obtained for all identified proteins in the proteome and secretome and a scoring system introduced to determine the subcellular localization of the proteins. This previously published scoring system [281], employs the abundance fold change between the secretome and proteome to determine if the protein was truly secreted, as the identification of proteins by mass spectrometry is concentration dependant. If the fold change between the secretome and proteome was determined to be 3-fold or higher a score of 1 was allocated, if the fold change was between 2 and 3-fold a score of 0.5 was allocated and if the fold change was less than 2-fold a score of zero was allocated. For accurate localisation, only proteins that obtained a score of 0.5 or 1 in all the NSAF, emPAI and total spectra values, or proteins which were only identified in the secretome (not

proteome) were determined to be bona fide secreted. All other proteins were removed from the analysis and determined to be present due to cell lysis or vesiculaiton.

5.3.6 Identification of putative virulence factors

The SA1 genome sequence was annotated using RAST, which assigns proteins to a specified subsystem based on abstract functional roles. For the identification of potential virulence factors, we looked for proteins assigned to the virulence, defence and disease, membrane transport, motility and chemotaxis subsystems, as well as looking through proteins unassigned to a specific subsystem. In addition, SA1 open reading frames were screened through MP3, a web server that uses a combination of Support Vector Machine (SVM) and Hidden Markov Model (HMM) to predict pathogenic proteins based on dipeptide composition (the fractions of amino acids and their local order) and Pfam domains (protein functional domains), respectively [185].

5.4 Results

5.4.1 Growth curve of *S. algae* SA1

Growth curves of *S. algae* SA1 are depicted in Figure 5-1. The mid-exponential growth phase was determined to occur at 90 minutes, while the early stationary growth phase occurs at 300 minutes.

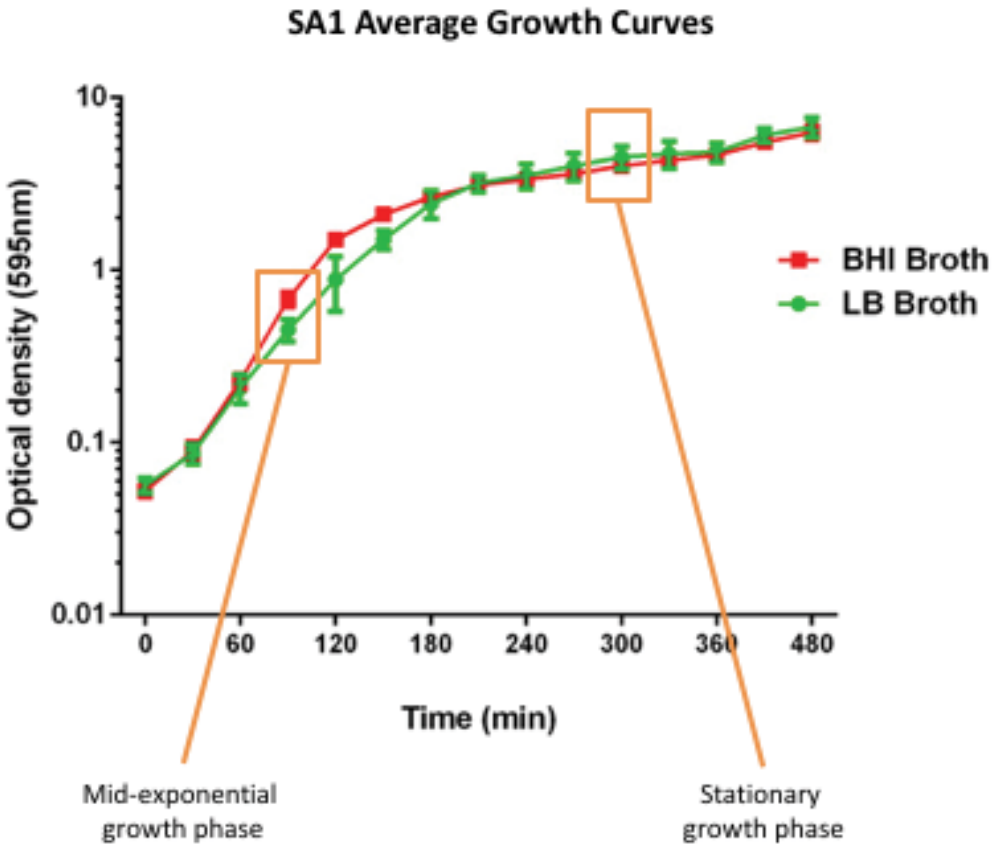

Figure 5-1 Growth curves of *S. algae* in LB broth and BHI broth.

5.4.2 Viability of SA1 cells

Live/dead staining was utilised to determine the percentage of cell lysis within the bacterial population (Figure 5-2). Only minimal levels of cell lysis was observed for SA1 under all tested conditions. For LB broth at mid exponential and stationary phase, the percentage of lysed cells was 0.09% and 1.6% respectively, while for BHI broth at mid-exponential and stationary time points the percentage of lysed cells was 1.8% and 1.2% respectively (Figure 5-3).

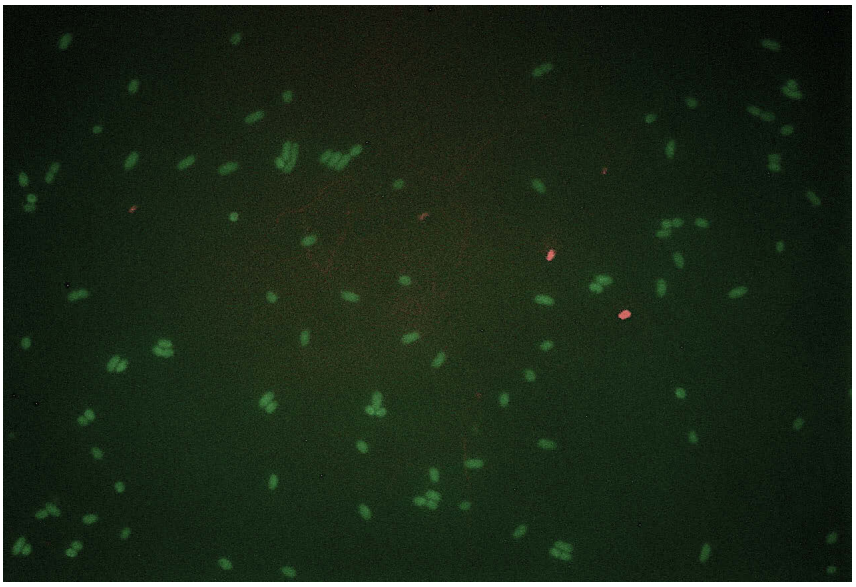

Figure 5-2 An example of the live dead staining of SA1.

Live cells appear green due to Syto 9, a cell membrane permeable dye; dead cells appear red due to the staining of propidium iodide, which is not cell membrane permeable and therefore only penetrates cells with compromised cell membranes.

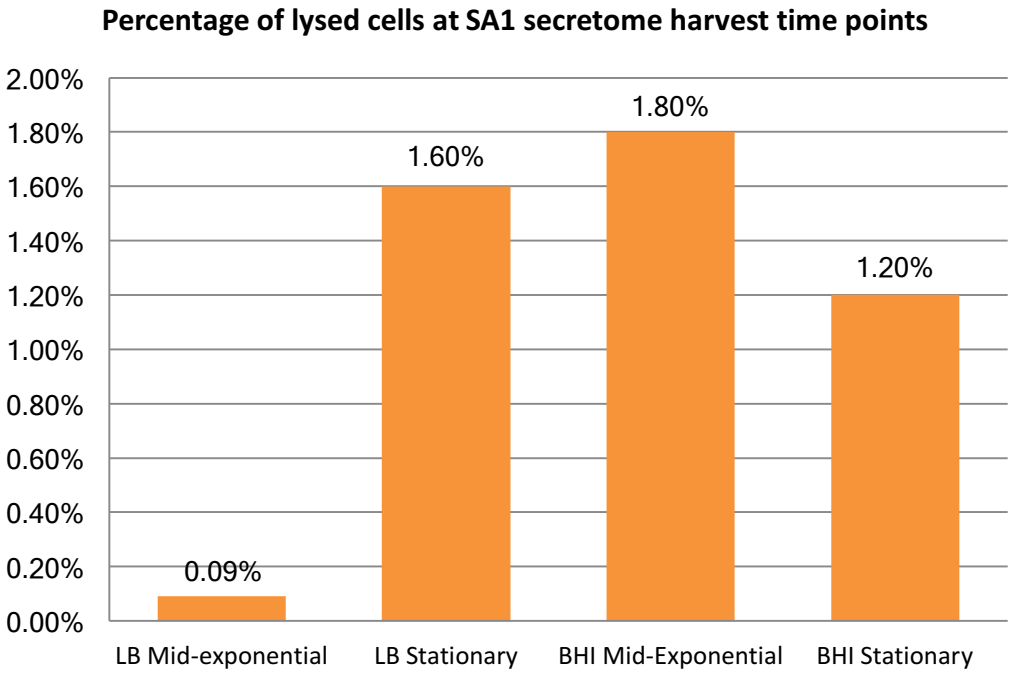

Figure 5-3 The percentage of SA1 cell lysis in LB broth and BHI broth and mid-exponential and stationary time points.

5.4.3 The SA1 secretome 1D-SDS-PAGE

Figure 5-4 depicts a 1D-SDS-PAGE of proteins recovered from the supernatants of *S. algae* grown in different medium. While the protein profiles between biological triplicates are indistinguishable from one another, differences are evident in the protein profiles representing the different medium conditions. This is of interest, as BHI broth is much more complex compared to LB broth, containing the components of numerous eukaryotic cells at high concentrations.

5.4.4 Identification and characterisation of the *S. algae* SA1 secretome

To improve the accuracy of the results, stringent criteria were applied and peptides from the proteins included in the secretome analysis had to be identified in two or more of the biological replicates and deemed to be truly secreted protein after classification from the label-free quantitative analysis (spectral count, emPAI and NSAF values compared to the equalised proteome) (Table S-11). The *S. algae* SA1 secretome identified 210 proteins by combining our analyses of mid- and stationary phase proteins (Figure 5-5, Table S-10). Fifty proteins were identified in mid-exponential cultures grown in LB broth whereas 125 proteins were identified in stationary phase cultures. Of these proteins, 30 were found in common between the two different growth phases. Only 25 proteins were identified in mid-exponential BHI broth and 94 were identified in stationary phase cultures. Of these proteins, 16 were found in common between the two different growth phases. Of all proteins identified in the *S. algae* SA1 secretome, none were identified in both media at both time points.

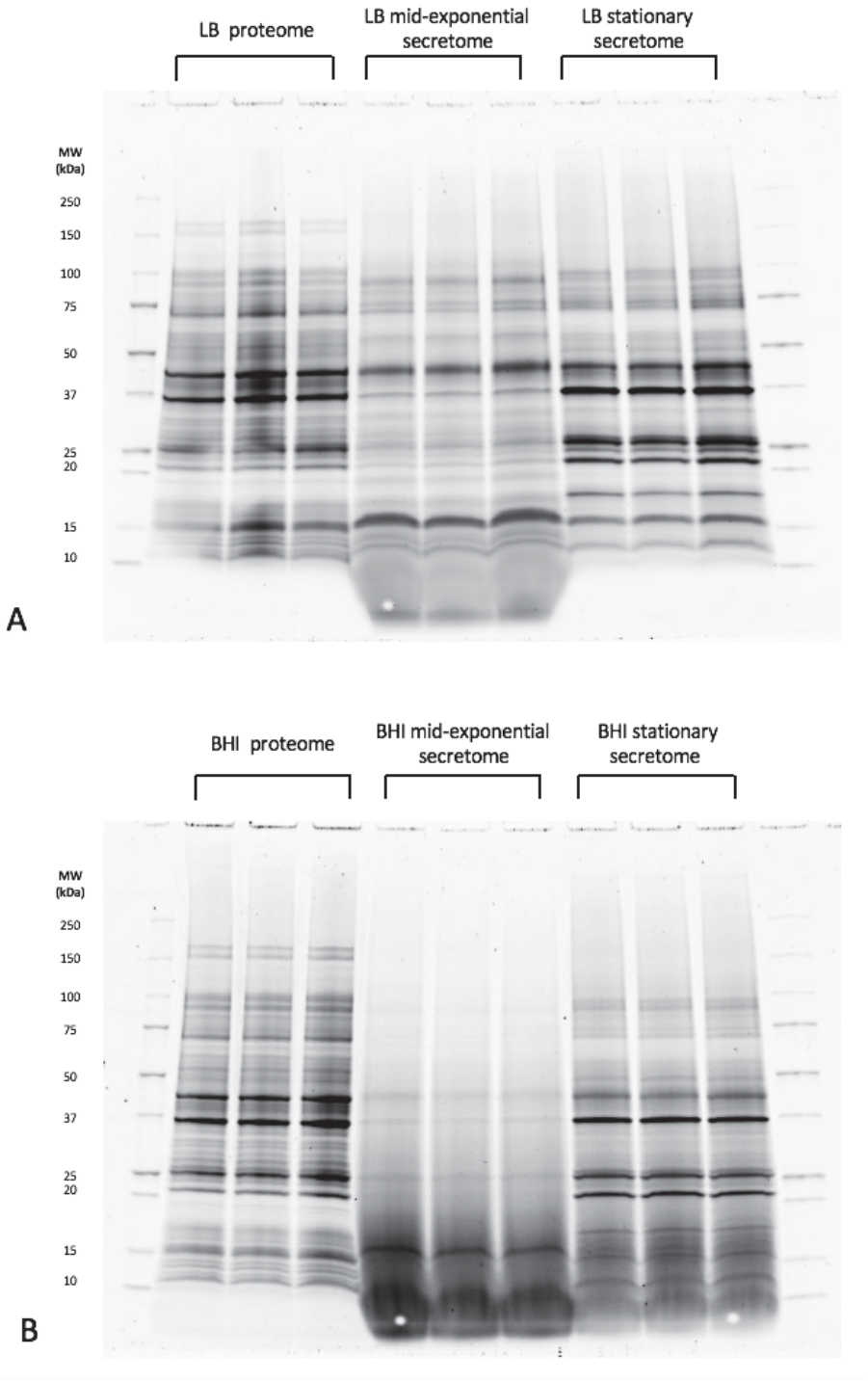

Figure 5-4 (A) 1D-SDS-PAGE of the SA1 proteome, mid-exponential and stationary secretome grown in LB broth. (B) 1D-SDS-PAGE of the SA1 proteome, mid-exponential and stationary secretome grown in BHI broth.

Each lane contains 120 µg of protein and each time point/growth condition represents a biological replicate.

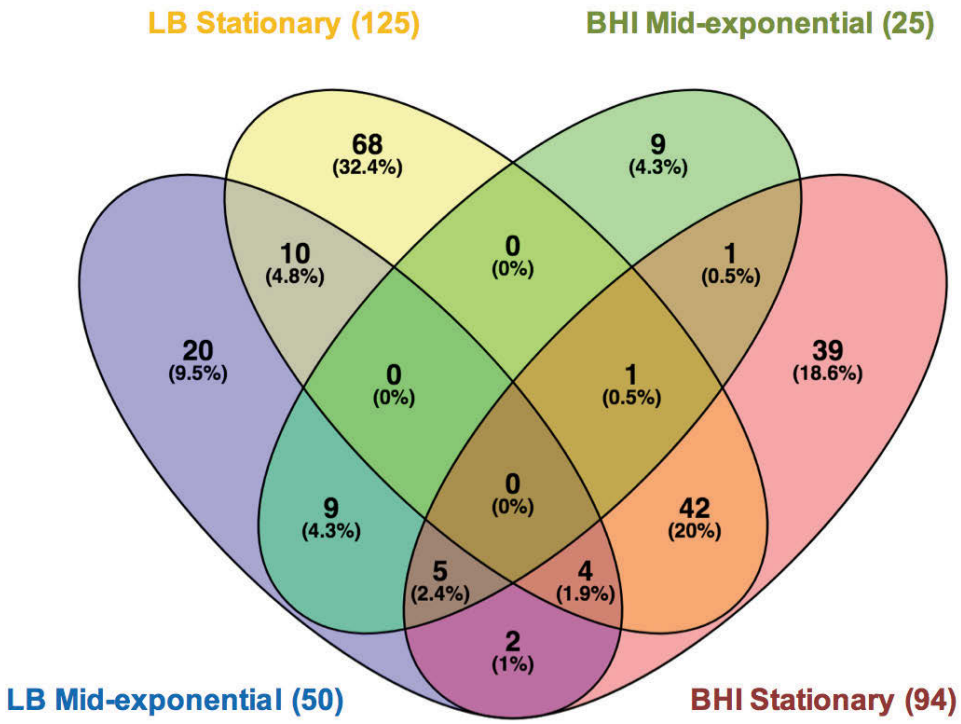

Figure 5-5 Venn diagram displaying the proteins identified in LB broth and BHI broth and mid-exponential and stationary time points.

The total number of proteins detected in each media is given after the media title (http://bioinfogp.cnb.csic.es/tools/venny/).

Only 90 of the 210 proteins were predicted to be secreted (43%) using a range of protein localisation prediction programs. Figure 5-6 depicts the number of proteins identified by PSORTb, SecreomeP, SignalP and predTAT. SecretomeP and predTAT identified the highest number of the 210 proteins. Interestingly, only 1 protein was predicted to be secreted by all four prediction tools. Of the proteins identified, classical protein secretion was seen in 67 of the proteins and non-classical protein secretion in 23 proteins, with the remaining 120 proteins determined not to be secreted (Table S-10).

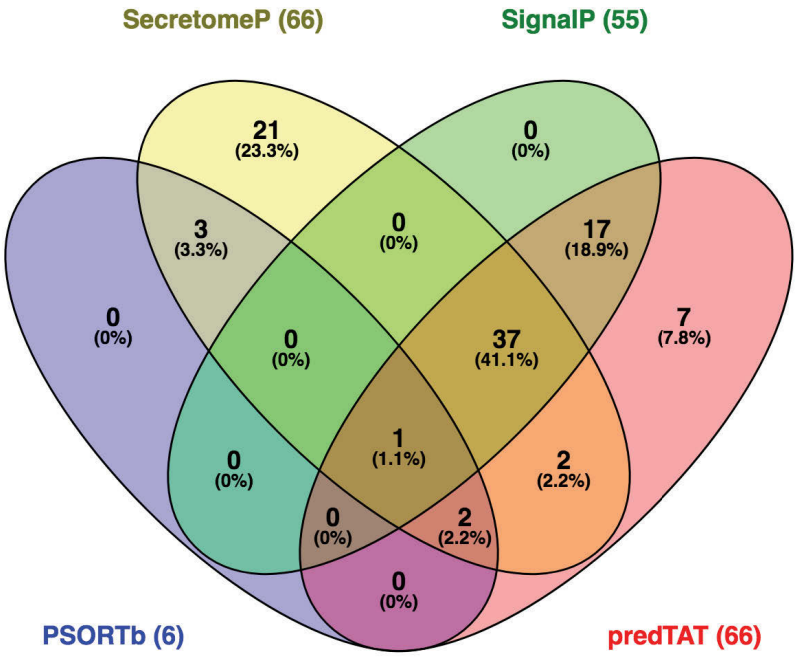

Figure 5-6 Proteins in the *S. algae* SA1 secretome identified by PSORTb, SecretomeP, SingalP and predTAT. The number following the prediction tool is the total number identified by that tool.

5.4.5 Identification of proteins involved in bacterial pathogenesis

The SA1 genome sequence was annotated using RAST, which assigns proteins to a specified subsystem based on abstract functional roles. For the identification of potential virulence factors, we looked for proteins assigned to the virulence, defence and disease, membrane transport, motility and chemotaxis subsystems, as well as looking through proteins unassigned to a specific subsystem. In addition, the *S. algae* SA2 proteins were screened through MP3, a server used to predict pathogenic proteins. This identified 46 proteins as pathogenic proteins, including proteins involved in bacterial pathogenicity, bacterial fitness and resistance to antibiotics (Table S-12), however, not all of these are known to be pathogenic in humans. Table 5-2 lists proteins identified in the SA1 secretome that may be involved in bacterial pathogenesis, along with the known role they play.

Table 5-2 Potential virulence factors in the *S. algae* SA1 secretome.

Protein	LB mid-exponential	LB stationary	BHI mid-exponential	BHI stationary	Predicted secretion	Potential role in virulence	Reference
ATP-dependent Clp protease ATP-binding subunit ClpX		✓			Not secreted	Expression of virulence factors Stress tolerance Resistance to antibiotics Colonisation	[282-285]
Chaperone protein DnaK			✓		Non-classical	Adhesion	[231-236]
Chitin binding protein		✓		✓	Classical	Expression of virulence factors Adhesion Protection against stomach acid	[227, 286-290]
DNA-binding protein HU-beta	✓		✓	✓	Not secreted	Potential virulence factor Invasion	[291, 292]
Flagellar hook-associated protein FliD		✓		✓	Not secreted	Expression of virulence factors Adhesion	[293, 294]
Flagellin protein FlaA		✓			Non-classical	Invasion Motility	[238, 239]
Periplasmic thiol:disulfide interchange protein DsbA		✓			Non-classical	Expression of virulence factors Invasion	[295-300]
Putative lipoprotein		✓		✓	Classical	Antibiotic resistance Colonisation	[301]
T1SS secreted agglutinin (RTX)		✓			Non-classical	Toxin Adhesion	[191-193, 227-229]
Thioredoxin				✓	Not secreted	Potential virulence factor	[302]
TPR repeat containing exported protein		✓		✓	Classical	Adhesion Translocation of virulence factors	[220, 221]
Uncharacterised protein ImpC		✓		✓	Not secreted	Adhesion Expression/translocation of virulence factors	

5.5 Discussion

S. algae is an emerging human pathogen known to cause gastroenteritis, cellulitis, otitis media and septicaemia [33, 37]. Members of the *Shewanella* genus, and more recently *S. algae,* are being considered for roles in bioremediation and microbial fuel cells due to their ability to diverse respiratory capabilities [37, 303, 304]. There is a pressing need to understand the mechanisms by which *S. algae* causes disease because the incidence of infections caused by this organism are on the rise, there are more frequent reports that it is acquiring resistance to antibiotics and global warming is expected to have an impact on how *Shewanella* colonises marine environments [305], particularly before it is used in large scale operations for bioremediation or microbial fuel cells.

The bacterial secretome is an ideal place to look for putative virulence factors in a novel pathogen. The secretome is likely to contain toxins, proteases, adhesins, defence proteins (antimicrobial proteins) and other immunogenic surface proteins needed to colonise and potentially invade host cells and manipulate the host immune response [270]. In this study, we analysed the *S. algae* SA1 secretome under different growth conditions (using LB broth and BHI broth, a media containing high concentrations of eukaryotic cell components to represent infection relevant conditions) at two different growth phases (mid-exponential and stationary). Whilst we also investigated the secretome of *S. algae* SA1 on a variety of agar plates, including LB agar, blood agar and thiosulfate-citrate-bile salts (TCBS) agar), the percent of cell lysis from these plates exceeded 30%, so our ability to identify bona fide secreted proteins was challenging (results not shown). Nonetheless the result is interesting because it implies that *S.*

algae protein expression is altered significantly when exposed to essentially the same medium in liquid and solid form. As depicted in Figure 5-4 and Figure 5-5, the *S. algae* SA1 secretome in LB and BHI broth at mid-exponential and early stationary growth phases change significantly. This was not unexpected, as previous studies have shown significant differences in the secretome under different growth conditions [306-308] and also between different growth phases [309].

The majority of proteins initially identified via mass spectrometry in the *S. algae* SA1 secretome were predicted to be cytoplasmic proteins. There is mounting evidence that many cytosolic proteins find their way onto the cell surface and display an alternate function(s) [272, 274, 310]. These are known as geographic moonlighting proteins [272, 273]. This is not an unusual observation in the field of proteomics, as many studies of both Gram-negative and Gram-positive bacteria report the presence of proteins with canonical functions in the cytosol (e.g. ribosomal proteins and metabolic enzymes) on the cell surface or in secretomes. What has changed over the past 15 years is how this data is interpreted. It is now clear that many proteins with known functions in the cytosol moonlight on the surface of bacterial pathogens [272, 273], performing important roles in pathogenesis and biofilm formation. These proteins can either be exported via an unknown mechanism(s) or are released due to cell lysis or vesicle formation and export. Live dead staining experiments showed minimal cell lysis within the *S. algae* SA1 bacterial populations in both LB and BHI broth (Figure 5-2; Figure 5-3).

We also interrogated our secretome datasets by comparing protein abundance in the secreteome with abundance in the proteome at the same growth phase using label-free quantitative analysis (emPAI, NSAF and spectral counting) [281]. A scoring system was implemented which examines the fold change in protein abundance in the secretome compared to the proteome. This scoring system allows for proteins only identified in the secretome (and not the proteome) or proteins identified in higher abundance in the secretome compared to the proteome to be used as a criteria for inclusion in the secretome. This system can be implemented as mass spectrometry identifies proteins in a concentration dependant manner. When comparing the results from spectral counting, emPAI and NSAF analysis, all three methods of label-free quantitative analysis showed similar results, and the 10 most abundant proteins in the proteome were not found in the secretome analysis. The application of this scoring system resulted in the identification of 210 proteins in the *S. algae* SA1 secretome, with 50, 125, 25 and 94 proteins present in the LB mid-exponential, LB stationary, BHI mid-exponential and BHI stationary time points respectively.

Of the 210 proteins identified, none were identified in both media at both time points, highlighting the importance of conducting secretome investigations in a variety of media over different growth phases. It has been previously reported that virulence factors are more prominent in late-exponential early stationary growth phases [311, 312], which explains the higher numbers of virulence factors in the SA1 early stationary phase secretomes. The majority were identified in the stationary growth phases, where cells are no longer rapidly dividing. The use of four bioinformatics tools to predict proteins secretion, PSORTb, SecretomeP, SignalP and predTAT, predicted 89

proteins to be secreted, with 67 of these classically secreted and 22 non-classically secreted. Each tool identified a significantly different subset of secreted proteins with only 1 protein being identified by all four tools. Each of these bioinformatic tools predicts protein secretion based on different algorithms and can be used for different purposes. Nonetheless, this highlighting the importance of using multiple bioinformatics predication tools for protein secretion.

We used MP3, a pathogenic protein prediction tool, to assist in the identification of potential virulence factors in the *S. algae* SA1 secretome. As listed in Table 5-2, twelve potential virulence factors were identified that are known to play roles in adhesion, colonisation, invasion, growth, pathogenesis, and assist in resistance to antibiotics in other bacteria. These proteins were more highly abundant in the stationary growth phases compared to the mid-exponential growth phases, and expressed more in LB growth media. Even though the growth curve of SA1 in both media follows a normal growth pattern, SA1 grown in BHI media expressed a higher proportion of proteins involved in the stress response, suggesting that the complexity of the growth media places SA1 in more stress. Furthermore, the 1D-SDS-PAGE in BHI broth appears to be smeared in both the mid-exponential and stationary growth phases in all biological replicates. This could be due to the presence of an increased number of bacterial proteases degrading the proteins or due to a high presence of nucleic acids [313], however the addition of proteases to the secretome at harvest time should inactivate most bacterial proteases. The complexity and protein richness of the media could also be why there are less proteins identified in the BHI media, as proteins secreted in low

abundance may not be identified via mass spectrometry if proteins from the media are present in a higher abundance.

The ability for bacteria to adhere and colonise niches both within the environment and within the host are crucial for their survival and ability to form biofilms. As seen in Table 3, SA1 secretes the T1SS secreted agglutinin (RTX), a cytotoxin produced by other Gram-negative such as *V. cholerae* that is a known virulence factor involved in colonisation of the small intestine in mice by causing actin cross-linking within the host [191, 192, 227-229, 314]. Other proteins that were identified and are known to play a role in the adhesion and colonisation of bacteria to host surfaces include DnaK [231, 235, 236] (binds plasminogen), chitin binding protein [287-289], flagellar hook associated protein FilD [293, 294], periplasmic thiol:disulphide interchange protein DsbA [296] and transient receptor potential (TRP) containing exported protein [221]. SA1 secretes a number of proteins that are known to be pivotal to the expression or translocation of virulence factors, including chaperone proteins DnaK, periplasmic thiol:disulphide interchange protein DsbA [295-300], tetratricopeptide repeat (TRP) containing exported protein [220, 221] and ATP-dependant protease ClpX [283].

In addition to identifying toxins and proteins involved in adhesion and colonisation of surfaces and host cells, *S. algae* SA1 secreted proteins are known to be integral for the invasion of host cells. DNA-binding protein HU-beta [291] and chaperone protein DnaK [232] are both known to play a role in the invasion of epithelial cells in addition to DNA-binding protein HU-beta [291] and periplasmic thiol:disulphide interchange protein DsbA [296] being involved in the invasion of macrophages. Furthermore, chitin

binding proteins are known to assist in the protection of bacteria against stomach acid in *V. cholerae* [290]. Lastly, *S. algae* also secreted proteins which play a role in the resistance to antibiotics, which aids in the survival of the bacteria. These two proteins were the ATP-dependant protease ClpX [283-285] and a putative lipoprotein [301], which have been shown to increase the resistance of *Staphylococcus aureus* to methicillin and antimicrobial peptides and *Borellia burgdorferi* respectively.

5.6 Conclusion

In conclusion, we identified an array of potential virulence factors secreted by *S. algae* SA1 under different growth conditions at different growth phases utilising mass spectrometry and label-free quantitative analysis. This is the first study to investigate expressed virulence factors in *S. algae*. Knowing that *S. algae* is emerging as a cause of disease in humans and is acquiring resistance to antibiotics, further studies are needed to investigate mechanisms of pathogenesis in model cell lines and in model animal hosts. Future efforts should focus on cloning, expressing and characterising recombinant versions of many of these putative virulence factors. Surfaceome studies should also be conducted to determine if proteins that are secreted into the medium also associate with the cell surface. The use of differential media in investigate the alteration in expression of proteins also aided in identifying potential virulence factors.

Chapter Six

Identification of a novel *qnrA* allele, *qnar8*, in environmental *Shewanella algae*

Chapter 6 – Identification of a novel *qnrA* allele, *qnrA8*, in environmental *Shewanella algae*

Declaration

I declare that the following publication included in this thesis in lieu of a chapter meets the following:

- More than 50% of the content in the following publication included in this chapter has been planned, executed and prepared for publication by me
- The work presented here has been peer-reviewed and accepted for publication
- I have obtained approval to include the publication in this thesis from the publisher
- The initial draft of the work has been written by me and any subsequent changes in response to co-authors and editors reviews was performed by me
- The publication is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in the thesis.

Publication title: The identification of a novel *qnrA* allele, *qnrA8*, in environmental *Shewanella algae*

Authors: Melvold, J. A., Wyrsch, E. R., McKinnon, J., Row Chowdhury, P., Charles, I. G. and Djordjevic, S. P.

Candidate's contribution (%): above 50 %

Journal name: Journal of Antimicrobial Chemotherapy

Volume/ page numbers: Volume 72, Issue 10

I declare that the publication above meets the requirements to be included in the thesis.

Candidate's name: Jacqueline Melvold

Candidate's signature:

Date: 13th October 2017

6.1 Compound abstract

Rationale: Quinlones are recognised as one of the most widely prescribed class of antibiotics used to treat infections caused by bacteria in humans, livestock and companion animals. The environemntal impact of quinolones is a cause of concern as metabolic breakdown products released from the body of target species provides a selection pressure that impacts the ecology of non-target bacterial populations, where it can influence natural mutation rates and lateral gene transfer. In addition, DNA supercoiling, which is affected by quinolone drugs, is known to play a role in thermotolerance among *Enteriobacteraceae.* Here, we report here the identification of a novel, chromosomally encoded *qnrA* allele, *qnrA8*, isolated from environmental *Shewanella algae* from the George's River, Sydney.

Methods: The genomes of *S. algae* SA1 and SA2 were sequenced using Illumina paired-end technology and annotated via RAST. BLASTN analysis and genome alignments (using Mauve) were performed to identify the closest relative of *qnrA8*. Antibiotic susceptibility was performed using standard procedures.

Results: The *qnrA8 allele* was found to be chromosomally encoded in the SA1 and SA2 genomes, and contains 31 silent SNPs and one that evokes an amino acid change in QnrA8. BLAST analysis revealed the immediate genetic environment that flanks the *qnrA8* gene in the *Shewanella* strains is remarkably similar to the plasmid-encoded *qnrA* genes found in multiple antibiotic resistant Enterobacteriaceae*,* providing support for the notion that these genes may have been sourced from aquatic *S. algae*.

qnrA8 did not confer resistance to quinolones or fluoroquinolones in strains SA1 and SA2.

6.2 Journal of Antimicrobial Chemotherapy Correspondance

Quinlones are recognised as one of the most widely prescribed class of antibiotics used to treat infections caused by Gram negative and Gram positive bacteria. [315] In humans, quinolones are used to treat infections of the urogenital, respiratory and gastroinestinal tracts as well as a range of anatomically-diverse infections in swine, poultry, cattle, companion animals and in aquaculture. [316] In the environment, fluoroquinolones break down slowly (half-life of approximately 100 days) and it is possible to measure trace levels of the drug in exposed environments. [317] The environmental impact of quinolones, particularly fluoroquinolones from humans, agriculture and from pharmaceutical production facilities, is a cause of concern as residues and metabolic breakdown products released from the body of target species provides a selection pressure that impacts the ecology of non-target bacterial, invertebrate and vertebrate populations, where it can influence natural mutation rates and lateral gene transfer. [316]

The cellular targets of quinolones are bacterial type II topoisomerases including DNA gyrase and topoisomerase IV. [318] DNA gyrase functions in the management of DNA supercoiling and topological stress while topoisomerase IV has a role in unlinking replicated daughter chromosomes. [318] The genes encoding QnrA, QnrB, QnrC, QnrD, QnrS and QnrVC are found on the chromosome or on plasmids and confer resistance to quinolones, and low-level resistance to fluoroquinolones. The Qnr proteins belong

to the pentapeptide repeat family and function to protect DNA gyrase and topoisomerase IV enabling bacteria to resist the actions of quinolones. [319]

QnrA is 218 amino acids in length and seven alleles have been described (*qnrA1-7*). The closest homologues of *qnrA* genes in clinically important Enterobacteriaceae are found in *Shewanella algae* (*S. algae*), [143] Gram-negative bacilli that live in diverse and unique aquatic environments. [33] Five alleles of *qnrA* (*qnrA2-5* and *qnrA7*) have been identified in *S. algae* [143, 145, 171] prompting suggestions that *qnrA* genes were transferred from *S. algae* into the Enterobacteriaceae. [42, 43] Here, we report the identification of a novel, chromosomally encoded *qnrA* allele, *qnrA8* (GenBank Accession Number KY554783), isolated from environmental *S. algae* strains SA1 and SA2 from the George's River, Sydney in 2011.

The *qnrA8* nucleotide sequence differs from the *qnrA1* sequence by 24 nucleotides (Figure 5-1). The majority of the polymorphisms encode silent mutations, however, $^{G}156^{D}$ was novel to this allele (Figure 5-2A). Personal correspondence with George Jacoby saw the sequence receive the unique designation of QnrA8. [320] BLASTN analysis using *qnrA8* nucleotide sequence from strain SA1 as the query, identified a *qnr* allele in *Klebsiella pneumoniae* plasmid IncA/C-LS6 (GenBank NC_021667.1) to be the closest homologue. [321] A Mauve alignment shows that the region within the *K. pneumoniae* plasmid IncA/C-LS6 flanking the *qnrA6* allele is similar to the region flanking *qnrA8* in SA1 and SA2 (Figure 5-2B). These observations are consistent with the hypothesis that *qnrA* alleles found on mobile genetic elements in the Enterobacteriaceae circulating in clinical environments have been captured from

environmental *S. algae.* [322] Further, our analyses indicate that IS*CR1* has played a role in capturing *qnr* genes, positioning them in close proximity to 3´ –CS of clinical class 1 integrons. [323] The IS*10* does not appear to be associated with the capture of the *qnrA* allele and is likely to have been acquired independently.

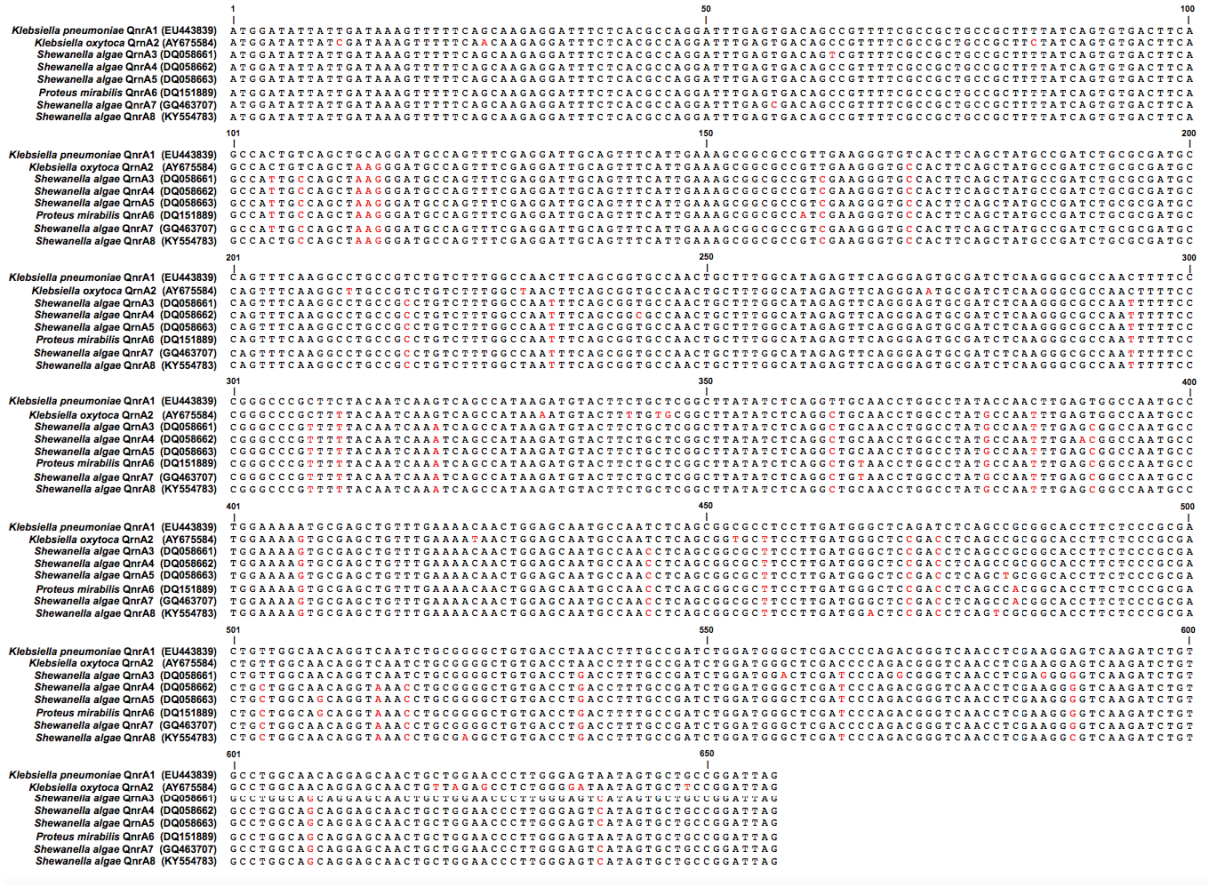

Table 6-1 The nucleotide sequence of *qnrA* genes 1-8. Differences in the nucleotide sequences are highlighted in red. GenBank accession numbers are in brackets.

The antimicrobial susceptibility of *S. algae* SA1 and SA2 were tested against 31 antibiotics using the Vitek 2 Compact System (Version 05.04 at Concord Hospital, Sydney) following the Biomerieux guidelines and the calibrated dichotomous sensitivity test (CDS) as outlined in the CDS Manual. [324] Notably, SA1 and SA2 were

susceptible to nalidixic acid (30 µg), ciprofloxacin (2.5 µg) and norfloxacin (2 µg). This observation is not without precedence as the susceptibility of *S. algae* to quinolones and fluoroquinolones carrying qnrA genes has been noted. [171] In *S. algae,* qnrA2 is expressed in response to cold shock (temperatures < 20°C) and not with DNA damaging agents, such as fluoroquinolones [145] while in *Escherichia coli*, *qnrA6* promotes bacterial fitness in the absence of antibiotics. [144] A link between bacterial supercoiling, to which DNA gyrase is vital, with thermotolerance and thermoresistance has been described. [146] Collectively these data suggest the qnr genes may have evolved to regulate DNA supercoiling in response the fluctuations in temperature, a role that would be advantageous as an evolutionary adaptation to seasonal changes in temperature in the environment where *Shewanella* spp. inhabit.

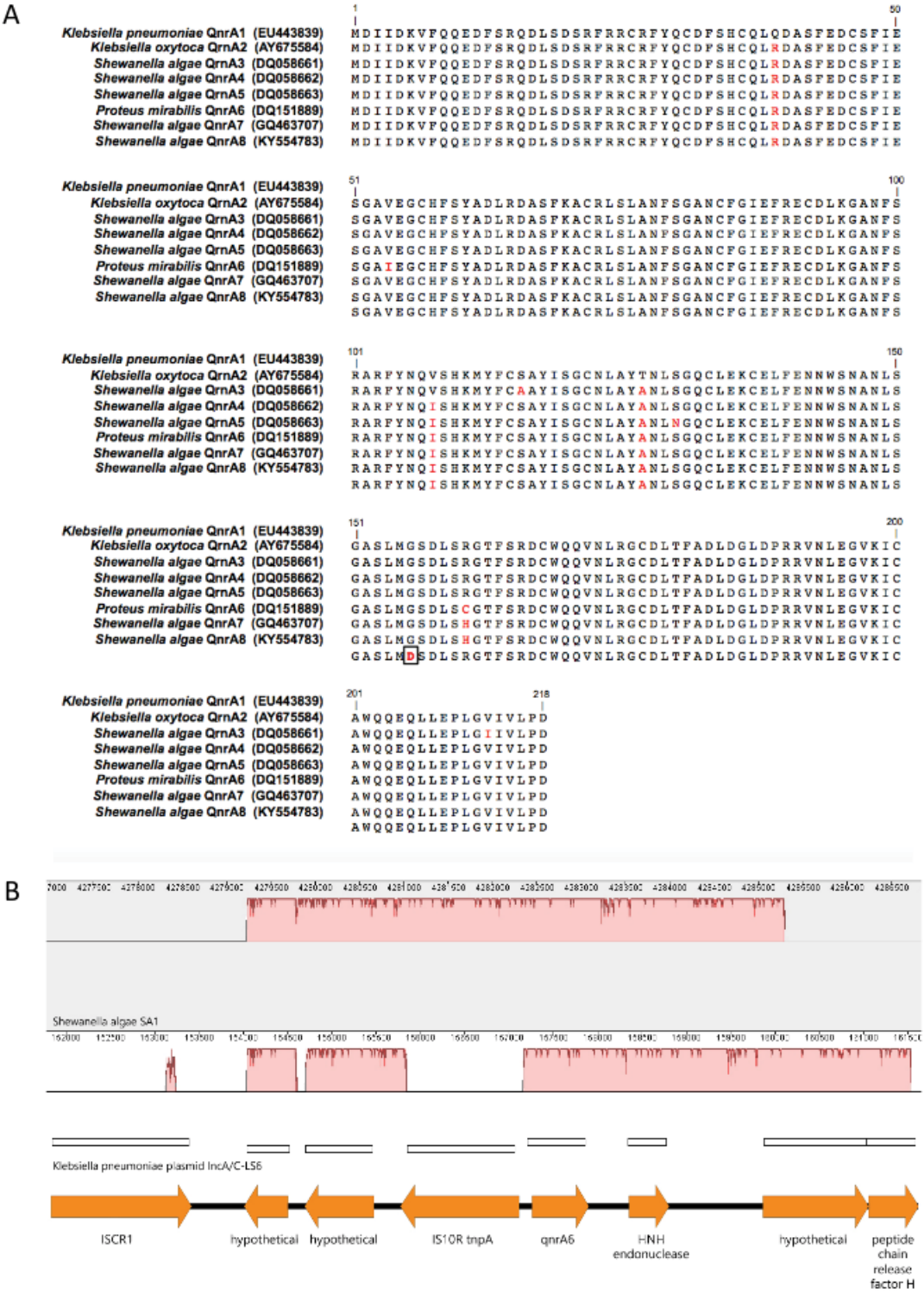

Table 6-2 (A) Amino acid sequence of QnrA alleles. (B) Mauve alignment of *S. algae* SA1 (top) chromosomal *qnrA8* and *K. pneumoniae* plasmid IncAC-LS6.

(A) Amino acid substitutions are shown in red. The boxed amino acid indicates a $^{G}156^{D}$ transition that is unique in Qnr8. GenBank accession numbers are shown in brackets. (B) Easyfig 2.2.2 was used visualise the plasmid annotation.

In summary, a novel, chromosomally-encoded *qnrA* allele, *qnrA8*, was identified in environmental *S. algae* strains SA1 and SA2 from Sydney, Australia. The immediate genetic environment that flanks the *qnrA8* gene in the *Shewanella* strains is remarkably similar to the plasmid-encoded *qnrA* genes found in multiple antibiotic resistant Enterobacteriaceae providing further support for the notion that these genes may have been sourced from aquatic *Shewanella* spp. *qnrA8* did not confer resistance to quinolones or fluoroquinolones in strains SA1 and SA2, but the gene may function to enhance bacterial fitness.

Chapter Seven

General discussion and concluding marks

Chapter 7 - General discussion and concluding remarks

The overarching aim of this thesis was to investigate the disease aetiology of the emerging human pathogen, *S. algae* (AIM ONE), and investigate the presence and expression of potential virulence factors and mechanisms of antibiotic resistance (AIMS TWO, THREE and FOUR). This has been achieved by using a systems biology approach, combining whole genome sequencing with proteomic studies to investigate the presence of potential virulence factors and the conditions in which they are expressed. Throughout the proteomic studies, we used a variety of media, both traditional laboratory media and complex media containing components of eukaryotic cells, to not only investigate the expression of virulence factors, but also the conditions required for their expression.

The combined contributions of these findings have added significant amounts of knowledge to the understanding the potential mechanisms by which *S. algae* can cause disease in the human host and resist antibiotic therapies. In addition, this thesis presents the first genome sequences of *S. algae* strains isolated from Sydney, Australia, along with their proteome, the functional output of the sequenced genome, and secretome, the portion of the proteome that is exported to the bacteria's external environment. These studies specifically identified putative virulence factors under specific and biologically relevant environmental conditions.

7.1 Collation of case studies representing infections caused by *S. algae*

It had previously been reported that *S. algae* are rare, opportunistic, secondary, community-acquired infections that primarily cause infections of the soft tissue [33, 325]. In addition, exposure to a body of water and the health status of the patient were major factors deemed to play a role in the contraction of *S. algae* infections [1]. Chapter 2 of this dissertation reviewed all of the published clinical literature about *S. algae* in an aim to investigate the aetiology of infections caused by *S. algae*. The evaluation of all case studies where *S. algae* was one of, or the causative organism(s) of infection suggested that many of the previously mentioned notions about *S. algae* infections are inaccurate. In reality, *S. algae* is most commonly associated with mono-microbial infections (>60%) causing, in order of prevalence, otitis media, cellulitis, gastroenteritis, and septicaemia. Furthermore, exposure to a body of water or the consumption of raw seafood as risk factors for *S. algae* were only true for 30% and 1.7% of cases respectively, and only 1.3% of patients were immunocompromised. Together, this highlights that *S. algae* is capable of causing mono-microbial disease in humans and the mechanisms of pathogenicity need to be investigated. In addition, with geography, season, and climate being known risk factors for *S. algae* infection, it should also then be noted that the impact of climate change is likely to influence the frequency of incidence of *S. algae* infections [139, 140, 305, 326].

The work presented in Chapter 2 indicated that *S. algae* should be included in all routine bacterial identification systems in hospitals, so that the infections caused by *S. algae* can be monitored and recorded. This would allow for epidemiological studies to be performed, giving the true representation of the burden of *S. algae* infections.

These data are currently lacking. In addition, it would also be ideal to gather and monitor information regarding the acquisition and evolution of antibiotic resistance of *S. algae*, knowing it has the ability to gain and transfer genes (including those involved in antimicrobial resistance such as *qnrA* [42, 143]) via horizontal gene transfer. Shewanella as a species is increasingly reported to have become resistant to multiple antibiotics [12, 34-36]. The recent report of a highly drug resistant *Shewanella xiamenensis* recovered from hospital eluent in Algeria is a cause for serious concern. In this case, drug resistance genes were associated with a variant of the mercury resistant transposon Tn1696 that was housed on a novel plasmid, identified as pSx1. The plasmid encoded a large number of antibiotic resistance genes including *blaOXA-416*, a beta-lactamase [141].

7.2 Genomic studies of *S. algae*

Genomic and phylogenetic studies offer numerous insights into emerging pathogens [327]. The second aim of this thesis was to sequence the genomes of *S. algae* SA1 and SA2, and use the annotated genome and bioinformatic tools to predict potential mechanisms of pathogenesis in this organism. This study is the first study to sequence genomes of *S. algae* isolates from Australia. Prior to this, only five other draft *S. algae* genomes were available on the NCBI database; four environmental isolates (BrY [328], C6G3 [81], JCM 21037 [55] and CSZ04KR) and one clinical isolate (MARS 14 [36]). Next generation sequencing using Illumina technology was employed, and two draft genomes of high quality were obtained and annotated. The genome sequences of SA1 and SA2 offered many insights into potential virulence factors present in *S. algae* genomes, including genes involved in the resistance to antibiotics, heavy metals and

toxins, as well as genes encoding virulence factors such as the RTX toxin, haemolysins, adhesins, proteases, and genes involved in motility, chemotaxis and biofilm formation. These findings were similar to those identified in the *S. algae* MARS 14 genome [36], suggesting that environmental *S. algae* isolates harbour similar mechanisms of pathogenicity to clinical isolates.

Phylogenetic analysis using PhyloSift showed that all *S. algae* genomes cluster together, with *S. algae* BrY having a slightly different evolutionary history. To compare the genome content of all *Shewanella* species and determine the core and accessory *Shewanella* genome, we performed an 'all vs. all' analysis. Accessory genomes are of importance as they contain customised genomic repertories, many of which can move laterally between bacterial species [217]. In this analysis, we included 26 genomes (with 15 different species) belonging to genus *Shewanella*. The pangenome contained 8,527 proteins; of this 20% make up the core *Shewanella* genome. When focused specifically on *S. algae*, there were 82 protein families unique to this species, the majority of which are annotated as 'hypothetical' proteins and have no known function. Final analysis of the SA1 and SA2 genomes via MP3, a pathogenic protein predictor [185], identified 1,393 and 1,361 proteins predicted to have a role in pathogenesis, representing 30.5% and 30.3% of the SA1 and SA2 genomes, respectively. This is similar to the protein predictions in other pathogenic bacteria such as *Mycobacterium tuberculosis* H37Rv (30.28%) and *Pseudomonas aeruginosa* B139-33 (26.2%), whereas non-pathogenic bacteria only have 10-13% of their proteins predicted to be pathogenic [185]. Of the 82 *S. algae*-specific genes, 49 (59.8%) encode proteins that are predicted to be pathogenic. The majority of these are hypothetical

proteins that have been poorly characterised. We speculate that some of these 82 hypothetical genes [329], in the *S. algae* genome may provide *S. algae* to cause disease in humans as opposed to other species in this genus. Further studies are needed to investigate this hypothesis.

Future work stemming from the genome sequencing of *S. algae* SA1 and SA2 will include closing gaps within the draft genomes, in order to attain a complete genome sequence of *S. algae*. Long-read sequencing platforms such as the PacBio or MinION sequencing technologies will be useful in this regard. Complete genome sequences offer the benefit of improved functional genomic studies, comparative genomic studies and more accurate biological insights into that organism [330]. In addition, the annotation of these genomes currently contains numerous unassigned or hypothetical proteins, labelled this way purely due to a lack of experimental studies aimed at determining their function. Further studies investigating the function of these genes, particularly those predicated to be pathogenic proteins, will offer further insight into the mechanisms of *S. algae* pathogenesis. Lastly, as a wide spread goal, the sequencing of more *S. algae* genomes, particularly clinical isolates, will offer further insight into this emerging pathogen, and allow for more comprehensive studies to be performed.

7.3 Proteomic analysis of *S. algae* to identify potential virulence factors

Proteomic studies allow for the linking of genotype to phenotype [331]. The third aim of this thesis was to investigate the expression of virulence factors in the *S. algae* proteome. This was performed utilising different growth media (both liquid and solid, and complex infection relevant media) in order to determine the conditions required

for the expression of putative virulence factors. While it is well known that environmental conditions, cell density and growth phase can influence the expression of virulence factors, the early stationary time point was specifically chosen as previous studies have determined the expression of virulence factors to be maximal at late exponential and early stationary growth phases [222, 223] as this is when bacteria are more physiologically active. Initially, the analysis of the SA1 differential proteome found 1,192 proteins, of which 92 were unique to specific media and only 195 found across all growth conditions, which emphasises the importance of investigating protein expression under a variety of growth media. The screening of the SA1 differential proteome against predicted pathogenic proteins found the expression of numerous virulence factors, particularly those that belong to the T6SS in Chapter 4. These proteins were found to be expressed in cells grown on solid agar media or in liquid broth containing components of eukaryotic cells. This highlights the importance of studying the differential proteome, as this has allowed us not only to identify potential virulence factors, but also the conditions required for their expression.

Whilst the use of complex media was successful in showing the differential expression of virulence factors, it would be of interest to determine which component of the media specifically induces expression. Future work could therefore investigate the expression of virulence factors using more specific media (e.g. LB broth with the addition of bile), and also different growth phases, such as early exponential or late stationary growth phases to investigate the changes in virulence factor expression changes over time. Currently, genetic systems that can generate targeted mutants in *S. algae* have not been reported but these would greatly facilitate studies to identify

putative pathogenesis factors. Furthermore, the prediction of potential pathogenic proteins using MP3 has also aided in identifying a number of hypothetical proteins involved in the virulence of *S. algae*. Determining their function and role in pathogenicity may help to elucidate how *S. algae* causes disease. Lastly, as a long term future study, the characterisation of identified virulence factors and their role in *S. algae* pathogenesis would be desired.

7.4 Analysis of the *S. algae* secretome to identify secreted virulence factors

The bacterial secretome is widely recognised to contain expressed virulence factors. The last aim of this thesis was to investigate the secretome of *S. algae* SA1. The secretome was investigated at two different growth phases (mid-exponential and early stationary) in two different growth media (LB broth and BHI broth), as the expression of secreted proteins is known to be dependent on environmental conditions. Both of these time-points and growth media produced minimal cell lysis (<1.8% as determined by live dead staining of cells). This, in addition to the label free quantitative analysis and comparison to the proteome, allowed for the study of bona fide secreted proteins. Interestingly, growth on solid media promoted cell lysis (> 30% cell lysis) and precluded studies to examine the secretome. The secretome analysis revealed the secretion of 17 potential virulence factors, which were expressed by cells in different growth media and at different time points. This highlights the value of studying the differential secretome and varied time points, as different virulence factors may be expressed at different times during the cell growth phases and may be of greater biological relevance.

Bioinformatic tools used in the prediction of secreted proteins can only predict proteins that are secreted based on the presence of N-terminal signal peptides previously characterised in known secretion pathways. Whilst these tools can be useful for the prediction of secreted proteins, they should not be used solely to determine bona fide secreted proteins. These tools do not take into account proteins that may have a moonlighting function, a canonical function in another cellular compartment [271]. Many have disregarded the presence of proteins annotated as cytosolic on the cell surface or secretome as an artefact of cell lysis, however there is now overwhelming evidence that many proteins annotated as cytosolic are multifunctional in the bacterial secretome [272, 273]. However, the lack of cell lysis in this study (which may be the major routes by which some cytosolic proteins are released) implies that proteins found in the *S. algae* SA1 secretome are genuinely secreted and may have moonlighting functions.

Future work stemming from the proteomic studies (both whole cell and secreted) include determining the exact role potential virulence factors play in the pathogenesis of *S. algae*. For the secretome, this could include obtaining the secreted products and placing them onto human cell lines to determine the effect of the secreted products (such as the RTX toxin) [332]. In addition, it would be ideal to investigate the potential of *S. algae* to invade human cells though cell invasion assays. Putative virulence factors could be cloned and expressed in *E. coli* and purified for biochemical analyses, structural studies, and binding studies. Lastly, determining the surfaceome of *S. algae* could also identify proteins involved in pathogenesis, as these proteins are the interface between the cell, the host and the environment. Identifying the components

of the surfaceome is crucial for understanding how bacteria interact with biotic and abiotic surfaces and execute bacterial pathogenesis, motility and cell-to-cell communication [333-337].

7.5 Concluding remarks

The work presented in this thesis has sought to address a number of gaps in knowledge regarding the pathogenesis of the emerging human pathogen *S. algae*. *S. algae* has the ability to cause monomicrobial infections in humans, ranging from infections of the skin and soft tissues, to blood borne and enteric infections. This thesis presents the first genome sequences of *S. algae* isolated from Sydney, Australia, and the first proteomic investigations which, combined, identify the presence and expression of potential virulence in this emerging human pathogen.

The work presented in this thesis has linked the *S. algae* genotype to the phenotype, giving a more holistic understand of the bacterium which is crucial to understanding any roles it has in pathogenesis. We identified a range of genes encoding putative virulence factors in *S. algae*, including toxins, haemolysins, adhesins, proteases and genes required for biofilm formation and motility/chemotaxis. Furthermore, the investigation into the expression of these proteins, via the differential growth media in the proteome and secretome, have highlighted that many of the genes encoding for these virulence factors require specific conditions for their expression.

The conclusion of this thesis certainly does not mark the conclusion of this work and there have been a number of identified future avenues, including (i) the addition of *S.*

algae to bacterial identification systems used in hospitals; (ii) the monitoring and reporting of infections caused by *S. algae* (iii) obtaining more clinical genome sequences and a complete genome sequence of *S. algae*; (iv) determining the exact role that identified virulence factors play in *S. algae* pathogenesis; and (iii) investigating the role of horizontal gene transfer in the transfer of genes involved in pathogenicity and antimicrobial resistance.

Supplementary Material

Disclaimer: Due to the size the supplementary tables, they are too large to be inserted into the thesis and are therefore available on the CD attached to the back cover of this thesis. The following tables can be found:

Chapter 3

Table S- 1 RAST annotation of the *S. algae* SA2 genome.

Table S-2 MP3 analysis of the *S. algae* SA2 genome to identify pathogenic proteins.

Abbreviations: SVM = Support Vector Machine; HMM = Hidden Markov models.

Chapter 4

Table S-3 RAST annotation of the *S. algae* SA1 genome.

Table S-4 'All vs. all' protein comparison of the genus *Shewanella*.

Worksheet headings (left to right):

All vs. all heat map analysis

Proteins identified unique to *S. algae*

Table S-5 Peaks output of proteins in *S. algae* SA1 proteome with protein scores, number of peptides, unique peptides and protein coverage.

+ represents a biological replicate.

Worksheet headings (left to right):

LB broth

LB agar

Blood agar

TCBS agar

BHI broth

Table S-6 *S. algae* SA1 proteome summary.

+ represents a biological replicate.

Worksheet headings (left to right):

SA1 proteome summary

LB broth comparison

LB agar comparison

Blood agar comparison

TCBS agar comparison

BHI broth comparison

TableS-7 MP3 analysis of the *S. algae* SA1 genome to identify pathogenic proteins.

Abbreviations: SVM = Support Vector Machine; HMM = Hidden Markov models.

Table S-8 MP3 analysis of the *S. algae* SA1 proteome.

Abbreviations: SVM = Support Vector Machine; HMM = Hidden Markov models.

Chapter 5

Table S-9 Peaks output of proteins in *S. algae* SA1 secretome with protein scores, number of peptides, unique peptides and protein coverage.

+ represents a biological replicate.

Worksheet headings (left to right):

LB mid-exponential

LB stationary

BHI mid-exponential

BHI stationary

Table S-10 *S. algae* SA1 secretome summary.

Abbreviations: NSAF = Normalized spectral abundance factor; emPAI = Exponentially modified protein abundance index.

Table S-11 Label free quantitative analysis of the *S. algae* SA2 secretome using spectral counting, the emPAI and the NSAF.

Abbreviations: NSAF = Normalized spectral abundance factor; emPAI = Exponentially modified protein abundance index.

Worksheet headings (left to right):

LB NSAF

LB emPAI

LB spectral counting

BHI NSAF

BHI emPAI

BHI spectral counting

Table S-12 MP3 analysis of the *S. algae* SA1 secretome.

Abbreviations: SVM = Support Vector Machine; HMM = Hidden Markov models.

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