IDENTIFICATION OF POTENTIAL THERAPEUTIC TARGETS FOR *STREPTOCOCCUS SUIS* INFECTION

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Abstract

Streptococcus (*S.*) *suis* is a commensal porcine pathogen that is the leading causative agent of bacterial meningitis and sepsis in young pigs globally. Systemic infection in swine is associated with considerable mortality and has significant animal welfare and economic consequences. This zoonotic pathogen causes similar clinical pathology in humans as seen in pigs, which can result in life-changing post-streptococcal sequelae. *S. suis* is recognised as an emerging zoonotic disease and is the primary cause of meningitis in human adults in South East Asia, with two recent outbreaks in China manifesting as Streptococcal toxic shock-like syndrome in patients. The virulence mechanisms by which *S. suis* transitions from a commensal species within the nasopharynx and tonsillar microflora to a pathogenic species proliferating within host blood and disseminating into peripheral organs and the brain are still largely unknown. Proposed virulence mechanisms are often extrapolated from current knowledge about the pathogenesis of similar infections caused by Group A and Group B Streptococci. Whilst *S. suis* infections are readily treatable with β-lactam antibiotics, reports of antimicrobial resistance are increasing and driving the need for alternative therapeutics with novel targets and mechanisms of action. Maintaining effective treatment options are critical as the genetic diversity of serotypes within the *S. suis* strain population has meant that an effective commercial vaccine has yet to be developed. Establishing a more comprehensive understanding of the mechanisms of *S. suis* pathogenesis must be achieved to identify molecular targets for novel therapies and diagnostic tools.

Transposon mutagenesis is frequently used in bacteriology to identify genes which are associated with increased fitness or essential for life. The Pragmatic Insertional Mutation Mapping System is a mapping-based tool which utilises the mutagen pG$^+$host::IS*S1* to identify essential genes in Gram-positive species in varying growth conditions. The PIMMS protocol was successfully used to elucidate genes which were essential for the growth of *S. suis* P1/7 in Brain-Heart Infusion (BHI) media with the addition of hydrogen peroxide (H_2O_2) to model a phagocytic respiratory burst *in vitro.* In total, 160 genes were identified as being essential for general survival and growth in media. Of these, functional annotation revealed that the majority of genes were associated with basic cellular functions including metabolism and information processing. Further analysis revealed that several genes were associated with the cell division cycle and the signal recognition particle pathway. A total of 35 genes were identified as important for survival and growth in the presence of H_2O_2. Statistical analysis revealed that a switch from carbohydrate metabolism to general metabolism occurred in the transition from the BHI to the H_2O_2 environment, evidenced by an overrepresentation of genes associated with general metabolism in the H_2O_2 phenotype. Genes associated with alternative metabolic pathways in the presence of H_2O_2 and the direct and indirect expression and secretion of

virulence factors were identified following further investigation. The FtsEX, complex, FtsY, Fhs, FolD, CcpA, SecE, putative signal peptidase I (SSU0212), OppD and putative haemolysin-III (Hly-III) (SSU0854) were identified as having promising novel therapeutic or diagnostic potential which require greater investigation and may be utilised in the control of future *S. suis* infections.

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Table of Contents

Table of Figures

Table of Tables

1.0 Introduction

1.1 *S. suis*: an important swine pathogen

1.1.1 *Classification of S. suis*

Streptococcus suis (*S. suis*) is a facultatively anaerobic, Gram-positive coccoid bacterium which occurs as single cells, in pairs or short chains *(Dutkiewicz et al., 2017a; Gottschalk et al*., 1989). Streptococci are classified based on colony morphology, haemolysis, biochemical reactions, and serological specificity. They are divided into three groups based on the haemolytic activity they exert on whole blood agar, a process by which bacteria lyse red blood cells to liberate haem compounds as a source of iron for growth and colonisation (Bates *et al.*, 2003): β-haemolytic (clear, complete lysis of red cells), α-haemolytic (incomplete, green haemolysis), and γ-haemolytic (no haemolysis) (Sharma and Gupta, 2014). Most *S. suis* strains appear as grey-white colonies and are classified as α-haemolytic (Huh *et al.*, 2011).

The peptidoglycan layer of *S. suis* is enveloped in a capsular polysaccharide (CPS) constituting of a layer of monosaccharides, amino acids and repeating antigen subunits (Lowry *et al.*, 2014; Sarkar *et al.*, 2014). Serological evaluation of the genetic diversity between CPS antigens using traditional co-agglutination assays has led to the characterisation of 29 true serotypes of *S. suis* identified to date, despite 35 originally being described (Auger *et al.*, 2018). Serotypes include 1-19, 21, 23-25, 27-30, 31 and 1/2 which expresses CPS antigens common to both serotype 1 and 2. Divergent serotypes 32 and 34 have since been reclassified as *Streptococcus orisratti* and serotypes 20, 22 and 26 as *S. parasuis* (Hill *et al.*, 2005; Estrada *et al.*, 2019). *S. suis* also possesses genotypic diversity within serotypes whose distribution varies worldwide. Multilocus sequence typing (MLST) can be used to assess the degree of genetic relatedness between isolates and had been used to determine the distribution of pathogenic and commensal-specific serotype and MLST patterns in countries globally to gain greater insight into the pathogenic potential of circulating strains (Estrada *et al*., 2019; King *et al*., 2002).

1.1.2 *S. suis as a commensal species in the healthy porcine microbiome*

It is now widely accepted *S. suis* contributes to the normal porcine nasal and oropharyngeal microflora and may also have niches within the gastrointestinal and reproductive tracts (Murase *et al.*, 2019). Reviews of tonsillar carrier rates suggest that colonisation is likely to be close to 100% (Goyette-Desjardins *et al.*, 2014). Discrepancies in tonsillar carrier rates have demonstrated significant ranges between herds and farms, for example in China detection

rates in clinically healthy animals ranged from 19.5-93.9%, however such broad ranges are likely due to sampling and culture based differences and findings cannot be extrapolated to the rest of the world (Zhang *et al*., 2009). Worldwide, serotype 2 is most frequently isolated from swine (27.9%) and is also most commonly associated with invasive disease in humans, followed by serotypes 9 (19.4%) and 3 (15.9%), however percentages vary between countries (Goyette-Desjardins *et al*., 2014).

S. suis has adapted to its porcine reservoir host and is a highly successful coloniser of mucosal surfaces of the upper respiratory tract, particularly the palatine tonsils, nasal cavities and submaxillary lymph nodes (Tharavichitkul *et al.*, 2014; Wileman *et al.*, 2019). Colonisation typically occurs in animals between 4-10 weeks of age; however, it has been suggested that colonisation may occur as early as five days post-partum and prior to weaning (Torremorell, Calsamiglia and Pijoan, 1998; Dutkiewicz *et al.*, 2017a).

Piglets heterogeneously acquire the bacteria as a result of close contact with carrier sows (Zhang *et al*., 2009). It is widely accepted that piglets can become colonised vertically via the ingestion of uterine and vaginal mucus secretions during parturition, however research into the role of the porcine vaginal and skin microbiomes in the colonisation of *S. suis* in neonates is lacking (Amass, Sanmiguel and Clark, 1997). As *S. suis* resides within the palatine tonsils, suckling may be another colonization route, however species level analysis of *Streptococcus* populations on the skin and teats of sows is yet to be established (Pena Cortes *et al.*, 2018) and there is no available literature which describes the detection of *S. suis* in milk.

The gastrointestinal tract may also be a niche for *S. suis* colonisation. At the time of weaning piglets undergo major intestinal microbial shifts as they transition from colostrum and milk to solid feed that contains carbohydrate, fibre, pre- and probiotics (Guevarra *et al.*, 2019). Organic acids, essential oils and enzymes which are included to feed to maximise cereal metabolism and improve gut barrier function may also contribute to microbiota shifts (Xu et al., 2020). Numbers of Lactobacilli and Enterobacteria decrease significantly whilst *S. suis* has been found to increase within the digesta within the stomach, jejunum and colon during this period (Su *et al.*, 2008; García *et al.*, 2016), providing increased *S. suis* colonisation potential.

Whilst *S. suis* is an endemic pathobiont in global commercial swine populations and predominately infects piglets, it has also been isolated in rare cases from clinically ill companion and production mammals including ruminants, horses, cats and dogs (Devriese and Haesebrouck, 1992; Salasia, Lämmler and Devriese, 1994; Roels *et al.*, 2009; Muckle *et al.*, 2010; Okwumabua *et al.*, 2017). *S. suis* has also been isolated from clinically ill fallow deer and birds, as well as wild European rabbits and boars (Devriese *et al.*, 1993; Sánchez del Rey *et al*., 2013; Sánchez del Rey *et al*., 2016). Although humans are not considered reservoirs,

colonisation by *S. suis* has been confirmed in those with close contact with both swine and associated pork products, at an estimated rate of 5%, although this figure is likely to be an underestimation (Strangmann, Fröleke and Kohse, 2002; Goyette-Desjardins *et al.*, 2014).

1.1.3 *Routes of infection and modes of transition in swine*

Despite being a frequent early coloniser of the porcine upper respiratory tract, *S. suis* is also most important global bacterial cause of meningitis in swine production and contributes to severe economic losses and compromised animal welfare globally (Haas and Grenier, 2018; Hopkins *et al.*, 2018; Vötsch *et al.*, 2018). Primary infections result in meningitis, polyarthritis, endocarditis and polyserositis (Dutkiewicz *et al.*, 2017a) which are associated with clinical signs such as fever, lameness (Dekker *et al.*, 2013), inflammation of joints, and rapid onset of neurological signs such as paddling (Besung *et al.*, 2019) and sudden death (Marois *et al.*, 2007). Systemic infections with *S. suis* are mainly associated with weaning piglets and show high morbidity with a poor prognosis, even with early treatment (Baums *et al.*, 2009). The mechanism for *S. suis* transition from a commensal species to a pathogenic species causing systemic infection in some piglets and not others is not fully understood (Hopkins *et al.*, 2018).

Clinical cases of *S. suis* infections most frequently appear in animals aged between 4 to 8 weeks of age and the highest mortality rates are typically observed 2-4 weeks after the weaning period (Cloutier *et al.*, 2003). Porcine weaning management practices including abrupt separation from the sow, transportation and handling and grouping of animals from different origins, are known to exacerbate stress and contribute to reduced immune competence and may increase *S. suis* infection risks (Campbell *et al*., 2013). The mortality rate may be as high as 30% in untreated piglets, with an average within-litter mortality of 14.4% (Hopkins *et al.*, 2018). Reported variation in clinical signs, including cessation of disease in some experimental infection studies has been attributed to differences in individual immune responses to infection as well genetic variation between strains (Berthelot-Hérault *et al.*, 2001), however differences in infectious doses and route of inoculation may also play a role.

S. suis can also accumulate in the environment and animals may be colonised from a number of sources including feed and water troughs contaminated with saliva, insect vectors, fomites such as manure covered boots and from cutaneous injuries resulting from castration, ear notching and tail docking (Dee and Corey, 1993; Lloyd *et al.*, 2016; Arai *et al.*, 2018; Hopkins *et al.*, 2018; Murase *et al.*, 2019). The routine application of animal manure as crop fertiliser may also provide a reservoir for *S. suis* and promote environmental persistence (Zhao *et al.*, 2014).

Despite this, transmission of *S. suis* is considered to mainly occur via the respiratory route, directly through nose to nose contact but can be transmitted horizontally in susceptible swine from both asymptomatic carrier herds and individuals with clinical disease (Dekker *et al.*, 2013). It is thought that infection begins once the bacteria penetrate host mucosa, enter the bloodstream and invade the spleen, liver, kidney, lung and heart, however the exact trigger and shift from commensal to invasive pathogen within the host and pathogenesis of infection is still largely unknown (Segura *et al.*, 2016). *S. suis* can also overcome tight junctions within the blood-brain barrier which promotes the infection of the central nervous system and inflammation, leading to the clinical manifestation of meningitis (Hoffman and Weber, 2009). Strains have also been demonstrated to translocate from the gastrointestinal tract to the mesenterial lymph nodes, liver, spleen and joints in susceptible pigs under transport stress (Swildens *et al.*, 2004). This indicates that the gastrointestinal tract cannot be excluded as a potential site of infection especially as the *S. suis* population within the gut microbiome have been found to increase in weaning piglets (Su *et al.*, 2008; García *et al.*, 2016).

In vivo interactions between *S. suis* and other endemic swine pathogens has been shown to exacerbate other infectious diseases that are also associated with significant economic losses (Valdes-Donoso *et al.*, 2018). Post-weaned piglets with existing *Mycoplasma* or *Pasteurella* infections are susceptible to *S. suis* induced suppurative pneumonia (Reams *et al.*, 1994). Co-infections with porcine reproductive and respiratory syndrome virus (PRRSV), porcine circovirus 2 (PCV2), and porcine respiratory coronavirus (PRCoV) have been found to result in the down regulation of the innate immune response and deregulation of the adaptive immune response which interferes with normal host defence mechanisms and predisposes animals to *S. suis* induced septicaemia and pneumonia (López and Martinson, 2017). Animals co-infected with H1N1 influenza virus and *S. suis* have also been shown to exhibit more severe clinical symptoms of pneumonia and increased mortality rates (Lin *et al.*, 2015).

1.2 The emergence of *S. suis* as a zoonotic pathogen

1.2.1 *Epidemiological trends of human S. suis infections*

S. suis is amongst ten porcine pathogens that are zoonotic to humans (Pappas, 2013). Since the first case was reported in the Netherlands in 1968, more than 1600 cases have been reported globally in 30 countries including China, Brazil and Vietnam which are some of the highest global pork producers (Arends & Zanen, 1988; Bojarska *et al.*, 2020; Feng *et al*., 2014; Hu *et al*., 2000; Wisselink *et al*., 2000). Despite also being significant pork producers, Canada and the United States have significantly lower reports of infections alongside South America, Australia and New Zealand (Lopreto *et al.*, 2005; Gottschalk *et al.*, 2010, 2013; Soares *et al.*,

2015; O'Dea *et al.*, 2018). The highest prevalence rate by far is in Southeast Asia, with the majority of cases associated with serotype 2, however serotypes with typically lower zoonotic potential including 1, 4, 5, 9, 14, 16, 21 and 31 have also been reported to cause severe systemic disease (Nghia *et al.*, 2008; Wertheim *et al.*, 2009; Taniyama *et al.*, 2016; Kerdsin *et al.*, 2017). Differences between human infection rates reported worldwide could be due to industrialisation and farming practices, with many more small scale farms in Asia, differences in slaughter practices and also serotype or strain specific variation (Huong, Ha, *et al.*, 2014). *S. suis* may be responsible for more than 90% of human bacterial meningitis cases in Southeast Asia, particularly in Vietnam, Thailand and China where high pork consumption and small-scale swine farming is endemic (Goyette-Desjardins *et al.*, 2014; van Samkar *et al*., 2015).

Meta analyses have identified that more than eighty percent of infections occur in healthy middle-aged adult males and infections appear to be most greatly associated with close occupational contact with infected pigs or pork products, particularly where appropriate personal protective equipment practices are not adopted (Zhu *et al.*, 2008; Wertheim *et al.*, 2009; van Samkar *et al.*, 2015; Rayanakorn *et al.*, 2018) An increased risk of infection has been reported for immunocompromised patients including those with alcoholism, diabetes mellitus, cancer and structural heart disease (Voutsadakis, 2006; Gomez *et al.*, 2014; van Samkar *et al.*, 2015; Mancini *et al.*, 2016). Highest occupational risks are found in swine farmers and transporters, abattoir workers, butchers and veterinarians (Strangmann, Fröleke and Kohse, 2002; Ibaraki *et al.*, 2003; Voutsadakis, 2006; Zalas-Wiecek *et al.*, 2013; Dutkiewicz *et al.*, 2017b), with occasional reports in those handling carcasses including chefs and hunters (Halaby *et al.*, 2000; Piech *et al.*, 2009; Malezieux *et al.*, 2014). Oral infection is rarely observed in Western countries, however *S. suis* is considered an emerging food-borne pathogen in Southeast Asia where consumption of traditional "high-risk" dishes containing raw blood and tissues increases the risk of infection alongside backyard slaughtering and the consumption of sick animals (Hanterdsith *et al.*, 2013; Huong *et al*., 2014; Dutkiewicz *et al*., 2017b).

Despite increased awareness of human *S. suis* infections within scientific and medical disciplines, the number of reported global infections reported in literature is continuing to rise (Huong, Ha, *et al.*, 2014). A lack of awareness amongst community members and policymakers about the economic burden of disease, lack of public health interventions for at-risk populations, underutilisation of diagnostic capacity in hospitals and poor continuity of care may contribute to this increase (Dutkiewicz *et al.*, 2017b; Rayanakorn *et al.*, 2018; Huong *et al.*, 2019). On the other hand, improved diagnostic capability may account for the increased numbers of *S. suis* infections in Southeast Asia (McNerney, 2015; Gomez-Torres *et al.*, 2017).

1.2.2 *Systemic inflammatory response syndrome, post-streptococcal sequalae and toxic-like shock syndrome*

Although, activation of the cellular immune response is a biologically protective mechanism which is associated with production of antibodies against invading pathogens and pathogen clearance, systemic inflammatory response syndrome (SIRS) may occur secondary to infection as a consequence of an exaggerated defence response (Chakraborty and Burns, 2020). SIRS is one of the most commonly reported clinical conditions resulting from *S. suis* infection in swine and humans, most likely due to the pathogens capacity to persist at high concentrations in the blood (Wertheim *et al.*, 2009; Kim *et al.*, 2018; Dai *et al.*, 2019; Ljungström, Andersson and Jacobsson, 2019; Minasyan, 2019). SIRS is a systemic disorder that is mediated by a downstream cytokine cascade resulting in vasodilation, increased vascular permeability, myocardial depression, reduced global oxygen supply and procoagulant activity; and ranges from sepsis, septic shock and multiple organ dysfunction syndrome (Tupchong, Koyfman and Foran, 2015; Hotchkiss *et al.*, 2016). In general, septic shock is associated with high morbidities including long-term chronic illness characterised by prolonged inflammation, immune suppression, cognitive and functional deficits in humans and has been described in patients following infections with *S. suis* (Zhu *et al.*, 2008; Hotchkiss *et al.*, 2016). During an outbreak of *S. suis* in the Sichuan Province in 2005, 24% of patients experienced septic shock, which led to death in 80% of cases (Lun et al., 2007a). In swine, sepsis directly impacts on economic burden and animal welfare, with attributed mortality rates resulting from *S. suis* sepsis thought to be as high as 20% (Cloutier *et al.*, 2003).

Other post-streptococcal sequalae associated with systemic *S. suis* infection and sepsis include profound or complete hearing loss and/or vestibular dysfunction which can occur uni- or bilaterally (Navacharoen *et al.*, 2009; Huong *et al.*, 2019; Rayanakorn *et al.*, 2019). Loss of sensorineural hearing in the high-frequency range and vestibular damage from bacterial toxins appears to occur early during the pathogenesis of meningitis (Sena Esteves *et al.*, 2017). Major complications include cognitive deficit, motor deficit, seizures, visual impairment, and hydrocephalus. Challenging behaviour, learning difficulties, hypotonia, diplopia and tinnitus are considered more minor sequalae that have also been reported (Edmond *et al.*, 2010; van Samkar *et al.*, 2015).

Streptococcal toxic shock syndrome is frequently associated with Group A Streptococcus (GAS) infections through expression of putative superantigens including SpeA, SpeC and SSA (Proft *et al.*, 2003). These antigens are not present in *S. suis*, however two large-scale outbreaks of human infections were associated with streptococcal toxic shock-like syndrome

(STSLS) in China during 1998 and 2005 with a 20% mortality rate and more than 200 occupation-associated infections recorded (Hu *et al*., 2000; Yu *et al*., 2006; Fittipaldi *et al.*, 2012; Wang *et al*., 2019; Wang *et al*., 2019; Zhang *et al*., 2017). *S. suis* related STSLS has been associated with the presence of an 89Kpb genomic pathogenicity island, acquired through horizontal gene transfer. More specifically, it was associated with the presence of *virD4* within a putative type IV-like secretion system and its upregulation is shown to be associated with the increased secretion of the cell death inducer and proinflammatory effector PrsA in murine models (Jiang *et al*., 2016; Lun *et al*., 2007b). This is thought to be a direct factor in STSLS sequelae and emphasises the importance of *S. suis* as an emerging zoonotic pathogen and increased virulence potential through acquisition of new genetic material.

1.3 Diagnosis of *S. suis* infections in swine and humans

1.3.1 *Diagnosis of S. suis in swine*

Presumptive diagnosis of *S. suis* infection in swine is often based on history, age of animals and clinical signs of acute meningitis which may include also red skin discoloration, anorexia, ocular-nasal exudate, diarrhoea, lameness, pyrexia, malaise and inflammation of joints (Goyette-Desjardins *et al.*, 2014; Hopkins *et al.*, 2018; Besung *et al.*, 2019). Confirmation of infection requires necropsy evaluation of gross and microscopic lesions and bacterial culture (Goyette-Desjardins *et al.*, 2014; Besung *et al.*, 2019). Tissue biopsies of affected tissues and histopathological analysis of the brain, heart, liver, intestines, kidney, and spleen are examined microscopically, with lesions in affected tissues likely to indicate meningitis, bronchopneumonia, endocarditis, myocarditis, pericarditis, erosion and enteritis, haemorrhagic hepatitis, glomerulonephritis, lymphoid depletion and haemorrhage and accumulation of inflammatory cells in the spleen; all pathologies that are compatible with systemic bacterial infection, meningitis and arthritis (Madsen *et al.*, 2001; Besung *et al.*, 2019).

Microbiological characterisation of isolated bacteria include assessing haemolytic activity and colony morphology, analysis of catalase, esculin hydrolysis, pyrrolidinyl arylamidase, sugar fermentation and Hippurate hydrolysis are often deployed to identify the causative pathogens in routine veterinary diagnostics, however assays may cause discrepancies between species of *Streptococcus* and lead to a misdiagnosis (Brigante *et al.*, 2006; Preethirani *et al.*, 2015).

Since *S. suis* is the primary pathogen that causes meningitis and septicaemia in pigs, the majority of work into the development of diagnostic tools has been centred around developing diagnostic PCR tests to identify specific serotypes of *S. suis* based on genes within the CPS cluster (Liu *et al.*, 2013) or multiplex PCR tests for other swine respiratory pathogens such as

those implicated in PRRSV (Lung *et al.*, 2017). More recently, the highly conserved glutamate dehydrogenase (Gdh) and the recombination/repair protein (RecN) encoding genes within the *S. suis* genome have been targeted but primers for these regions are not routinely included in current assays (Tarini *et al.*, 2019).

1.3.2 *Diagnosis of S. suis infections in humans*

S. suis infections in humans predominately manifest as meningitis (van Samkar *et al.*, 2015) with patients presenting with fever/neck stiffness/altered mental status or headache and requiring prompt analysis of cerebrospinal fluid and/or blood, to distinguish the pathogen from other aetiological agents or non-infectious causes of meningeal inflammation (Hoffman and Weber, 2009; Viallon *et al*., 2016). Quantitative (q) PCR is preferentially used for simultaneously screening for the major bacterial pathogens responsible for causing meningitis in humans which include *S. agalactiae, S. pneumoniae, Neisseria meningitidis, Haemophilus influenzae, Listeria monocytogenes* as well as 16S rRNA sequencing from other potential bacterial agents (Wagner *et al.*, 2018; Albuquerque *et al.*, 2019). Given *S. suis* is an emerging pathogen, it is often not included in PCR based diagnostics (El Bashir, Laundy and Booy, 2003). Despite this, many low-income countries lack infrastructure for infectious disease management and the ability to rapidly and accurately identify infections in molecular microbiology facilities which may result in misdiagnosis (Wertheim *et al.*, 2009; Ahmed *et al.*, 2015). This may have historically contributed to the underreporting of *S. suis* infections in humans (Wertheim *et al.*, 2009).

Achieving diagnosis is not always limited by PCR techniques and other nucleotide-based technologies such as DNA microarray and ribotyping have been used to characterise *S. suis*, however they are not always routinely used in conventional diagnostics (Xia *et al.*, 2018). Loop-mediated isothermal amplification (LAMP) which targets 16S rRNA has been used to identify clinical isolates containing the 89Kpb pathogenicity island and may be more cost effective as point of care diagnosis (Njiru, 2012) and for epidemiological monitoring of strains (Zhang *et al.*, 2013). However, it should be noted that few strains carry the pathogenicity island and it would not be applicable for wide scale screening.

Next generation sequencing (NGS) technologies in combination with tailored bioinformatic workflows to analyse genomic information are emerging as a promising single, universal pathogen detection method for infectious disease diagnostics (Gu, Miller and Chiu, 2019; Salazar *et al.*, 2020). Pathogens are identified by recombining sequencing reads and aligning them to reference databases (Simner, Miller and Carroll, 2018). When NGS and PCR are used synergistically, pathogen genome regions of interest at very low levels within samples which

can be selectively enriched before sequencing and can successfully detect multiple pathogens in clinical samples which may be missed by routine techniques (Anis *et al.*, 2018). NGS technologies for diagnosis of *S. suis* infections may be particularly valuable when patients have received prior antibiotic therapy and have been demonstrated to detect pathogenic DNA in culture-negative blood five days post-venepuncture (Dai *et al.*, 2019). Despite having greater sensitivity than traditional technologies, the use of NGS for diagnostic purposes require further development as they still lack specificity and are associated with high cost reagents (Gu, Miller and Chiu, 2019; Torchia *et al.*, 2019).

1.4 Antibiotic susceptibility and resistance

Chemotherapy is still the most important strategy for the treatment of acute *S. suis* bacterial infections (Yao *et al.*, 2014). Successful treatment relies on the effectiveness of antibiotics (Yongkiettrakul *et al.*, 2019), with *S. suis* susceptibility to β-lactam antibiotics including penicillin, ampicillin ceftriaxone and cetiofur with fluoroquinolones and trimethoprim-sulfonamides also critically important for treatment (Burch and Sperling, 2018; Yongkiettrakul *et al.*, 2019). Despite being largely susceptible to β-lactam antibiotics, a low level of resistance to penicillin has been reported in some *S. suis* strains, which could be attributed to spontaneous mutations in genes encoding binding proteins (Haenni *et al.*, 2018; Zhang *et al*., 2008).

The overuse of antibiotics in human and veterinary medicine, including the empirical treatment with broad-spectrum antibiotics to treat Streptococcal infections, has contributed to the emergence of multi-drug resistant genotypes and dissemination of resistance genes. The prophylactic and metaphylactic use of tetracyclines, macrolides and aminoglycosides in swine agriculture has contributed to high rates of resistance in *S. suis* strains world-wide but geographical variations exist (Barton, 2014; Tall *et al.*, 2016; Lekagul, Tangcharoensathien and Yeung, 2019; Rodrigues, 2020). In addition to differences in the prophylactic usage of antimicrobials, variations may also result from differing farming practices including animal stocking densities, biosecurity and perception that the use of antibiotics contributes to profitability through use as growth promoters (Stevens *et al.*, 2007; van der Fels-Klerx *et al.*, 2011; Eltayb *et al.*, 2012; Raasch *et al.*, 2018). Whilst the use of antibiotics as growth promotors was prohibited in Europe in 2006, the USA in 2017, Australasia and Brazil have implemented partial bans on drug classes which have critical importance in human medicine (Brüssow, 2017; Manyi-Loh *et al.*, 2018). Key policies in many countries within Southeast Asia have been introduced but enforcement remains a challenge, as does local compliance to reduce prophylactic usage (Goutard *et al.*, 2017).

1.5 Pathogenesis of *S. suis* infections

When pathogens breach barrier surfaces such as the skin or mucosal surfaces, pattern recognition receptors of the immune system are rapidly stimulated by microbe associated molecular patterns (Gasteiger *et al.*, 2017). Stimulation results in a downstream cascade of proinflammatory cytokines and chemokines which control the release of effector immune cells and recruit them to the site of infection (Sokol and Luster, 2015). Proinflammatory cytokines such as tumour necrosis factor (TNF) and interleukin (IL)-1 and IL-6 also act as endogenous pyrogens which mediate fever (Netea, Kullberg and Van der Meer, 2000). Myeloid effector cells include polymorphonuclear neutrophils, dendritic cells and macrophages which are involved in the clearance of pathogens via phagocytosis, release of granules that contain enzymes and toxic compounds and formation of extracellular traps (Rosales *et al.*, 2017; Hirayama, Iida and Nakase, 2018). This rapid, non-specific activity which recognises and destroys pathogens during the early stages of infection is known as an innate immune response (Hirayama, Iida and Nakase, 2018). Whilst the innate immune response has limited capacity for memory, cell mediated/adaptive immunity is associated with immunological memory and the ability to respond to and clear a pathogen with greater specificity (Ratajczak *et al.*, 2018). Cell mediated immunity is characterised by the binding, activation and proliferation of T and B leukocytes which are activated by pathogen antigens expressed on antigen-presenting cells following phagocytosis (Janeway *et al*., 2001). During this process B cells mature into memory cells and express surface immunoglobulins (antibodies) which become antigen receptors but can also secrete immunoglobulin into the circulating blood which can rapidly recognise and produce a more fine-tuned response to re-invading pathogens (Bonilla and Oettgen, 2010; Hoffman, Lakkis and Chalasani, 2016).

S. suis causes severe systemic infection in both swine and humans of similar pathology but typically characterised by septicaemia and meningitis (Kim *et al.*, 2018). In order to cause disease, *S. suis* successfully colonises the host, breaches epithelial barriers, achieves and maintains a high level of bacteraemia, invades organs and stimulates exaggerated inflammation (Fittipaldi *et al.*, 2012). The ability for *S. suis* to transition from a commensal organism and cause severe systemic infection through resisting the innate immune response has been associated with more than 100 putative adhesins and virulence factors, which can be classed into four main groups. These include surface components or secreted elements, enzymes, transcriptional factors and/or regulatory systems and transporter factors or secretion systems (Gottschalk and Segura, 2000; Dutkiewicz *et al.*, 2018). Specifically, those factors that have been linked to adhesions and invasion of epithelial cells, resistance to phagocytosis, stimulation of proinflammatory cytokines and interaction with the blood-brain barrier (BBB) have been the target of most pathogenesis-based research. Despite an increasing number of

studies, the pathogenesis of *S. suis* infection is yet to be fully elucidated and disease physiology is hypothesised and based on the similarity in pathology caused by Group B Streptococcus infections in humans (Mitchell and Mitchell, 2010; Calzas *et al.*, 2017).

1.5.1 *Immune evasion and modulation: the capsular polysaccharide (CPS)*

When a pathogen invades, host cells recognise pathogen-associated molecular patterns (PAMPs) through pathogen recognition receptors, which activates the innate and acquired immune responses, which in turn serve to eliminate and recognise pathogens (Shishido *et al.*, 2012; Xia *et al.*, 2019). In the absence of specific antibodies that are produced by the humoral response following previous exposure, *S. suis* resists phagocytosis and intracellular killing by leukocytes, following epithelial invasion and entry into the blood. Survival in blood is attributed to the capsular polysaccharide (CPS), as non-capsulated mutants are rapidly cleared during challenge within *S. suis* swine and murine models (Chabot-Roy *et al.*, 2006; Houde *et al.*, 2012). Bacterial CPS are a diverse class of high molecular weight polysaccharides found in many species of Gram-positive and Gram-negative bacteria which prevent desiccation and shield bacterial cell wall proteins from host immune cell recognition (Cress *et al.*, 2014). The *S. suis* CPS components express similarities to host sialic acids (Van Calsteren *et al.*, 2010) that are responsible for the identification of invading pathogens, and regulation of host inflammation (Pillai *et al.*, 2012; Varki and Gagneux, 2012; Chang and Nizet, 2014). Similarly to Group B Streptococcus, the sialic acid moiety of the *S. suis* CPS may facilitate immune system invasion through the molecular mimicry of host sialic acids and downregulate leukocyte activation (Pillai *et al.*, 2012; Chang and Nizet, 2014). Sialic acid linkages may also mediate bacterial attachment to the surface of phagocytes in the blood and dissemination to organs via “phagocyte hitchhiking”, as sialidase-treated bacteria have reduced adhesion levels to murine macrophages *in vitro* (Segura and Gottschalk, 2002; Sofias *et al.*, 2020).

The CPS of some strains have been found to block nitric oxide (NO) production and bacterial phagocytosis in contrast to non-encapsulated mutants (Houde *et al.*, 2012). The increase in internalisation is supported by Lecours *et al.*, (2012) who also demonstrated that non-encapsulated mutants induced the secretion of proinflammatory cytokines from murine Toll-like-receptors and dendritic cells, in contrast to a significant reduction in parental strains, therefore supporting the role for the CPS in immune quiescence and phagocytosis resistance. *S. suis* may still be engulfed by phagocytic cells, however bacteria have been demonstrated to remain viable 24 hours following phagocytosis which may also contribute to the dissemination of *S. suis* within both dendritic cells and porcine brain microvascular endothelial

cells (Segura *et al.*, 2006). Persistence has historically been attributed to the CPS, however muramidase-released protein (MRP) may also be accountable (Meijerink *et al.*, 2012). Since the role of Lactosylceramides (LacCers), a ceramide lipid found in the microdomains of neutrophils (Bacic, Fincher and Stone, 2009) have been implicated in inducible nitric oxide synthase (iNOS) gene expression and nitric oxide (NO) production, the blocking of LacCers by the CPS may also contribute to a diminished immune response by dysregulating NO production, therefore impacting downstream signalling pathways which lead to proinflammatory cytokine production, T cell recruitment, apoptosis and cell signalling that are ordinarily modulated by NO (Pannu *et al.*, 2004; Vig *et al.*, 2004; Wink *et al.*, 2011).

1.5.2 *Epithelial translocation and immune perturbation: Suilysin (SLY) and Dipeptidyl peptidase IV (DPP-IV)*

To reach the bloodstream of the host, *S. suis* must colonise and disrupt the integrity and navigate across mucosal barriers (Bercier, Gottschalk and Grenier, 2020). Pathogenic Streptococci are characterised by the release of cytotoxic cholesterol-dependent pore-forming cytolysins (haemolysins) during infection (Meng *et al.*, 2016). Suilysin (SLY), is an extracellular protein expressed by *S. suis* but is antigenically related to the streptolysin O and pneumolysin of *S. pyogenes* and *S. pneumoniae* respectively (He *et al.*, 2014). SLY specifically bind to cholesterol rich receptors on host cells, and are associated with paracellular translocation across the epithelial layers, lysis of immune cells and stimulation of macrophages to secrete large quantities of proinflammatory cytokines which increase the permeability of the blood brain barrier (BBB) (Billington, Jost and Songer, 2000; Lun *et al.*, 2003; Vötsch *et al.*, 2020). SLY-producing strains of *S. suis* stimulate the released of lactate dehydrogenase, an enzyme involved in cell lysis, and increasing permeability between the tight junctions between epithelial cells in a number of studies of porcine epithelial cell line infection studies (Vanier *et al.*, 2004; Bercier, Gottschalk and Grenier, 2020)

Inflammation is a hallmark of *S. suis* infection and SLY may potentiate the inflammatory response by macrophages and the breakdown of the BBB (Tanabe, Gottschalk and Grenier, 2008; Vanier *et al.*, 2009). This has led to the hypothesis that cytokines released by the BBB may modulate the increased inflammatory activity of astrocytes or glial cells which in turn increase the permeability or adhesion properties of brain microvascular endothelial cells (BMECs) and facilitate intracranial release of cytokines, chemokines and ROS which contribute to the development and severity of meningitis (Vadeboncoeur *et al.*, 2003; Yau *et al.*, 2018). Several studies have demonstrated that SLY induces the upregulation of inflammatory cytokines IL-6, IL-8 and tumour necrosis factor-α (TNFα) in cell line models

including porcine and human BMECs, human monoblastic leukaemia cells, and porcine alveolar macrophages (Lun *et al.*, 2003; Vadeboncoeur *et al.*, 2003; Vanier *et al.*, 2009).

Intracellular adhesion molecules (ICAM) are expressed on endothelial cells and promote the adhesion and transmigration of leukocytes across the BBB (Glushakova *et al.*, 2018). Purified SLY was shown to significantly increase the upregulation of ICAMs on human THP-1 monocytes but failed to upregulate ICAM-1, vascular cell adhesion molecule-1 (VCAM-1) and E-selectin on human endothelial cells (Al-Numani et al., 2003).

SLY has also been linked to the lysis of red blood cells and the release of extracellular haemoglobin has been associated with the recruitment of leukocytes, platelets and red blood cells to vessel walls, oxidation of lipoproteins and vascular injury (Belcher *et al.*, 2010). Human erythrocytes also exhibited greater susceptibility to lysis, followed by equine, ovine, bovine and porcine red blood cells (Gottschalk, Lacouture and Dubreuil, 1995). This reinforces the need for caution when extrapolating findings from studies using differing *S. suis* strains, cell lines and animal species and highlights the requirement for studies which include a high diversity of *S. suis* strains and standard experimental designs that produce reproducible and comparative results in order to fully elucidate the virulence factors that are associated with the pathogenesis of infection (Auger *et al.*, 2017).

Other surface-bound and secreted proteases may also contribute to immune modulation and persistence in the central nervous system (CNS) by degrading antimicrobial peptides that stimulate an inflammatory response. *S. suis* produces four major proteases which include arginine aminopeptidase, chymotrypsin-like protease, casein protease and the dipeptidyl peptidase IV (DPP-IV) which have been hypothesised to hydrolyse host proteins to attenuate immune responses and destroy tissues (Jobin and Grenier, 2003). Dipeptidyl peptidase IV (DPP-IV) is a serine exopeptidase secreted by eukaryotic cells plays an important role in degrading cytokines, chemokines and neuropeptides to regulate normal inflammation, immunity and vascular function (Fadini and Avogaro, 2011) DPP-IV is also widely expressed by prokaryotic cells, including *S. suis* DPP-IV, which cleaves peptides and proteins with X-Proline or X-Alanine at their N-termini (LeBel *et al.*, 2018). This includes a porcine host defence peptide that is produced by porcine leukocytes and is pivotal in swine innate immune defence against infections (Lee *et al.*, 2005).

Many studies examining the role of putative virulence factors in *S. suis* mediated meningitis have been performed on murine models or porcine and human tissue culture methods and have produced conflicting data (Takeuchi *et al.*, 2013; Meng *et al.*, 2016). Therefore, more research is required to characterise virulence factors that are essential for the pathogenesis of *S. suis* infections in swine to obtain a greater understanding of how and why *S. suis* may

transition from a commensal bacteria to cause systemic infection as well as identifying targets for a commercial vaccine and novel therapeutics.

1.6 Current vaccine development against S. suis

There is no current commercially available vaccine against *S. suis* for veterinary or human use. The requirement for an efficacious commercial vaccine against *S. suis* for veterinary application has been recognised however, the high genotypic, phenotypic and geographic variability of strains, combined with an incomplete understanding of the molecular mechanisms of *S. suis* infection and persistence, has hampered vaccine development (Segura, 2015; Pian *et al.*, 2016). Despite the complex epidemiology of *S. suis,* research has been predominately performed using serotypes 2 and 9 as they are the most frequently associated with invasive disease in swine. There is enormous strain diversity even in individual serotypes which makes vaccine development to cover all serotypes very difficult (King *et al.*, 2002; Rieckmann *et al.*, 2019).

Several vaccines have been trialled against *S. suis* that have focused on conferring protection to piglets as they are the most affected age group. Field trials have focused on serotype-specific inactivated whole-cell autogenous bacterins which are isolated from clinically diseased animals, purified and administered to sows or piglets (Baums *et al.*, 2010). Formalin processing and heat treatments which lead to cross-link instability, structural rigidity and denaturation of proteins in the process of processing bacterins may be a factor contributing to commercial vaccine development failure alongside reports of poor disease control and higher production costs (Meeusen *et al.*, 2007; Thaysen-Andersen *et al.*, 2007; Segura, 2015; Borkar and Goenka, 2019). Research suggests that inactivated vaccines lack the ability to confer immunity to heterologous strains and may require continuous adaptation to include the most appropriate serotypes, which may have geographical limitations given the diversity in serotypes throughout differing regions and countries. It is likely that this would make the generation of a universal serotype or capsular based vaccine unlikely to be successful (Meeusen *et al.*, 2007). Furthermore, in the case of *S. suis,* there may be greater challenges associated with specific vaccine effects during field studies as it is a commensal bacteria in which colonization may occur in up to 100% of individuals within a herd (Arai *et al.*, 2018).

Experiments using live attenuated vaccines may confer immunity to *S. suis* and overcome drawbacks associated with bacterin-based measures, however they can be associated with unpredictable methods of attenuation, with the potential for evolutionary reversion back to virulence (Meeusen *et al.*, 2007; Bull, 2015; Li *et al.*, 2018). One live attenuated serotype 2 strain was found to provide piglets with 100% cross-reaction protection against the same

serotype but only 60% protection against serotype 7 strains, highlighting the continued problem of generating vaccines with cross-serotype protection (Hu *et al.*, 2019).

Characterising highly conserved immunogenic factors that elicit heterogenous protection against all *S. suis* serotypes may contribute to a vaccine with greater application (Feng *et al.*, 2018). Prepartum vaccination of sows with inactivated recombinant surface antigen conferred cross-immunity to heterologous strains of *S. suis* in challenged neonatal piglets (Hsueh *et al.*, 2017). However, immunoglobulin was only detectable until 6 weeks of age which was attributed to the short half-life of colostrum-derived immunoglobulin.

Many vaccines including those described here are still in experimental phases and require additional studies to evaluate their long-term protective capacity (Feng *et al.*, 2018). Prophylactic vaccination of swine with a commercial vaccine against *S. suis* may directly reduce the requirement for antibiotic use in agriculture and indirectly by reducing the number of subsequent human infections, therefore identifying suitable vaccine targets is paramount (Lipsitch and Siber, 2016). Currently, vaccine development strategies may target single genes or proteins that have been empirically identified, however the pathogenesis of *S. suis* infection is multifaceted and using techniques that enable the identification of multiple genes associated with infection and persistence can be utilised.

1.7 Identification and prediction of bacterial gene essentiality and virulence factors

1.7.1 *Transposon sequencing*

Transposon elements (TEs) are mobile genetic elements that naturally exist in all bacterial genomes, have a vital role in evolution and can provide resistance to antibiotics and heavy metals (Blackwell, Iqbal and Thomson, 2019). TEs have the capacity to move within or between chromosomes and replicons in cells and mobility is mediated by a transposase enzyme which hydrolyses the double strand and promotes transposon insertion into the genome (Munoz-Lopez and Garcia-Perez, 2010) (Nesmelova and Hackett, 2010). Disrupting genes by artificially inserting transposons, known as transposon mutagenesis, has been used to elucidate genes involved in pathogenesis of bacterial infections (Lin *et al.*, 2014) and provides a mechanism to exploit the function of transposons to achieve a greater understanding of how bacterial genotypes contribute to observed phenotypes (Van Opijnen and Camilli, 2013; Peng *et al.*, 2017). High-throughput approaches facilitate simultaneous sequencing of many transposon mutants grown under differing environments alongside massive parallel sequencing, to identify genes important for growth or survival and potentially linked to host pathogenesis. Approaches involve the construction of transposon insertion

libraries where the majority of non-essential genes contain high-density insertions before the library is then cultured within the phenotype environment. Mutant frequency can then be determined by comparison of growth conditions, for example growth in bacterial media compared with growth in blood or serum for a pathogen that can cause septicaemia, such as *S. suis.* Bacterial DNA is then subjected to massive parallel sequencing, in particular focussed on the genome/transposon junctions which allows for the quantification of essential and conditionally essential genes involved (Van Opijnen and Camilli, 2013).

Four main approaches for mapping the essential genome have been developed. They include insertion sequencing (INSeq), transposon insertion sequencing (TN-Seq), high-throughput insertion tracking by deep sequencing (HITS) and transposon-directed insertion site sequencing (TraDIS) (Gawronski *et al.*, 2009; Goodman *et al.*, 2009; Langridge *et al.*, 2009; van Opijnen, Bodi and Camilli, 2009). Each mapping approach is similar, however there are subtle differences in applicable transposon or insertional elements, preparation protocols and sequencing approaches, PCR product sizes, library design and software tools used to bioinformatics analysis (Van Opijnen and Camilli, 2013). Genome-wide mapping approaches have been successfully used to identify niche-specific essential genes in several significant *Streptococcus* species. These include *S. pneumoniae* (Verhagen *et al.*, 2014)*, S. pyogenes* (Le Breton *et al.*, 2015)*, S. agalactiae* (Hooven *et al.*, 2016), *S. uberis* (Blanchard *et al.*, 2016) *S. mutans* (Shields *et al.*, 2018) *S. equi* (Charbonneau *et al.*, 2020) and *S. suis* (Arenas *et al.*, 2020).

High throughput mapping approaches are often associated with laborious, timely and complex laboratory and bioinformatics protocols in order to produce mutants, isolation and sequencing of DNA fragments flanking insertions (Blanchard *et al.*, 2016)*.* Successful transformation often only occurs in bacterial species which have high transformation frequencies (Maguin *et al.*, 1996). Perhaps one of the most significant challenges of attempting to randomly insert transposons into bacterial genomes is that many transposons demonstrate preference for insertion at differing locations on genes based on their nucleotide sequences which may result in insertion biases and incomplete coverage of a region (Munoz-Lopez and Garcia-Perez, 2010; Green *et al.*, 2012). To overcome some limitations of conventional mapping approaches, a more accessible transposon insertion mapping pipeline was developed utilising the thermosensitive replicon pG^{+}host::IS*S1* transformant which integrates randomly into Gram-positive Streptococcal, Enterococcal and Lactococcal genomes (Maguin *et al.,* 1996), species which have previously been notoriously difficult to manipulate (Blanchard *et al.*, 2015a). The Pragmatic Insertion Mutation Mapping System (PIMMS), has low insertion bias and mutagenesis with pGhost9:IS*S1* can be achieved with little manipulation of cells and inter-

strain competition, further reducing bias (Blanchard *et al.*, 2016). The PIMMS laboratory protocol alongside a bespoke bioinformatic pipeline has been successfully used to analyse genes essential for survival of *Streptococcus uberis,* a pathogen responsible for bovine mastitis and is widely applicable to any bacterial species that can be mutated with pGhost9:IS*S1* including *S. suis* (Blanchard *et al.*, 2016; Tomazi *et al.*, 2019).

Several housekeeping genes have been already identified as important for cellular homeostasis in *S. suis.* They include *sodA* which confers protection against superoxide radicals (Merkamm and Guyonvarch, 2001), enzymes and proteins associated with amino acid synthesis (*aroA),* phosphorylation (*thrA*) and protein assembly for normal cell growth (*cpn60*) (Katinka *et al.*, 1980; Hemmingsen *et al.*, 1988; Sun *et al.*, 2005). Others are associated with nutrient acquisition such as Dpr which is involved in iron acquisition and homeostasis and Gki which regulates glucose kinase activity (Pulliainen *et al.*, 2005). The mechanisms of survival and dissemination of *S. suis* in porcine and human blood are poorly understood, therefore genome-wide transposon mutagenesis and high throughput sequencing could identify conditionally essential genes which may be important for pathogenesis. One recent TNSeq based approach investigated a *S. suis* infection model in gnotobiotic piglets and identified a number of conditionally essential genes important for survival in porcine brain, blood, serum and cerebral spinal fluid through direct inoculation of *S. suis* transposon libraries. The majority of genes identified were involved in metabolic and transport processes, translation, ribosomal structure and biogenesis, transcription, replication, recombination, repair and cell wall and envelope biogenesis (Arenas *et al.*, 2020). Using similar mapping approaches to identify similarities and differences in genes required for survival in human infections through in vitro studies in blood, serum or other biologically relevant fluids will likely provide greater insight into the pathogenesis of both human and swine *S. suis* infections.

1.8 The role of hydrogen peroxide (H_2O_2) in control of bacterial infection

Given that *S. suis* must survive and proliferate within the human or porcine host to cause septicaemia, the function of host phagocytic cells is critical for pathogen clearance. The phagocytic respiratory burst utilises endogenous reactive oxygen species (ROS) to target phagocytosed pathogens, of which, H_2O_2 is a fundamental precursor to reactive intermediates which have bactericidal capacity (Odobasic, Kitching and Holdsworth, 2016; Yang, Huang and Xu, 2016; Nguyen, Green and Mecsas, 2017). Many bacteria have developed strategies that facilitate resistance to killing by neutrophils and the elimination of ROS during respiratory bursts, including indirect detoxification of ROS and indirect repairing damaged molecular and

cellular targets (Nguyen, Green and Mecsas, 2017). Indirect resistance mechanisms can include proteins that are involved in the repair of DNA or bacterial proteins damaged by ROS during a respiratory burst, metal ion transporters involved in metal homeostasis and oxidative stress resistance and have been already been identified in a number of pathogens including Group A Streptococci (Henningham *et al.*, 2015).

Recently, the genes associated with survival of *S. equi* in blood and against the action of hydrogen peroxide have been identified in a TIS based genomic comparison study (Charbonneau *et al.*, 2020). Fourteen genes were identified that were ubiquitous for bacterial survival in both whole blood and hydrogen peroxide and included those associated with energy production and conversion, cell replication, recombination, and repair. It is likely that similar genes may impact the survival and dissemination of *S. suis* in swine and human blood and provide potential targets for understanding key host/pathogen infection dynamics.

1.9 Research aims and objectives

The aim of the project was to characterise genes determined as essential for *S. suis* survival and growth in the presence of hydrogen peroxide using PIMMS bioinformatics-based comparative analysis and determine the potential functional roles of encoded proteins and potential role in *S. suis* survival and virulence.

2.0 Materials and methods

2.1 Bacterial strains and growth conditions

Unless otherwise stated, all reagents were obtained from Sigma-Aldrich. *S. suis* P1/7 (ATCC BAA-853) (Holden *et al.*, 2009) and 5 mutant *S. suis* P1/7:pGh9:*ISS1* pools (mutant pools (MP) 1, 2, 3, 4 and 5) containing approximately 20,000 bacteria were provided by Dr Sharon Egan at the University of Nottingham. Strains were routinely cultured in Brain-Heart Infusion (BHI) broth (Oxoid) or on solid media at 37°C for 16 hours in the presence of 0.5µg/ml erythromycin (Ery).

2.2 Minimum inhibitory concentration (MIC) of hydrogen peroxide (H_2O_2)

To determine the concentration of H_2O_2 required to exert a selective pressure on *S. suis*, the MIC of H_2O_2 in BHI was determined. In a 96-well plate, an overnight culture of *S. suis* was diluted such that each well contained 10^5 cfu/ml. Doubling dilutions of BHI containing H_2O_2 (Sigma Aldrich), ranging from 50-0.78 µM were added to wells, alongside controls containing BHI only and no H_2O_2. Bacterial concentrations were confirmed by overnight growth of serially diluted cultures on BHI agar. The experiment was conducted in triplicate and repeated twice. The plate was incubated at 37°C for 15 hours in a Varioskan LUX microplate reader. Optical density (OD) readings were taken every hour at 600nm and prior to readings, the plate was shaken at 300spm for 5 seconds to agitate samples.

2.3 Survival and growth of *S. suis* P1/7: pGh9:ISS1 mutant pools in hydrogen peroxide

A 6.25 mM dilution of H_2O_2 was prepared in BHI and inoculated with approximately 10^5 CFU/ml of each *S. suis* mutant pool, with a control generated for each sample omitting H_2O_2. Cultures were grown at 37°C for a maximum of 6 hours with bacterial concentrations determined hourly by plating serial dilutions at each time point and for the initial inoculum to determine bacterial concentration. Samples were stored in 25% glycerol (v/v) at -80°C for each time point.

2.4 Preparation of Streptococcal Chromosomal DNA from *S. suis*

2.4.1 *Cell harvesting*

Enriched mutants were harvested from 0.5µg/ml erythromycin BHI agar plates using 15ml of 0.9% sterile saline solution (Oxoid, UK) and sterile wedge spreader (Fisher Scientific, UK) and

an equal volume of sterile 50% glycerol added to the remaining suspensions and inverted to mix and stored at -80°C.

2.4.2 *Extraction of genomic DNA from cells*

Genomic DNA was extracted from 1.0ml of harvested cultures. Cultures were centrifuged at 13,000 x *g* for 5 minutes at room temperature. The supernatant was removed, and cell pellet washed in 1.0ml of Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM disodium EDTA, pH 8.0). The supernatant was removed, and cell pellet resuspended in 500µl of fresh cell wall disruption buffer (30U/ml mutanolysin; 10mg/ml lysozyme in TE buffer). Samples were incubated at 37°C for 30 minutes. Following incubation, 40µl of cell lysis buffer (SDS (20% w/v in 50 mM Tris, 20 mM EDTA pH 7.8) and 120µg Proteinase K were added to cells and gently inverted to solubilise cells into a clear solution. Cells were incubated for a further 60 minutes at 37°C. An equal volume of saturated 6.0M NaCl solution was added to the cell solution and agitated for 15 seconds to precipitate cell wall material. Samples were centrifuged at 17,000 x *g* for 10 minutes to obtain a firm pellet. Approximately 1.0ml of supernatant was removed and added to a fresh microcentrifuge tube and centrifuged as above, repeated twice with 700 µl and 500 µl of supernatant respectively to remove protein contaminants. DNA was precipitated by the addition of 100% ice cold ethanol (Fisher Scientific, UK) and incubated on ice for 60 minutes. Precipitated DNA was centrifuged at 13,000 x *g* for 5 minutes. The ethanol was carefully discarded, and the DNA pellet washed twice with 70% ice cold ethanol and centrifuged as above. DNA pellets were air-dried overnight and carefully re-suspended in 50 µl of TE buffer containing 20µg/ml RNAse A and incubated at 4°C for 16 hours followed by incubation for 30 minutes at 37°C to remove RNA.

2.4.3 *Quantifying DNA concentration*

DNA concentrations were quantified using Qubit® dsDNA Broad Range (BR) Assay (Fisher Scientific, UK). Assay components were equilibrated to room temperature and Qubit® working solution was created by diluting the Qubit® dsDNA BR reagent (Fisher Scientific, UK) at a ratio of 1:200 in Qubit® dsDNA BR buffer (Fisher Scientific, UK), ensuring that the final volume of working solution in each assay tube was 198 µl for each DNA sample and 190 µl for each standard used. The final volume in each assay was made up to 200 µl by adding respective Qubit® standards or DNA. Samples were vortexed for 3 seconds, incubated at room temperature for 2 minutes and quantified using a Denovix DS-11 FX+ Fluorometer (Cambridge Biosciences).

2.5 Generation of DNA fragments for PIMMS based bacterial sequencing

2.5.1 *DNA shearing and purification*

Approximately 4µg of each sample of genomic DNA sample was suspended in 200ul of TE buffer and fragmented in 3Kb MiniTUBEs (Covaris, UK) using focused-ultrasonic using a Covaris M220 focused ultrasonicator. Following shearing, DNA samples were transferred into a Lo-Bind microcentrifuge tube and purified using Agencourt AMPure XP Solid Phase Reversible Immobilisation (SPRI) beads (Beckman Coulter, UK) at a ratio of 1:1.8 of DNA:beads according to manufacturer's instructions. Briefly, samples were slowly mixed by pipetting and incubated at room temperature for 5 minutes and collected on a magnetic rack. The supernatant was removed and beads were washed twice with 70% BioUltra grade ethanol, with DNA eluted in 85ul of molecular grade water.

2.5.2 *DNA fragment repair*

The purified DNA fragments were blunt end repaired using NEBNext End Repair Module (New England Biolabs, UK). 1x NEBNext End Repair buffer and 5% NEBNext End Repair Enzyme mix were added to each sample and incubated at 20°C for 30 minutes using a Thermocycler PCR block (Thermofisher, UK). DNA was purified using SPRI beads previously described and eluted into 50 µl of molecular biology grade water.

2.5.3 *DNA re-circularisation and inverse PCR*

To circularise the DNA fragments, DNA was incubated with 1x ligase buffer, 1000 U of T4 DNA ligase (NEB, UK) and incubated at 15°C for 16 hours. The DNA was purified using the NucleoSpin® Gel and PCR Clean-up kit (Macherey & Nagel, Germany) according to the manufacturer's instructions, however at the elution step, samples were eluted using 30µl of pre-heated (70°C) elution buffer. Re-circularised DNA was quantified using Qubit BR assays previously described. PCR was performed using 100ng of re-circularised DNA, 1x Phusion HF buffer (NEB), 200µM of dNTPs, 0.5 µM of each primer (forward: p064 5'-AGAACCGAAGAATTCGAACGCTC-3' and reverse: p082 5'-CCAACAGCGACAATAATCACATC-3'). The PCR programme for inverse PCR reactions followed: 98°C for 5 minutes, paused and 1U of Phusion High Fidelity DNA Polymerase (NEB), was added to samples. This was followed by 35 cycles of 98°C for 10 seconds, 63°C for 30 seconds and 72°C for 1 minute and one cycle of 72°C for 8 minutes. The PCR products were

purified using SPRI beads using the method described above by combining 50 µl of each DNA sample to 90 µl of SPRI beads. DNA was eluted into 30 µl of molecular biology grade water.

2.6 Nucleotide sequencing

Triplicate PCRs of each sample were pooled into individual tubes containing a final concentration of 500ng of DNA. Extracted DNA from the *S. suis* P1/7 mutant pools (n=5) grown in BHI (input pools), and mutant pools (n=5) grown in H_2O_2 (output pools) were sequenced at the Leeds Next Generation Sequencing Facility using 2x150PE reads using NextSeq 500 to obtain approximately 16 million reads per pool.

2.7 Bioinformatics analysis Bioinformatics analysis

2.7.1 Quality Control

Libraries were constructed using the standard NEB Ultra workflow (New England Biolabs, USA). The resulting raw FASTQ sequence data was assessed for sequence quality using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) to ensure the data was of sufficient quality (>Q30).

2.7.2 *Raw data processing*

The PIMMS 2.0 bioinformatic pipeline (Blanchard *et al.,* 2015) was used to process sequence reads and map them to the *S. suis* P1/7 genome. The *S. suis* P1/7 genomic sequence information was downloaded from NCBI (accession number: AM946016.1). Matched reads were defined as sequence reads containing the IS*S1* and *S. suis* P1/7 genomic information and were mapped to the reference genome to confirm the locations of mutations within the genome for each sequence analysed. A true insertion was defined as one with ≥3 occurrences within each mutant pool, to be confident that detections had not occurred by chance. Bioinfographics of the distribution of pGh9:IS*S1* insertions in BHI and H_2O_2 were subsequently created using GraphPad Prism version 9.0.0 for Windows (GraphPad Prism, 2020).

2.7.3 *Functional annotation of essential genes*

Phenotypically essential genes were determined for both input and output pools and bioinfographics were created using Venny 2.1.0 (Oliveros, 2007). The essential genes from the genome protein annotation file were parsed using an in-house script, to create an essential

gene FASTA file for further analysis. To enable genes to be grouped according to the functions of encoded proteins and give a broad indication of their function, BlastKOALA was used to obtain KEGG Orthologies (KO) (Kanehisa, Sato and Morishima, 2016). The essential gene FASTA files were uploaded to BlastKOALA with taxonomy ID 218494. KEGG genes were searched in the family_eukaryotes + genus_prokaryotes database and assigned a K number which corresponded to a Brite Hierarchy, indicating functionality. Further assessment was performed to assess the distribution of the C terminal centile positions for every unique insertion point to identify the presence of truncated genes within the BHI input and H_2O_2 output pools. Likely subcellular protein location was determined for genes identified as being essential for growth in H_2O_2 using PSORTb 3.0 bacterial protein subcellular localisation software (Yu *et al.*, 2010) and SignalP 5.0 (Almagro Armenteros *et al.*, 2019).

2.7.4 Statistical analysis for identification of overexpression of genes in specific phenotypes

EdgeR Bioconductor software (Robinson, McCarthy and Smyth, 2009) was used to conduct statistical analysis to identify putative genes with an increased fitness value using a false discovery rate threshold of <0.05. Metabolic pathway analysis was performed using BLASTKOALA and the categorised output for each phenotype was assessed for enrichment of specific genes within the same metabolic pathways using a chi-squared statistical analysis in R (R Core Team, 2020). Differences were considered significant between metabolic pathways in the two growth conditions if the p value was <0.05.

2.7.5 *Essential genes shared with other bacterial species*

The protein sequences from the essential genes was parsed through the Database of Essential Genes (DEG) (Zhang *et al*., 2004) to identify whether putative function was shared with other bacterial species. Amino acid sequences from essential genes of interest were uploaded and compared to all essential genes within DEG. Genes were considered as having shared putative function when the percentage identity of amino acid sequences was greater than 70% and the query length was greater than 75% of the original amino acid sequence. Unique Clusters of Orthologous Genes (COG) numbers for the genes identified were allocated a functional category using the COG database (Galperin *et al.,* 2020).

3.0 Results

3.1 Analysis of *S. suis* P1/7 survival and growth in hydrogen peroxide

S. suis P1/7 was incubated for 15 hours in BHI media containing H_2O_2 at concentrations ranging from 0.78 µM to 50 µM in a 96-well plate. Optical densities (ODs) were measured at an absorbance of 600nm to measure survival and growth of bacteria at every hour. Analysis of the resulting growth curves indicated that bacterial growth was inversely associated with increasing concentrations of H_2O_2 and no growth of bacteria was observed at concentrations above 25µM (see Figure 3.1**)**. A concentration of 6.25µM was chosen to assess the effect of H_2O_2 as it was shown to impact but not completely prevent bacterial growth.

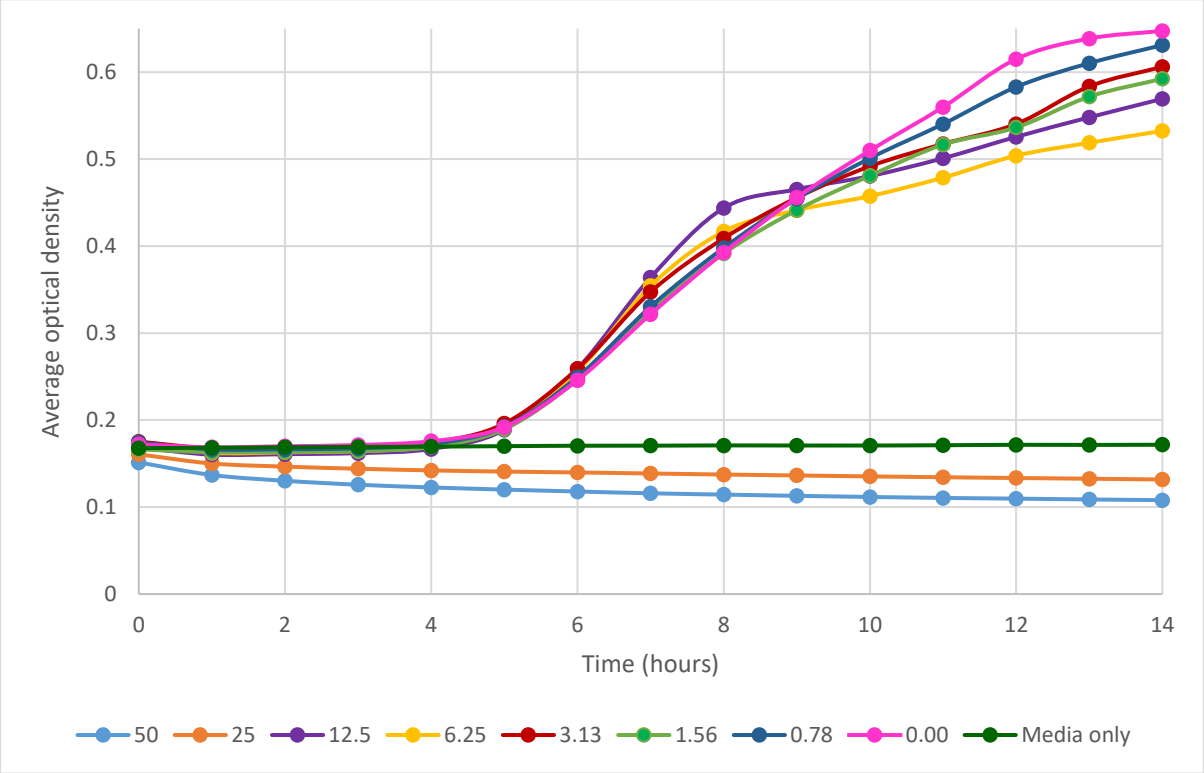

Figure 3.1: Growth assessment for *S. suis* P/17 in the presence of hydrogen peroxide.

The growth of *S. suis* was assessed in BHI media in the presence of H_2O_2 to assess impact on bacterial survival. Cultures were analysed in triplicate and media controls omitting bacteria were performed for each concentration of H_2O_2. No bacteria growth was observed above a concentration of 25 µM of H_2O_2.

3.2 Sequencing and PIMMS

After parsing the sequencing data for quality, it was analysed through the PIMMS pipeline to determine the number of bacterial mutants that could be detected in each growth condition. A total of 84,518,709 sequencing reads were analysed for the BHI cultured bacterial populations, 635,998 which contained transposon/genome junction information and individual *S. suis* mutant DNA. A total of 23,208 unique mutations were identified within this population with the exact location of the IS*S1* element mapped within the bacterial chromosome. Within the cultured populations grown in BHI containing H_2O_2, a slightly higher total of 97,180,970 sequencing reads were identified, containing 1,383,668 sequence reads with both transposon/genome junction information and a higher total of 42,157 unique mutations also observed within this population.

3.3 Distribution of pGh9:IS*S1* insertions in BHI and H_2O_2

The location of each transposon insertion site was used to generate a graphical representation of identified mutation within *S. suis* P1/7 genome for bacteria cultured in BHI media (input pool), to determine any potential for transposon insertion site bias (Figure 3.2). Consistent distribution of genomic mutations was observed across the bacterial genome, with areas where mutations could not be identified indicating the likely location of genes essential for growth within this environment. A similar profile was observed for the H_2O_2 (output pool) data where no overrepresentation of specific genomic mutations was observed for a particular genomic site, indicating that there was no site-specific bias or overrepresentation of individual bacterial mutants within the population as a whole (Figure 3.3).

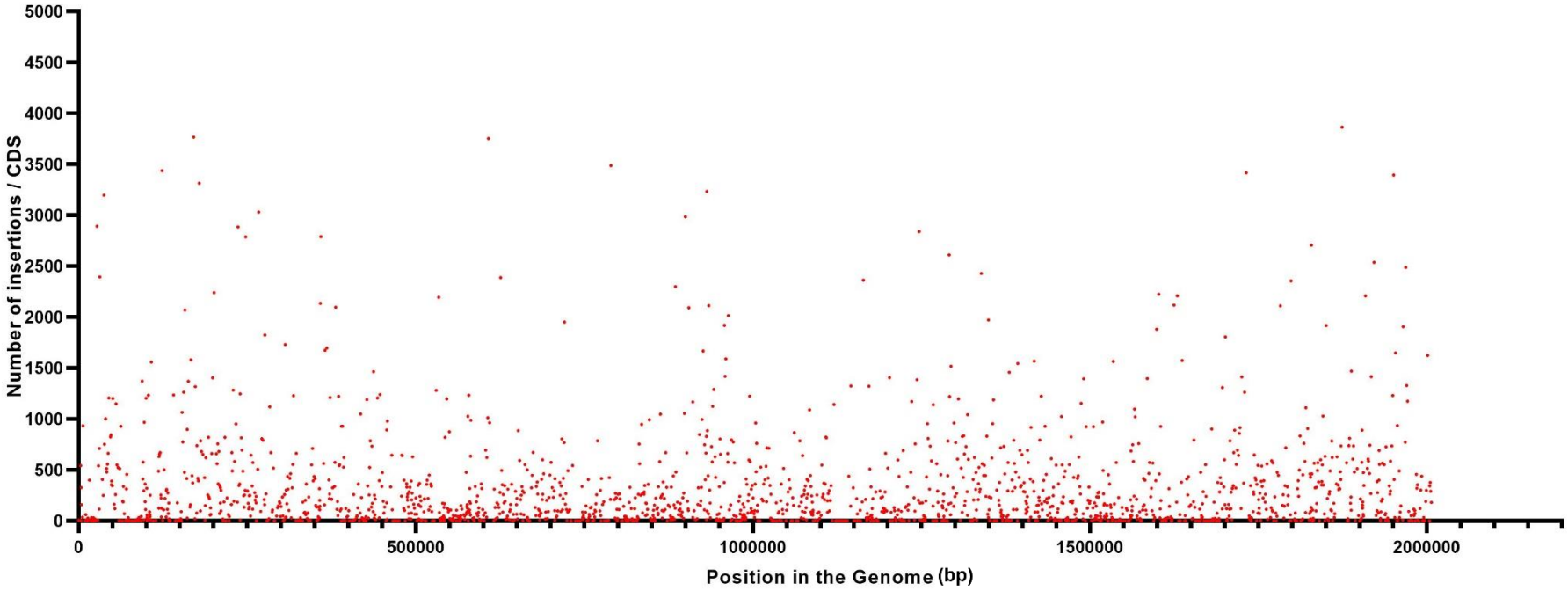

Figure 3.2 Genomic representation of *S. suis* P1/7 bacterial mutants identified after growth in BHI

The graph represents a non-linear relationship between gene loci and the total number of mutations detected within bacterial colonies grown in BHI media. A total of 2052 loci including tRNA were identified. Represented in red, mutations were identified throughout the genome and areas where no coloured points are displayed represent regions of the genome where no bacterial mutants could be identified. Several mutations were represented at high numbers (4000), indicating that some mutations were highly represented within the overall population.

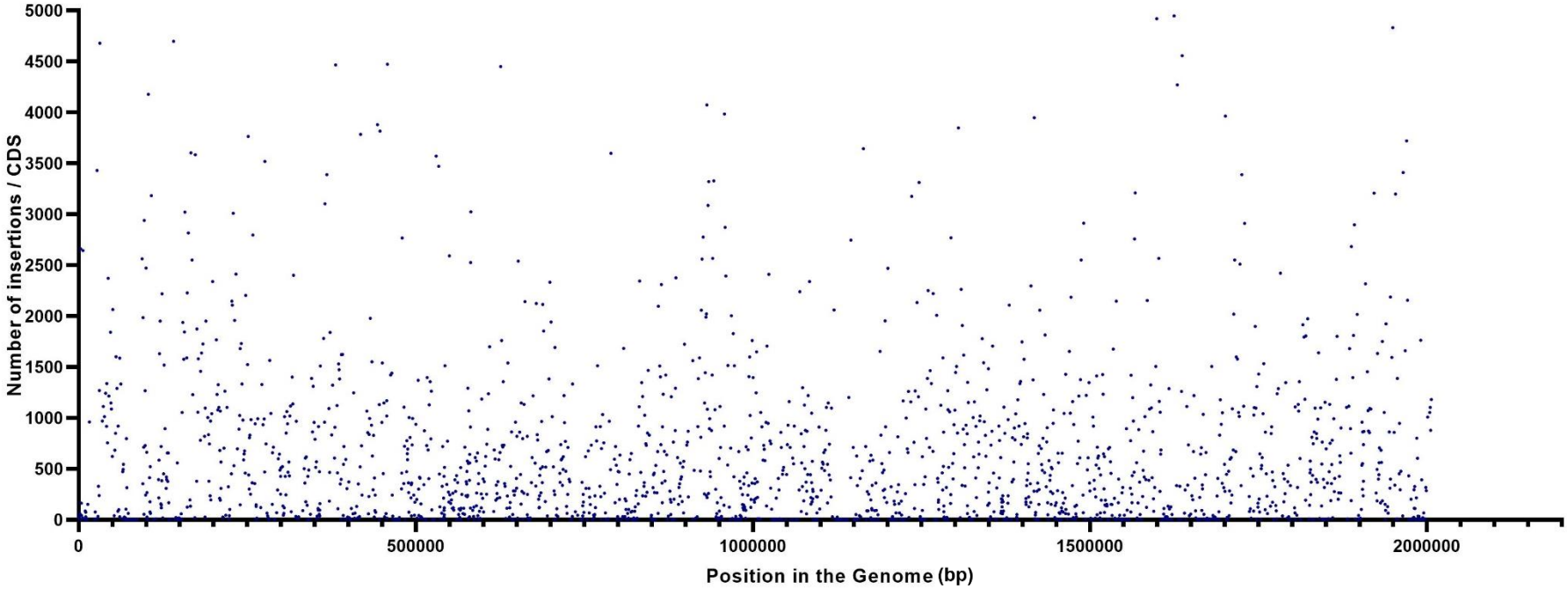

Figure 3.3 Genomic representation of *S. suis* P1/7 bacterial mutants identified after growth in H_2O_2

The graph represents a non-linear relationship between gene loci and the total number of mutations detected within bacterial colonies grown in BHI with additional H_2O_2. A total of 1969 loci including tRNA were identified. Represented in blue, mutations were identified throughout the genome, however areas where no coloured points are displayed represents bacterial mutants which were unable to be identified in a number of loci. Several mutations were represented at high numbers (4000-5000), indicating that mutations were highly represented in some bacteria.

3.4 Essential genes in the *S. suis* genome

To identify phenotypic differences between input and output populations, a comparison was performed using PIMMS 2.0. A total of 2052 coding sequences including tRNA genes were identified within the annotated *S. suis* P1/7 genome. Genes were deemed essential for bacterial growth in specific phenotypes when no IS*S1* insertions were identified throughout the coding sequences of bacterial mutant pools. In the BHI phenotype, a total of 160 genes were identified as being essential. A total of 35 genes were identified as being essential for the H_2O_2phenotype and 198 genes identified as essential for growth in both conditions (Figure 3.4).

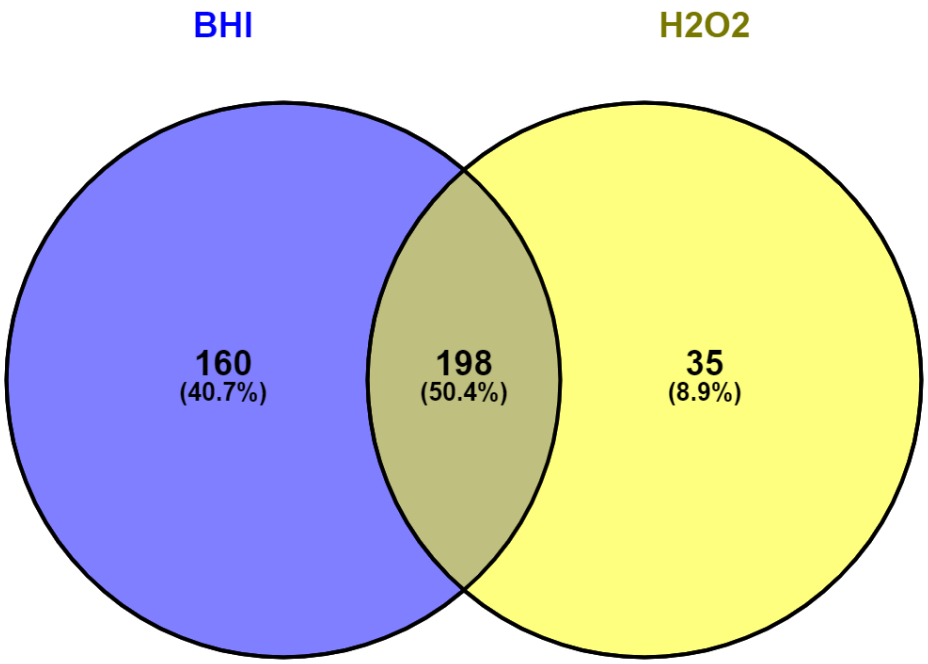

Figure 3.4 Comparison of the number of essential genes found after growth of *S. suis* in BHI and in the presence of H_2O_2.

Phenotypically essential genes identified from *S. suis* grown in BHI (represented in blue), H_2O_2 (represented in yellow) and essential genes common to both phenotypes (centre). A total of 2052 genes were identified in the bacterium, of which 160 were essential for growth in exclusively BHI, 198 essential for growth in both phenotypes and 35 for growth exclusively in H_2O_2.

3.5 Analysis of essential genes for *S. suis* growth

A total of 159 out of 160 genes identified as essential for growth in BHI were functionally annotated using BlastKOALA. The majority of which were related to metabolism of carbohydrates, nucleotides, lipids, cofactors and vitamins and amino acids (n=54), followed by genetic information processing (n=38), signalling and cellular processes (n=11) and environmental information processing (n=5). A total of 51 genes could not be assigned a KO number and 42% (n=67) had no functional annotation (Table 3.1).

In total, 194 genes out of 198 essential for growth in both BHI and H_2O_2 were given functional annotation using BlastKOALA (Table 3.2). Most genes were associated with genetic information and processing (n=85), followed by metabolism (n=60) including glycan biosynthesis and metabolism (n=6) and cellular and signalling processes (n=6). A total of 28 genes could not be assigned a KO number, 3 genes were poorly characterised and 38% (n=73) had no functional annotation.

Finally, all 35 genes essential for growth in H_2O_2 were annotated. Most genes were related to genetic information processing (n=10), followed by metabolism of carbohydrates, nucleotides, lipids, cofactors and vitamins and amino acids (n=10), environmental information processing (n=3) and cellular processes (n=2) . A total of 8 genes could not be assigned a KO number and 54% (n=19) had no functional annotation (Table 3.3). Additionally, genes belonging to protein families associated with signalling and cellular processes (n=7) and metabolism of energy (n=6), terpenoids and polyketides (n=4) were identified in the input pools but not in output pools.

Table 3.1: Essential coding sequences for *S. suis* grown in BHI and their associated Brite hierachies using KEGG mapper reconstruction result from BlastKOALA.

Brite Hierarchy	Counts	Gene
Protein families: genetic information processing	37	SSU0009, SSU0020**,** *hisS,* SSU0705, SSU0721, SSU079, *ffh, gyrA,* SSU1141, *rplA,* SSU1168, SSU1186**,** *era,* SSU1227, *gyrB,* SSU1387, SSU1461, *mraW, nusB, rpsF,* SSU1642, *argS*, *gidA,* SSU1958, *recF, trsA rplB,* rpoB, *priA, ileS, lysS, holB, rimM, cca, engB, glyS,* SSU165
Genetic information processing: unclassified	1	SSU0545
Protein families: signalling and cellular processes	10	SSU0219, SSU0292**,** *fba, asnS,* SSU0709, *dpr,* g*lnQ5,* SSU0159, SSU0377**,** *ftsZ*
Signalling and cellular processing: unclassified	1	*mecA*
Environmental information processing	5	SSU0577, SSU1121**,** *vicK*, SSU1853, SSU1867
Protein families: metabolism	10	*revA, csp2K, addB, spxA, pepB,* SSU1108, SSU1116, SSU1431, *oppB,* SSU1677
Carbohydrate metabolism	10	*prsA, treA, glmS, ddlA,* SSU1256, *galE,* SSU1395, *atoB, accD, pgi*
Glycolysis/ Gluconeogenesis	1	*Ldh*
Lipid metabolism	2	*fabH, oppC*
Metabolism of cofactors and vitamins	4	SSU0054, SSU0919, *engC, gltX*
Metabolism of terpenoids and polyketides	4	*mvaK1*, *mvaD,* SSU1119, *uppS*
Amino acid metabolism	7	SSU0335, SSU0365, *hom, thrB,* SSU0996, SSU1129, *csdB*
Nucleotide metabolism	5	*thyA,* SSU0797, *apt, pyrH,* SSU1778
Energy metabolism	6	SSU0063, *cysM, potB, potC, oppF, cysE*
Metabolism: unclassified	5	*murD, folD, ftsW,* SSU1329, *fabK*
Unknown	51	SSU0010, *rplX,* SSU0213, *mvaK2, thiD*, *rplS,* SSU0334, *murG,* SSU0432, *cps2B*, SSU0528, SSU0529, *argR,* SSU0645, *dpfB, addA ribC, pheT,* SSU1110, SSU1114, SSU1115, *rmlA,,* SSU1152A, SSU1161, SSU1166, *murF, secG, rpmGB,* SSU1294, SSU1321, *ezrA,* SSU1341, SSU1396, SSU1399, SSU1440, SSU1458, *ppaC,* SSU1525, SSU1537, SSU1542, *serS, fabE, scrB,* SSU1622, SSU1626, *rbfA, oppA*, *iscU, hasC, nrdI,* SSU1953
Total	159	

Essential genes were characterised (indicated by gene names in italics) and uncharacterised (indicated by unique SSU numbers) genes based on their KO numbers.

Table 3.2: Essential coding sequences for *S. suis* growth in BHI and H_2O_2 and their associated Brite hierachies using KEGG mapper reconstruction result from BlastKOALA..

Brite Hierarchy	Counts	Gene
Genetic information processing	84	*infA,* SSU0112, *groES, fus, glnR,* SSU0162, *hrcA, grpE, recU,* SSU0436, SSU0439, SSU0729A, SSU0780, SSU0792, *infC, ccpA, prfB,* SSU1536*rpsJ, rplC, rplV, rpsC, rplP, rpsQ, rplN, rplE, rpsN, rpsH, rplF, rplR, rpsE, rpmD, rplO, secY, rpmJ, rpsM, rpsK, rpoA, rpsL*, *rpsG, rpmF, dnaK, gatC, gatA, rpoZ, dnaE,* SSU0505B, SSU0704, *parE, rplU, rpmA, rpsP, rplL, rplJ, rpsT, pheS, lig, ftsY, rnc,* SSU1045, *alaS, rplT, rpmI, rrf, rplK, vicR, rpsU,* SSU1348, *metK,* SSU1545, *ftsL, efp, rpsR, nusA,* SSU1728, *rpsO, proS, tRNA-Cys, rpe,* SSU1935, *veg, rplI*
Genetic information processing: unclassified	1	*nrdH*
Protein families: signalling and cellular processes	5	SSU0437, SSU1122, SSU1544, *ftsA, rpoD*
Signalling and cellular processes: unclassified	1	SSU1112
Protein families: metabolism	5	SSU0720, SSU1120, SSU1123, SSU1124, *eno*
Environmental information processing	12	SSU0113, SSU0703, *potA,* SSU0951, *pstC, pstS, pstI, ptsH, ftsX, ftsE, pbpX,* SSU1952
Amino acid metabolism	4	SSU0440, *dapB,* SSU1131, *ptsK*
Metabolism of other amino acids	3	SSU1517, *gnd,* SSU1613
Carbohydrate metabolism	12	*plr, tpi, pfk, pyk, murB, prsA2,* SSU1320, *nadE,* SSU1530, SSU1541, *accA, thiN*
Energy metabolism	8	*atpC, atpD, atpG, atpA, atpH, atpF, atpB, atpE*
Lipid metabolism	3	SSU1200, *accC,* SSU1954
Nucleotide metabolism	7	*hpt, gmk, tmk, tdk,* SSU1044, *cmk, tRNA-Arg*
Metabolism of cofactors and vitamins	6	*coaC, dyr,* SSU1486, *alr,* SSU1689, SSU1784
Glycan biosynthesis and metabolism	6	SSU1117, *rmlB, rmlC,* SSU1548, *oppD,* SSU1672
Metabolism: unclassified	6	SSU0065, SSU0435, SSU0560, SSU1109, SSU1292, *rpmE*
Poorly characterised	3	SSU0743, *glr,* SSU1644
No KO number assigned	28	SSU0004, SSU0064, SSU0084, SSU0086, SSU0087, SSU0121A, SSU0121B, SSU0254A, SSU0373, SSU0438, SSU0522, SSU0530, SSU0619, *cpsY,* SSU0769, SSU0770, SSU0820, SSU0825, SSU1111, SSU1113, SSU1118, SSU1291, *ssb,* SSU1648, SSU1657, *rgpG, rpsD,* SSU1937
Total	153	

Essential genes were characterised (indicated by gene names in italics) and uncharacterised (indicated by unique SSU numbers) genes based on their KO numbers

Table 3.3: All essential coding sequences for *S. suis* grown in H_2O_2 and their associated Brite hierachies using KEGG mapper reconstruction result from BlastKOALA.

Brite Hierarchy	Counts	Gene
Protein families: genetic information and processing	10	SSU0013, SSU0712, *prfA,* SSU0857, SSU0853*,* SSU1293, *ptsK, nusA,* SSU0505A, *vicX,*
Genetic information processing	2	*rplD, rnz,*
Environmental information processing	3	*prsA, oppD,* SSU1078
Cellular processes	2	*accA,* SSU1608
Protein families: metabolism	2	*recR,* SSU1866
Carbohydrate metabolism	1	*gcaD*
Nucleotide metabolism	1	SSU1823
Lipid metabolism	1	SSU1077
Metabolism of cofactors and vitamins	1	SSU0321
Amino acid metabolism	3	*adk,* SSU1906, SSU1442
Metabolism: unclassified	1	SSU0018
No KO number assigned	8	*rpmC,* SSU0568, SSU0679, SSU0854, *coaA,* SSU1137, *rnpA, rplI*
Total	35	

Essential genes were characterised (indicated by gene names in italics) and uncharacterised (indicated by unique SSU numbers) genes based on their KO number

3.6 Truncated genes important for *S. suis* P1/7 growth in H_2O_2

Analysis was performed on the output data to identify additional genes which may be conditionally essential in the presence of H_2O_2, where mutations could only be identified in the final 15% of the C-terminal sequence of the gene. This identified a further 17 'truncated' genes from the H_2O_2 output pool where the comparative gene in the BHI population showed multiple mutations detected throughout the gene sequence (Table 3.4). Similarly, to BHI, the majority of genes in the H_2O_2 output pool were related to genetic information and processing (n=8) as well as environmental information processing (n=2) and metabolism (n=3). Some genes were poorly characterised (n=2) or could not be assigned a KO number (n=2). Furthermore, 65% (n=11) were not functionally annotated and 1 was a pseudogene. Additionally, genes essential for xenobiotic biodegradation and metabolism (n=1) was only identified in the input pool (Table 3.1).

Table 3.4: Truncated genes important for *S. suis* P1/7 growth in H_2O_2 and their associated Brite hierarchies*.

Brite hierarchy	Counts	Gene
Protein families: genetic information and processing	8	*pth, rplQ,* SSU0158, SSU0212, SSU0713*, *trmD*, SSU1757, SSU1775
Environmental information processing	2	SSU0114, SSU0883,
Carbohydrate metabolism	1	SSU1290
Energy metabolism	1	*fhs*
Metabolism of other amino acids	1	*metG,*
Poorly characterised	2	SSU0473, *sufD*
No KO number assigned	2	SSU0798, SSU1569
Total	17	

Essential genes were characterised (indicated by gene names in italics) and uncharacterised (indicated by unique SSU numbers) genes based on their KO numbers.

*Each protein coding sequence only contained insertions within the 15 percentile of the C terminus.

3.7 Analysis of subcellular location of genes essential for growth in H_2O_2

The subcellular location of corresponding proteins identified as conditionally essential for growth in H_2O_2 was determined using PSORTb 3.0. The majority of genes were identified as likely to be cytoplasmic proteins (n=18), localised within the cell. A total of 8 cytoplasmic membrane proteins were identified including inner membrane (SSU0854), molecular chaperone (*prsA*), oligopeptide transport (oppD), zinc transport (SSU1866) and unknown cytoplasmic membrane proteins (SSU1906, SSU0018, SSU1077). An extracellular protein of unknown prediction was also identified (rpmC) (Table 3.5). A total of 9 sequences had exclusively unknown prediction where no subcellular localisation could be determined (SSU0568, SSU0505A, SSU0071, SSU1939 SSU1823, SSU0853, SSU1293, SSU1442, SSU0712).

A number of genes were identified as essential for growth in both BHI media and H_2O_2 as indicated by * in Table 3.7. A total of 36 genes were identified as likely cytoplasmic membrane proteins within the H_2O_2 phenotype (*rplO,* SSU0093, SSU0437, SSU0440, SSU0087, SSU0440, SSU0560, SSU0703, SSU0729A, *potA,* SSU0770, SSU0780, SSU0951, *pstC, pstS, atpD, atpF, atpB, atpE, ftsY,* SSU1111, SSU1112, SSU1113, SSU1118, SSU1122, SSU1123, *ftsX, ftsE,* SSU1292, *glr,* SSU1544, SSU1548, *oppD, rplM,* SSU1952, SSU1954), with only one extracellular protein (SSU0087) identified (Table 3.5). A total of 140 genes were cytoplasmic proteins and 22 genes had unknown prediction (SSU0004, SSU0065, SSU0084, SSU0086, SSU0092, SSU0121A, SSU0121B, SSU0254A, SSU0278, SSU0324, SSU0436, SSU0505B, SSU0522, SSU1043, SSU1045, SSU1117, SSU1132, SSU1291, SSU1503, SSU1657, SSU1692, SSU1936). PSORTb analysis of truncated genes for growth in H_2O_2 identified 4 cytoplasmic membrane proteins of no known localisation (SSU0114, SSU0212, SSU0473, SSU1775) (Table 3.7).

SignalP 5.0 was used to confirm the presence of signal peptides, indicating translocation from the cytoplasm. Analysis of genes essential for growth in H_2O_2 and both H_2O_2 and BHI both identified *prsA* (marked with a #) as having a lipoprotein signal peptide (Sec/SPII- lipoprotein signal peptides transported by the Sec translocon and cleaved by Signal Peptidase II) (Table 3.5).SignalP analysis of the truncated genes for growth in H_2O_2 identified 1 secretory signal peptides (Sec/SPI- secretory signal peptides transported by the Sec translocon and cleaved by Signal Peptidase I) (SSU0883) and 1 lipoprotein signal peptide (SSU0798) (Table 3.6).

3.8 Additional functional statistical analysis

EdgeR statistical analysis was performed to identify putative genes which had an increased fitness value. No genes were found to have statistically significant impact on fitness. A chi-

squared statistical analysis of genes essential for growth in BHI and H_2O_2 was performed using R to identify enrichment of metabolic pathways. The results indicated that genes required for pathways including information processing, signalling and cellular processes, environmental information processing, glycolysis, metabolism of lipids, cofactors and vitamins, terpenoids, amino acids and nucleotides, energy metabolism, glycan biosynthesis and unknown processes were significantly associated with growth in both BHI and H_2O_2 phenotypes (Table 3.7). However, the analysis also identified an overrepresentation of essential genes associated with general metabolism required for growth in H_2O_2, compared to genes for carbohydrate metabolism in BHI, indicating a switch from carbohydrate metabolism to general metabolism in the H_2O_2 phenotype (Table 3.7).

Table 3.5: Subcellular localisation of genes identified as essential genes for growth in the presence of H_2O_2. A total of 45 positive localisations were identified based on PSORTb analysis.

Gene	Function	Subcellular localisation	Total insertions in BHI	Unique insertions in BHI
SSU0018	Rod shape-determining protein *MreC*	Cytoplasmic membrane	6	1
rpmC	50S ribosomal protein L29	Extracellular protein	5	1
SSU0854	Haemolysin-III related membrane protein	Cytoplasmic membrane: inner membrane	30	3
SSU1077	Conserved hypothetical protein	Cytoplasmic membrane	7	1
#*prsA*	Foldase protein *prsA* precursor	Molecular chaperone, lipoprotein signal peptide	10	2
oppD	Oligopeptide transport ATP-binding protein o*ppD*	Cytoplasmic membrane: oligopeptide transport	3	1
SSU1866	Metal cation ABC transporter membrane protein	Cytoplasmic membrane: zinc transport	3	1
SSU1906	Putative membrane protein	Cytoplasmic membrane	4	1
*rplO**	50S ribosomal protein L15	Cytoplasmic membrane	0	0
SSU0093*	ABC transporter ATP-binding protein	Cytoplasmic membrane	0	0
SSU0437*	Putative membrane protein	Cytoplasmic membrane	0	0
SSU0440*	Acetyltransferase (GNAT) family protein	Cytoplasmic membrane	0	0
SSU0087*	Putative phosphoribosylaminoimidazole carboxylase (fragment)	Extracellular protein	0	0
SSU0440*	Aetyltransferase (GNAT) family protein	Cytoplasmic membrane	0	0
SSU0560*	Cell envelope-related transcriptional attenuator domain protein	Cytoplasmic membrane	0	0
SSU0703*	Putative membrane protein	Cytoplasmic membrane	0	0

SSU0729A*	Conserved hypothetical protein (fragment)	Cytoplasmic membrane	0	0
*potA**	Putative spermidine/putrescine ABC transporter ATP-binding protein	Cytoplasmic membrane	0	0
SSU0770*	DegV family protein	Cytoplasmic membrane	0	0
SSU0780*	Putative exported protein	Cytoplasmic membrane	0	0
SSU0951*+	Putative phosphate ABC transporter permease protein	Cytoplasmic membrane	0	0
*pstC**+	Putative phosphate ABC transporter permease protein	Cytoplasmic membrane	0	0
*pstS**+	Putative phosphate ABC transporter, extracellular phosphate-binding lipoprotein	Cytoplasmic membrane	0	0
*atpD**	ATP synthase beta chain	Cytoplasmic membrane	0	0
*atpF**+	ATP synthase B chain	Cytoplasmic membrane	0	0
*atpB**+	ATP synthase A chain	Cytoplasmic membrane	0	0
*atpE**	ATP synthase C chain	Cytoplasmic membrane	0	0
*ftsY**	Putative cell division protein FtsY	Cytoplasmic membrane	0	0
SSU1111*+	Putative glycosyl transferase	Cytoplasmic membrane	0	0
SSU1112*+	Putative membrane protein	Cytoplasmic membrane	0	0
SSU1113*+	Putative glycosyl transferase	Cytoplasmic membrane	0	0
SSU1118*	Putative membrane protein	Cytoplasmic membrane	0	0
SSU1122*+	Putative polysaccharide export ABC transporter permease protein	Cytoplasmic membrane	0	0
SSU1123*+	Putative glycosyltransferase	Cytoplasmic membrane	0	0
*ftsX**+	Putative cell division protein	Cytoplasmic membrane	0	0
*ftsE**+	Putative cell division ATP-binding protein	Cytoplasmic membrane	0	0
SSU1292*	Putative membrane protein	Cytoplasmic membrane	0	0
*glr**	Glutamate racemase	Cytoplasmic membrane	0	0
SSU1544*	Putative thioredoxin reductase	Cytoplasmic membrane	0	0
SSU1548*	Putative helicase	Cytoplasmic membrane	0	0
*oppD**	Oligopeptide transport ATP-binding protein OppD	Cytoplasmic membrane	0	0

SSU1672*	Putative D-alanyl-D-alanine carboxypeptidase	Cytoplasmic membrane	0	0
*rplM**	50S ribosomal protein L13	Cytoplasmic membrane	0	0
SSU1952*	Putative tRNA (5-methylaminomethyl-2-thiouridylate)-methyltransferase	Cytoplasmic membrane	0	0
SSU1954*	ABC transporter ATP-binding protein	Cytoplasmic membrane	0	0
Total	45			

*Essential genes for growth in both H_2O_2 and BHI

#Identified to contain a signal peptide by SignalP

T**able 3.6: Subcellular localisation of truncated genes for growth in H_2O_2 by PSORT and SignalP.** A total of 4 positive localisations were identified based on PSORTb analysis and 2 positive localisations identified based on SignalP analysis.

Gene	Function	Subcellular localisation	Insertions mapped in BHI	Insertions mapped in H_2O_2
SSU0114	ABC transporter permease protein	Cytoplasmic membrane protein	17	10
SSU0212	Putative signal peptidase I 4	Cytoplasmic membrane protein	16	27
SSU0473	Putative membrane protein	Cytoplasmic membrane protein	18	41
SSU1775	Putative preprotein translocase *SecE* subunit	Cytoplasmic membrane protein	1	67
SSU0883	Putative glutamine ABC transporter, glutamine-binding protein/permease protein	Secretory signal peptide	10	15
SSU0798	Putative lipoprotein	Lipoprotein signal peptide	39	6

Table 3.7: Chi-square analysis of genes essential for growth in BHI and H_2O_2, with significant P-values in bold.

Brite Hierachy	BHI ChiSq	p value	H_2O_2 ChiSq	p value
Information processing	159.811479	**0.00049975**	188.990616	**0.00049975**
Signalling and cellular processes	3.09065657	0.11044478	0.1542939	0.84007996
Environmental Information Processing	16.9514169	**0.00049975**	6.63586378	**0.0089955**
Metabolism	2.92143133	0.10094953	4.10581599	**0.04797601**
Carbohydrate Metabolism	6.00903855	**0.01749125**	2.69922481	0.14942529
Glycolysis	6.00697496	**0.01949025**	5.23918478	**0.017991**
Lipid Metabolism	0.00361263	1	0.11374772	0.78710645
Metabolism of Cofactors and Vitamins	14.1654344	**0.001999**	12.7337825	**0.00349825**
Terpenoid Metabolism	0.01158951	1	2.47907611	0.15892054
Amino Acid Metabolism	10.5794723	**0.001999**	10.0896454	**0.00749625**
Nucleotide Metabolism	0.27555375	0.67166417	0.13733693	0.72513743
Energy Metabolism	32.3223711	**0.00049975**	20.053576	**0.0009995**
Glycan Biosynthesis	0.70780107	0.45927036	0.02894736	1
Unknown	115.518384	**0.00049975**	39.7266848	**0.00049975**

3.9 DEG analysis

The protein sequences from genes identified as essential for growth in the presence of H_2O_2 was parsed through the Database of Essential Genes (DEG) (Zhang *et al*., 2004) to identify whether putative function was common to known genes in different bacterial species. Following DEG analysis, a total of 16 genes which were essential for growth in H_2O_2 and shared H_2O_2 and BHI phenotypes were identified as having shared putative function with at least one other species of bacteria (see Appendix 2). Homology was described as a percentage amino acid match. *Streptococcus (S.) mutans* UA159 grown on blood agar, *S. pyogenes* NZ131 grown in Todd-Hewitt medium and *S. agalactiae* A909 and *S. pneumoniae* grown in rich medium were consistently represented with homologous genes identified. Some genes shared similarity with other Gram-positive bacterial species including *Bacillus* (*B.) subtilis* and *B. thuringiensis* BMB171 grown in rich medium as well as Gram-negative *Staphylococcus aureus subsp. aureus* MSSA476 grown in MHBII medium and *Mycoplasma pulmonis* UAB CTIP grown in rich medium.

A total of 94% (n=15) of genes shared putative function with genes in *S. agalactiae* A909. Furthermore, 69% (n=11) of genes appeared in both *S. agalactiae* A909 and *S. mutans* UA159. A total of 56% of genes (n=9) were identified in *S. pyogenes* NZ131 and 38% (n=6) in *S. pneumoniae.* Genes *rpIO* and *atpD* were additionally found in *Staphylococcus aureus subsp. aureus* MSSA476. *AtpD* was identified in *Mycoplasma pulmonis* UAB CTIP and *B. thuringiensis* BMB171 and *rpIO* in *B. subtilis.*

4.0 Discussion

S. suis is a highly adapted, commensal zoonotic pathobiont which contributes to the normal porcine nasal and oropharyngeal microflora (Vötsch *et al*., 2018). *S. suis* opportunistically infects young animals and is the leading cause of meningitis, endocarditis and sepsis in young pigs (Dutkiewicz *et al.*, 2017a) which is associated with considerable animal welfare and economic implications (Wileman *et al.,* 2019). *S. suis* is also responsible for increasing numbers of human infections with similar pathology in South East Asia where high pork consumption and small-scale swine farming are rooted in tradition (Goyette-Desjardins et al., 2014; van Samkar et al., 2015). Despite receiving greater scientific interest in recent years, the mechanisms by which *S. suis* breaches host barrier surfaces, maintains a high level of bacteraemia and causes systemic disease are still largely unknown (Fittipaldi *et al.*, 2012) and research methods into understanding the putative role of virulence factors have been inconsistent and produced conflicting data (Takeuchi *et al.*, 2013; Meng *et al.*, 2016).

The first line of treatment for *S. suis* is broad-spectrum β-lactam antibiotics (Burch & Sperling, 2018) and enforcement of the use antibiotics for prophylactic purposes in some countries and antimicrobial resistance remains a continuing challenge and with increasing demand for new therapeutic agents for disease control (Goutard *et al.*, 2017). Furthermore, attempts to produce an effective commercial veterinary vaccine to reduce the requirement for conventional antibiotics have been hampered by an incomplete understanding of the pathogenesis of infection and high variability of strains (Segura, 2015; Pian *et al.*, 2016). Since gaining a greater understanding of the pathogenesis of infection is the key to vaccine and therapeutic development, this project aimed to identify genes which may be important during host phagocytic respiratory burst through comparative growth experiments of individual *S. suis* mutants grown in the presence of hydrogen peroxide and using the transposon sequencing approach known as PIMMS (Blanchard *et al.,* 2016)

4.1 PIMMS-based analysis of genes essential for *S. suis* growth

The *S. suis* pan-genome is the complete genetic repertoire which constitutes both the core-genome and accessory genome (Carlos Guimaraes *et al.*, 2015). The accessory genome is a sub-set of non-essential genes which may not be consistently expressed in all *S. suis* isolates but rather are associated with niche-adaptive, pathogenic and antibiotic resistant functions and *S. suis* may survive and proliferate in the absence of these genes under certain phenotypic conditions (Saunders *et al.*, 2005; Segerman, 2012). On the other hand, the core-genome is highly conserved and shared by all individuals of the same species and codes for proteins involved in basic cellular homeostasis, translation and replication. This should

represent genes that are essential for general replication for *S. suis* and mutations in such genes would likely result in the inability for the bacteria to survive and proliferate (Segerman, 2012; Juhas *et al.*, 2014; van Tonder *et al.*, 2014; Carlos Guimaraes *et al.*, 2015; Martínez-Carranza *et al.*, 2018). As gene essentiality is condition-dependent, it is possible to identify genes of interest that are involved in the pathogenesis of *S. suis* infection including hypothetical proteins which have not previously been established as essential through the process of elimination (Moule *et al.*, 2014).

Transposon elements (TEs) are mobile genetic elements that naturally exist in all bacterial genomes and have a vital role in evolution and resistance to antibiotics and heavy metals (Blackwell, Iqbal and Thomson, 2019). TEs have the capacity to move within or between chromosomes and replicons in cells; known as transposition or retrotransposition depending on whether DNA or RNA is concerned (Munoz-Lopez and Garcia-Perez, 2010). Transposon mutagenesis provides a method by which bacterial genomes can be randomly mutated utilising TEs and have been used extensively for the study of bacterial pathogenesis to further understand how bacterial genotypes contribute to observed phenotypes (Lin *et al.,* 2014). High-throughput approaches have been used to facilitate simultaneous sequencing of many transposon mutants alongside massively parallel sequencing (MPS). Approaches involve the construction of transposon insertion libraries where most of or all non-essential genes contain high-density insertions before the library is cultured *in vitro* or *in vivo.* Mutant frequency can be determined at the start and end of growth conditions via MPS of the transposon junctions, which allow quantification of essential and conditionally essential genes involved (Van Opijnen and Camilli, 2013). In addition the normal growth of mutants defective in genes that are previously hypothesised to be essential, could reveal alternative biochemical pathways for fulfilling essential functions and give rise to novel therapeutic and vaccine targets (Goodall *et al.*, 2018; Charbonneau *et al.*, 2020).

High throughput mapping approaches are often associated with laborious, timely and complex laboratory and bioinformatics protocols in order to produce mutants, isolate and sequence DNA fragments flanking insertion elements (Blanchard *et al.*, 2016). Successful transformation often only occurs in bacterial species which have high transformation frequencies (Maguin *et al.*, 1996). Perhaps one of the most significant challenges of attempting to randomly insert transposons into bacterial genomes is that many transposons demonstrate preference for insertion at differing locations on genes based on their nucleotide sequences, which may result in insertion biases and incomplete coverage of a region (Green et al., 2012; Munoz-Lopez & Garcia-Perez, 2010). Some conventional sequencing approaches are associated with additional complexity, for example during the annealing steps of PCR in the Transposon Directed Insertion Sequencing (TraDIS) protocol , fluorescence is lost and the transposon-

specific sequencing primer requires a PhiX Control Library spike-in to prevent sequencing failure (Barquist *et al.*, 2016) which is associated with additional cost and risk of contamination of microbial isolate genomes (Mukherjee *et al.*, 2015). Finally, conventional sequencing approaches are often associated with the need for advance programming pipelines in order to map sequences and analyse data which are not universally accessible.

To mitigate the limitations of conventional mapping approaches, PIMMS was developed with the intention of providing a more accessible transposon insertion mapping pipeline which utilises standard inverse PCR techniques to generate libraries and novice-friendly bioinformatics pipeline which takes less than 10 minutes to complete using a desktop computer (Blanchard *et al*., 2015). Another advantage of the PIMMS pipeline is that it utilises the pG$^+$host::IS*S1* transformant. This transposon element has been shown to integrate randomly into Streptococcal, Enterococcal and Lactococcal genomes (Maguin *et al*., 1996), which have previously shown notoriously low transposition frequencies. The pG$^+$host:: transformant has also recently been utilised in a TraDIS-based approach with modified IS*S1* libraries to elucidate genes required for the fitness of *S. equi* subsp. *equi* in equine blood and hydrogen peroxide (Charbonneau *et al.*, 2020).

In the analysis of the distribution of mutants within the *S. suis* P1/7 mutant libraries used within this study, the distribution of mutants in both the bacterial media and H_2O_2 phenotypes identified no specific insertion site bias with a similar representation of mutants identified throughout the genome in both environments (Figures 3.1 and 3.2). This provided a visual representation of those sections within the genome where no mutations could be generated without being lethal for bacterial survival. The results confirmed minimal presence of insertion bias and was consistent with previous studies utilising this approach for the mastitis pathogen *S. uberis* (Blanchard *et al.,* 2016) and zoonotic pathogen *S. agalactiae* (Santi *et al*., unpublished).

4.2 Analysis of essential genes for *S. suis* survival and growth

Essential genes are defined as those necessary for the survival and maintenance of basic cell function (Koonin, 2000). Genetic technologies and studies of gene essentiality for survival and pathogenesis can be widely applied to many biological fields (Martínez-Carranza *et al.*, 2018) and have facilitated a shift from culture-based to genome-based vaccinology research (Rinaudo *et al.*, 2009).

4.2.1 *Genes essential for survival and growth of S. suis are predominately associated with metabolic pathways involved in basic cellular function*

To create the input pool of *S. suis* P1/7 and obtain an accurate representation of genes essential for general survival and growth, bacteria were cultured in BHI media which provided the optimal environment and nutrition for reproducible growth and mimic normal selection pressures associated with growth such as natural competition for resources (Bonnet *et al.*, 2020). In total, results from the PIMMS analysis identified 160 genes which were essential for growth in BHI. Of these, functional annotation analysis revealed that the majority of genes were involved in basic cellular functions including metabolism (of carbohydrates, nucleotides, lipids, cofactors, vitamins, and amino acids), and information processing (genetic, signalling, environmental and other cellular processes). In a study of *S. uberis* on an alternative bacterial media, Todd Hewitt Agar (THA), a number of comparative genes that were also identified as essential in the current study of *S. suis* P1/7 that were associated with basic cellular functions including cell division and cell cycle (*ftsA, recU*), regulation and cell signalling (HisS), DNA metabolism (HolB and GryA) and those related to the cell wall and capsule (Glr, MurF, DdlA, MurG, RmlB, MurE, PbpX, GlmS, RmlA) to name a few (Blanchard *et al.*, 2016). These metabolic pathways associated with standard cellular processes are consistent with others identified in similar studies of *S. pyogenes* gene essentiality where essential genes were associated with key cellular processes and metabolic pathways and were conserved within the core genome (Le Breton *et al.*, 2015).

In addition, 42% of the essential genes identified in the BHI input pool had no functional annotation and could only be deemed as hypothetical proteins (Table 3.1). Putative gene function are hypothesised based on inferred sequence homology with known proteins, location within the genome and surrounding genes and also relevant gene-gene interactions, however the exact function remains unknown (Zhao *et al.*, 2013; Hanoudi, Donato and Draghici, 2017). When the complete genome of *S. suis* P1/7 was first published, more than 800 genes were annotated as having putative function (Holden *et al.*, 2009) and this lack of knowledge is common, especially in species such as *S. suis* where genomic based research analysis is still relatively in its infancy (Wood *et al.*, 2012). Many genomic studies performed on *S. suis* have thus far focused on elucidating genes and proteins involved in the pathogenesis and virulence of disease from clinical isolates of the most invasive serotypes of *S. suis* (Fittipaldi *et al.*, 2010; Tohya *et al.*, 2016; Zhu *et al.*, 2018). Since this project is the first to identify genes essential for general survival, it is unsurprising that a large proportion of proteins have not yet been functionally annotated.

4.2.2 *Essential genes associated with the cell division cycle*

Bacterial cells can reproduce using multiple mechanisms including budding, hyphal growth, daughter cell formation and through the formation of multicellular baeocytes. However the role binary fission, where cells double in size, clone genetic information and divide into two daughter cells is the most common method of propagation and best understood by scientists (Chien, Hill and Levin, 2012). The genetic mechanisms that regulate and drive bacterial cell division are complex. Prior to separation, bacteria assemble essential proteins to the division site into an intricate complex called a “divisome” which ensures that cytokinesis occurs at the correct time (Rowlett and Margolin, 2015). The tubulin-like GTPase *FtsZ* protein is a critical and conserved structural component of the divisome which initiates cell division through the formation of a Z-ring in the centre of the cell (Mahone and Goley, 2020). Upon formation, approximately a dozen known additional essential proteins (FtsA, ZipA, FtsE, FtsX, FtsK, FtsQ, FtsL, FtsB, FtsW, FtsI, FtsN, and AmiC*)* are recruited to the cell division site. This is known as a septal ring which mediates DNA replication, DNA segregation, division site selection, invagination of the cell envelope and synthesis of a new cell wall (Weiss, 2004). Notably, 8 genes implicated in the divisome were identified as being essential for the survival of *S. suis*. They included *ftsZ, ftsA, ftsL, ftsW, fftsX*, *ftsE*, *EzrA* and *PbpX* (see Tables 3.1 and 3.2).

FtsA modulates the assembly state of FtsZ and connection of the Z-ring to the cytoplasmic membrane (Weiss, 2004) and FtsW is a polymerase implicated in the biosynthesis of the peptidoglycan cell wall alongside penicillin binding proteins including PbpX (Strobel *et al.*, 2014; Taguchi *et al.*, 2019). Recent advances in research in the role of FtsL, a component of the conserved FtsQLB complex, have demonstrated that it has an essential role in the recruitment of FtsW and the activation of peptidoglycan synthesis (Park, Du and Lutkenhaus, 2020). Furthermore, the membrane complex FftsEX is an ABC transporter located in the cell membrane. FtsE is an ATPase associated with the transmission signals that regulate the activity of cell wall hydrolases in the periplasm, whereas FtsX interacts with peptidoglycan hydrolase at the septum (Alcorlo *et al.*, 2020). Finally, EzrA is a negative regulator of Z-ring assembly by regulating the correct formation of Z-rings in the mid-cell and ensuring that only one ring forms per cycle (Levin, Kurtser and Grossman, 1999).

The mechanisms of cell division and proteins involved are largely conserved across bacteria (Rivas-Marin *et al.*, 2020) and a localisation dependency pathway has been determined with 10 of the essential proteins (FtsZ→FtsA/ZipA→FtsK→FtsQ→FtsL/FtsB→FtsW→FtsI→FtsN), suggesting that the divisome assembles based on a hierarchy (Goehring and Beckwith, 2005) and the functioning

and localisation of proteins cohesively is dependent on those which are upstream and downstream from each other (Gonzalez *et al.*, 2010). Therefore, the divisome has become a target for the development of alternative antimicrobials. The removal or inactivation of proteins within the divisome through the creation of mutants has been demonstrated to significantly impair cell division and often be fatal in Gram-positive and Gram-negative species. In *S. pneumoniae*, the partial depletion of FtsA resulted in the delocalisation of the Z-ring, cell expansion and lysis (Mura *et al.*, 2017). Furthermore, depletion of FtsL in *E. coli* resulted in wrinkled-colony morphology and arrested cell division (Gonzalez *et al.*, 2010) and mutations in FtsEX blocked septal peptidoglycan synthesis and blocked the initiation of constriction and ongoing constriction events (Du, Pichoff and Lutkenhaus, 2020). This could be described as being phenotypically similar to the activity of semisynthetic antimicrobial cephalexin, which has broad spectrum activity against Gram-positive and Gram-negative bacteria (Speight, Brogden and Avery, 1972). Ciprofloxacin has been demonstrated to exhibit bacteriostatic activity when DNA gyrase is inhibited (Silva *et al.*, 2011), an essential enzyme necessary for the supercoiling of chromosomal DNA during cell division, however mutations in this gene have led to an increase in quinolone resistance, including in studies of *S. suis* (Escudero *et al.*, 2007; Collin, Karkare and Maxwell, 2011). The latest finding by Du et al., 2020 indicates that the FtsEX complex could be a possible target for novel broad-spectrum antimicrobials, particularly since they are located within the cytoplasmic membrane (Table 3.5). Additionally, since FtsZ is implicated as the central protein responsible for initiating cell division, it has become an attractive antimicrobial target and compounds such as peptides, polyphenols and synthetic small molecules have been investigated with promising effects (Han *et al.*, 2020) and low cytoxicity towards host cells, evidenced through a lack of disruption to human erythrocytes and a low binding capacity to mammalian tubulin (Kunal *et al.*, 2020). The inhibition of FtsZ in *B. subtilis* by small-molecule synthetic peptides lead to the impairment of Z-ring assembly, the distribution of rings which deviated from the mid-cell, hyperaccumulation of FtsZ and long undivided cells (Araújo-Bazán *et al.*, 2016). Targeting of this protein by similar molecules may provide an alternative treatment strategy for *S. suis* porcine infections.

The divisome functions in a complex web of interactions, and not just linearly (Figure 4.1). New evidence suggests that many proteins in the divisome may undergo subtle confirmational changes or complete bypass of pathways when Fts proteins are overexpressed or mutated. Often the bypassing mechanism involves several proteins and varying domains which are up and downstream of each other, and the mechanisms of recruitment are still unclear (Gonzalez *et al.*, 2010; Du and Lutkenhaus, 2017). Fts proteins have also been demonstrated to share overlapping functions with proteins outside of the divisome (Mura *et al.*, 2017) and have regions that are dispensable in the recruitment of subsequent targets (Gonzalez *et al.*, 2010).

This highlights the need for more research into the potential of identifying novel antimicrobials which can block the cell division pathway.

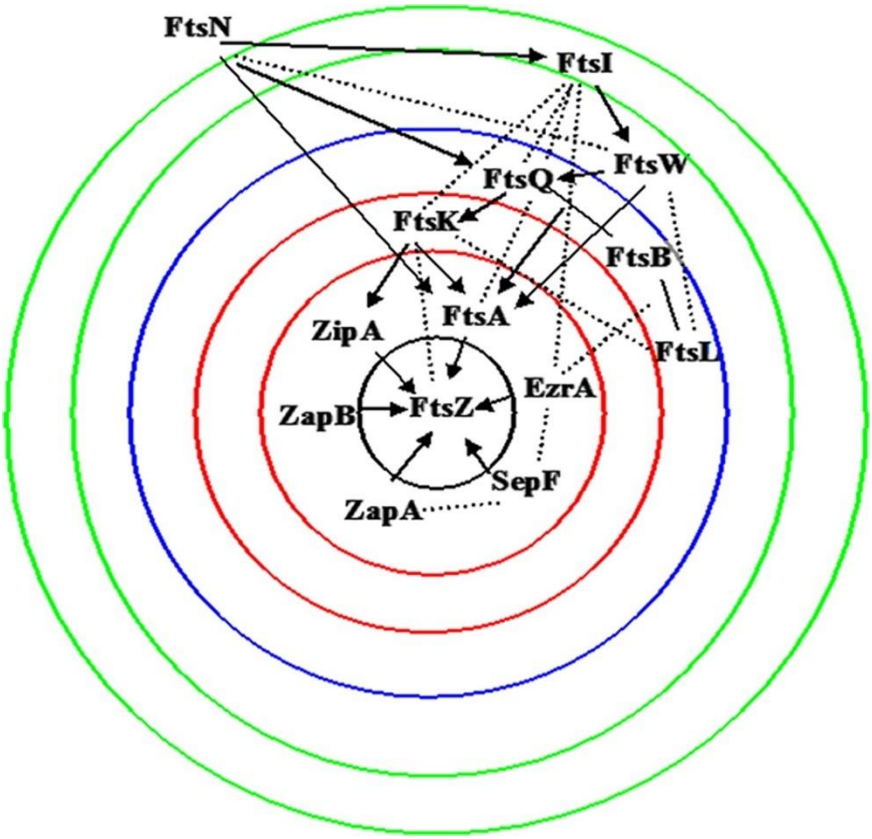

Figure 4.1 Schematic representation of the hierarchy of proteins associated with the bacterial divisome and their multi-pathway interactions (from Misra et al). Misra et al Microbiological Research 2018 208:12-24.

4.2.3 *Essential genes associated with the Signal Recognition Particle (SRP) pathway*

The BHI and H_2O_2 growth conditions were both very similar phenotypes, given that survival was compared in BHI media with and without the addition of H_2O_2. Genes that were identified as essential for growth within both phenotypes were validated as likely essential nature of these for general growth and survival. Similarly to those genes identified as essential for growth in BHI, most genes were associated with cellular processes including genetic information processing, metabolism and signalling (Table 3.2).

The genes *Ffh*, *FtsY* involved in the Signal Recognition Particle (SRP) pathway were identified across both conditions. The SRP pathway is a universally conserved protein targeting system which is associated with membrane protein assembly (Angelini, Deitermann and Koch, 2005) and facilitates the localisation of proteins to their correct cellular destinations (Akopian *et al.*, 2013). The ability of bacteria to survive and colonise is dependent on the correct functioning of the SRP and has been demonstrated to cause significant dysfunction in several species of yeast and bacteria by disrupting protein organisation and localisation. The bacterial SRP contains universally conserved membrane proteins Ffh and Ftsy (Park *et al*., 2002), where Ffh recognises exposed sequences and binds them to the SRP RNA and interacts with the SRP receptor, FtsY (Bernstein *et al.*, 1989). FtsY is a peripheral membrane protein which, promotes the release of proteins from the ribosome (Draycheva *et al.*, 2016) and directs them to either the cytoplasmic reticulum or plasma membrane (Faoro *et al.*, 2018).

Targeting the SRP pathway may significantly reduce the virulence of those Streptococci in which Ffh is dispensable for growth. Research exploring the function of Ffh found deficiencies severely impaired growth of *S. mutans* in the absence of environmental stress (Hasona *et al.*, 2005) and altered the metabolic processes involved in pH homeostasis, including pyruvate dissimilation and sugar transport which have been hypothesised to facilitate an increased acid tolerance when grown in slightly acidic conditions at around pH 5 (Crowley *et al*., 2004). Ffh was found to be essential for bacterial survival within this study of *S. suis* unlike for *S. mutans*, therefore it would be unlikely that an attenuated *S. suis* mutant could be generated for future functional studies since the mutation would likely be lethal to the bacteria.

Research carried out on *S. pyogenes* has also identified secreted extracellular virulence factors which are dependent on the SRP pathway for secretion, namely the haemolysin SLO and SPN which is associated with the escape of bacteria from neutrophil extracellular traps (Hynes and Sloan, 2016). The deletion of the pathway resulted in mutants which were highly attenuated in both zebrafish and murine infection models (Rosch *et al.*, 2008). The *S. suis* FtsY shares significant homology with *S. pyogenes, S. sanguinis* and *S. agalactiae* (see

Appendix 2), therefore the SRP may be universally associated with the secretion of extracellular virulence factors in haemolytic Streptococci. Fragment-based drug discovery have also identified 3 fragments which have affinity for the NG domain of FtsY in a number of Gram negative pathogens including *E. coli* and *Acinetobacter baumannii* (Faoro *et al.*, 2018). Although the mechanism of action was not elucidated, it may provide a starting point for the development of high affinity $FtsY_{NG}$ binders as an antimicrobial alternative for antibiotic resistant bacterial strains. More research into the role of the *S. suis* SRP is required to elucidate the pathogenesis of *S. suis* in greater detail, however the SRP may be a promising target for therapeutics which reduce the virulence of many Streptococcal species.

4.3 Analysis of essential genes for *S. suis* survival in H_2O_2

4.3.1 *H_2O_2 modulates a host phagocytic respiratory burst in vitro*

Endogenous reactive oxygen species (ROS) are an essential part of the innate host defence against infection, associated with the rapid elimination of invading microbes (Vatansever *et al.*, 2013; Belambri *et al.*, 2018). When phagocytes including neutrophils and monocytes are recruited to sites of infection, they engulf and kill pathogens (Slauch, 2011; Yang, Huang and Xu, 2016). and produce large concentrations of superoxide via hyper activation of NADPH-dependent phagocytic oxidases (NOX-2) which target phagocytosed pathogens during a phenomenon known as a “respiratory burst” (Babior, 1984; Slauch, 2011; Singel and Segal, 2016; Xu *et al.*, 2016). Superoxide reacts rapidly with itself and dismutes to oxygen and H_2O_2 (Babior, 1984; Singel and Segal, 2016) and whilst not primarily involved in bacterial killing, when catalysed by myeloperoxidase, the combination of H_2O_2 with halides and pseudo halides including chloride (Cl^-), bromide (Br^-), thiocyanate (SCN^-), tyrosine or nitrite (NO_2^-) produce reactive intermediates which have potent bactericidal capacity through the damage of DNA, protein and lipid molecules (Odobasic, Kitching and Holdsworth, 2016; Yang, Huang and Xu, 2016; Nguyen, Green and Mecsas, 2017).

Since H_2O_2 is pivotal in the phagocytic respiratory burst, it can been used as a model for *in vitro* studies which investigate the pathogenesis and immune evasion of pathogens which cause bacteraemia, such as *S. equi* which causes strangles in horses (Charbonneau *et al.*, 2020) and has been used to examine the impact of increasing H_2O_2 on phagocytic activity against *Klebsiella pneumoniae* (Phan *et al.*, 2011).

4.3.2 Further investigation of essential genes related to virulence

S. suis express a plethora of virulence factors which facilitate epithelial and blood brain barrier (BBB) translocation, survival and persistence in blood and perturbation of host immune responses, which results in severe systemic infection, meningitis and septicaemia with similar pathology in swine and humans (Kim *et al.*, 2018). The ability of *S. suis* to initially resist phagocytosis and respiratory burst and stimulate exaggerated inflammation and sepsis may be one of the hallmarks of its pathogenesis. Roles of the CPS and its sialic acid moiety has been implicated in the downregulation of leukocyte activity (Chang and Nizet, 2014), blockage of NO production and resistance to phagocytosis (Houde *et al.*, 2012). Furthermore, the role of pore-forming cytotoxin haemolysin III, also known as suilysin, in the lysis of immune cells and stimulation of the hypersecretion of proinflammatory cytokines that increase the permeability of the BBB is well documented (Billington, Jost and Songer, 2000; Lun *et al.*, 2003; Vötsch *et al.*, 2020). Despite an increasing number of studies aimed at elucidating the genetic basis of pathogenesis, disease physiology is often still hypothesised and extrapolated from infections caused by Group B Streptococcus which has similarity pathology (Mitchell and Mitchell, 2010; Calzas *et al.*, 2017). There is greater requirement for research which aims to elucidate *S. suis*-specific virulence factors which highlight explanations as to how the bacterium opportunistically causes infections. New genes identified as specific for the evasion of the immune response may also be identified as potential targets for novel therapeutics or antimicrobials.

In the study, PIMMS comparative analysis revealed that 35 genes were identified as being exclusively essential for survival and growth in the presence of H_2O_2. Most were related to genetic information processing, metabolism including those pertaining glycan biosynthesis and cellular processes (Table 3.3). Since H_2O_2 was used to model a phagocytic respiratory burst, a number of genes were identified as contributing to virulence; namely *PEP, Fsh, secE, CcpA, oppD* and putative Haemolysin III and putative Signal Peptidase I (SSU0212).

4.3.2.1 *Fundamental genes associated with alternative metabolic pathways and adaption to host environment*

When pathogenic bacteria cause invasive disease, they are exposed to changes in temperature, oxygen concentrations and pH values (Härtel *et al.*, 2011) and the host immune system actively restricts the availability of nutrients and cofactors, which creates a hostile growth environment (Richardson et al., 2015). Research has demonstrated that the ability of *S. suis* to survive in the blood, CFS and brain tissue during the pathogenesis of disease due to adaptations in metabolic activity. Analysis of *S. suis* metabolic adaptations in porcine blood

identified an increased expression of genes associated with pathways involving the transport of alternative carbohydrate sources including glycogen metabolism and the pentose phosphate pathway which functions parallel to glycolysis (Ramos-Martinez, 2017). This is consistent with the findings in the current study that *S. suis* switched from carbohydrate metabolism in enriched media to general metabolism in the presence of H_2O_2 (Table 3.7) and supports evidence that H_2O_2 impairs the functioning of the Kreb's Cycle involved in aerobic respiration of glucose (Tretter and Adam-Vizi, 2000). The pentose phosphate pathway produces NADPH and ribose 5-phosphate (R5P) (Ge *et al.*, 2020) which are utilised for anabolic processes including the synthesis of cell wall constituents, vitamins, co-enzymes, nucleic acids and amino acids in the absence of ATP and oxygen (Campbell, 2006; Pal et al., 2013). It has also been implicated in rapid adaption to oxidative stress in *E. coli,* where NADPH flux is rapidly re-routed through the pentose phosphate pathway upon exposure to H_2O_2 in order to increase the availability of $NADP^+$ for reactions such as glutathione-dependent defence against reactive oxygen species (Christodoulou *et al.*, 2018).

S. suis has been found to be auxotrophic for genes which encode enzymes required for the biosynthesis of amino acids arginine, histidine, glutamine, leucine and tryptophan (Willenborg *et al.*, 2015) and relies on the synthesis of oxaloacetate from the Kreb's Cycle as an essential precursor of aspartic acid, threonine, lysine, β-alanine (pantothenate), nicotinamide, nicotinate, α-ketoglutarate, and purines (Richardson *et al*., 2015). Arginine, glycine and aspartic acid have been implicated in the structure of extracellular cysteine protease, also known as Streptococcal pyrogenic exotoxin B which is a critical virulence factor in group A Streptococci (Stockbauer *et al.*, 1999). Furthermore, *S. suis* cysteine protease ApdS has been demonstrated to participate in immune system evasion through the cleavage of key antimicrobial peptides which are secreted by neutrophils. The cleavage of antimicrobial peptides led to impaired neutrophil chemotaxis the inhibition of the formation of neutrophil extracellular traps and formation of reactive oxygen species (Xie *et al.*, 2019). Since R5P is a precursor for aromatic amino acids including tryptophan, the current study highlights the requirement for greater research into alternative pathways that *S. suis* may utilise to synthesise amino acids that are associated with secretory exotoxins, oxidative stress tolerance and immune evasion independent of oxaloacetate (Litwack, 2018).

Streptococcal species such as *S. suis* and GBS are also auxotrophic for genes involved in purine metabolism and have evolved alternative pathways to biosynthesise purines via carbon-metabolism *de novo*. Upregulation of genes required for purine biosynthesis in human blood growth studies of *S. pneumoniae* has been reported (Orihuela *et al.*, 2004) leading to the suggestion that there may be a deficiency in purine availability within host fluids and tissues *in vivo* (Rajagopal *et al.*, 2005). One possible pathway could be the formation of

phosphoribosylpyrophosphate from R5P which initiates the purine synthetic pathway and produces precursors for guanine nucleoside monophosphates (Litwack, 2018). Purine biosynthesis genes have been previously demonstrated to be essential in the virulence of both Gram-positive and Gram-negative pathogens including *Salmonella typhimurium* (Mahan, Slauch and Mekalanos, 1993)*, Shigella fexineri* (Cersini *et al.*, 2003) and *Brucella abortus* (Alcantara *et al.*, 2004). In addition, 5,10-methylene-tetrahydrofolate dehydrogenase/cyclohydrolase (FolD) and 10-formate-tetrahydrofolate synthetase (Fhs) are a catabolic enzymes which catalyse N^{10}-formyltetrahydrofolate (N^{10}-fTHF) in purine nucleotide biosynthesis and the formylation of the initiator tRNA which initiates protein synthesis in bacteria (Sah *et al.*, 2015). Until recently, it was unclear as to why some bacteria possess both FolD and Fhs given shared reported function. However, evidence demonstrated that the function of FolD in *E. coli* was replaced when Fhs from *Clostridium perfringens* was integrated into the genome (Sah *et al.*, 2015). This is consistent with the findings in the current study that FolD was expressed in the BHI only phenotype but only Fhs was expressed in the H_2O_2 phenotype, indicating a potential switch between pathways when bacteria were exposed to H_2O_2 (see Tables 3.1 and 3.4). Critically, functional Fhs has been demonstrated to be essential for virulence of *S. suis* in both murine and porcine infection models. Mutant strains were reported at lower bacterial loads in the blood, brain, liver and spleen and minor clinical signs of infection and higher murine survivals rates compared to wild type and control infection groups. In addition, similar infection studies in piglets showed mutant strains displayed reduced capacity for colonisation in the heart, liver, spleen, lung, kidney, brain, blood and CFS (Zheng *et al*., 2016). Overall, this provides evidence that *S. suis* may regulate virulence via fundamental metabolic pathways (Rajagopal *et al.*, 2005) and the need for further investigation to elucidate whether the switch between FolD to Fhs consistently occurs in *S. suis* and if it is mediated by exposure to phagocytic respiratory burst.

As previously discussed, the regulation of processes related to sugar uptake and metabolism by Streptococci are crucial for fitness in host tissues and fluids. Carbon catabolite repression (CCR) controls hierarchical sugar utilisation and bacterial growth rates and CcpA is the major transcriptional regulator in Gram-positive bacteria (Iyer, Baliga and Camilli, 2005). In addition to being critical for sugar metabolism, CcpA has also been demonstrated to contribute to sophisticated adaptive and virulence capacity in several species of bacteria, including *S. pneumoniae* where CcpA mutants were severely attenuated in their ability to colonise murine nasopharynx and lung tissue, indicating a potential role in the fitness on mucosal environments (Iyer, Baliga and Camilli, 2005). Furthermore, CcpA may also contribute to acid resistance in *S. mutans* (Abranches *et al.*, 2008). Considering the fact that oral Streptococci are typically not as inherently resistant to acidification (Quivey, Kuhnert and Hahn, 2001), this finding may

provide greater explanation as to not only how *S. suis* has adapted to its tonsillar niche, may also provide an insight into the pathogenesis of enteric infection with *S. suis* in humans after the ingestion of raw pork products (Huong, Hoa, *et al.*, 2014). Studies of *S. suis* CcpA deletion mutants showed reduced expression of the surface-associated virulence factors Surface Antigen One (Sao) associated with cell wall anchoring (Xia *et al.*, 2019), Enolase (Eno) associated with BBB permeability (Sun *et al.*, 2016), Serum Opacity Factor (Ofs), shown to mediate adhesion to host cells and disrupt high-density lipoproteins in the blood (Courtney and Pownall, 2010), and Suilysin (Sly) which promotes epithelial translocation (Bercier, Gottschalk and Grenier, 2020). Furthermore, mutation resulted in the reduced expression of 18 genes related to capsular polysaccharide and cell wall synthesis, resulting in reduced capsule thickness and reduced resistance to neutrophil killing. It was therefore proposed that genes regulating the capsule may expressed when *S. suis* enters the blood, since it is a glucose-rich environment (Willenborg et al., 2011). In the current study, *CcpA* was expressed in both the BHI and H_2O_2 phenotypes (see Table 3.2), but since *CcpA* is associated with fundamental carbohydrate metabolism in Gram-positive species, it is unsurprising that it was identified in the enriched BHI media, but it does highlight the integral link between nutrient utilisation and virulence for *S. suis* pathogenesis.

4.3.2.2 *Genes associated with the expression and secretion of virulence factors*

An essential component of Streptococcal pathogenesis is the secretion of virulence-associated proteins from the bacterial cytosol across phospholipid membranes and into host tissues or the environment (Green & Mecsas, 2016). Secreted proteins have many functions to enhance the virulence of cells, including adherence and invasion of host cells, innate and adaptive immune evasion and the killing of host cells including tissue and blood cells (Sharma *et al.*, 2017). Protein translocation and secretion relies on dedicated apparatus, and putative preprotein translocase SecE subunit (SSU1775) from the Sec secretion pathway was identified as essential for survival in the presence of H_2O_2 (See Table 3.6).

The Sec protein translocation pathway is a highly conserved cytoplasmic membrane complex. Briefly, it consists of SecB which prevents premature protein folding in the cytoplasm, the ATPase SecA, SecDF which may act as a molecular chaperone and SecYEG which forms a stable complex in the cytoplasmic membrane (Beckwith, 2013). SecYEG is a protein-conducting channel which consists of three integral membrane proteins, SecY and SecE which interact with SecG (Veenendaal, Van Der Does and Driessen, 2004). Sec-dependent translocation of proteins across the membranes is mediated by the Signal Recognition Particle

Pathway previously discussed (Cranford-Smith and Huber, 2018), therefore this supports the findings in the current study that SecY and SecG were found to be essential in the BHI input pool (see Tables 3.1 and 3.2).

Virulence factor translocation via the Sec pathway are well documented in Gram-negative bacterial species including *Listeria monocytogenes* (Desvaux and Hébraud, 2006)*, Vibrio cholorae, K. pneumoniae* and *Yersinia enterocolitica* (Korotkov, Sandkvist and Hol, 2012). Sec protein homologues, also known as auxiliary proteins, have been identified in several species of Gram-positive bacteria and whilst not considered essential for viability (Bensing and Sullam, 2002). In Streptococci, *S. gordonii* SecA2 and SecY2 have been implicated in the ability of the bacteria to bind to human platelets, suggesting it may be may contribute to platelet aggregation during pathogenesis (Bensing and Sullam, 2002; Fitzgerald, Foster and Cox, 2006). More recently, *S. suis* has been demonstrated to have SecA2 and SecY2 which constructs fimbria-like proteins involved in adhesion to host cells (Zhang *et al.*, 2018), highlighting the potential role in pathogenesis. Furthermore, *S. suis* with *secE* mutations have shown reduced fitness in pigs (Arenas *et al.*, 2020) and a recent investigation found that physical blocking of the Sec complex led to cell death as a result of the degradation of translocator components and upregulating proteins involved in cell apoptosis upon prolonged secretion stress (Van Stelten *et al.*, 2009).

A putative signal peptidase I (SSU0212), was also identified as an essential gene for survival in the presence of H_2O_2 (See Table 3.6). Type I signal peptidases (Type I SPase) are indispensable enzymes which catalyse the cleavage of signal peptide sequences from preproteins before being translocated across the cell membrane and reach their appropriate specific secretion pathways (Tuteja, 2005). Type I Spases interact closely with the SecYEG pathway, since it cleaves the preproteins as they emerge (Auclair, Bhanu and Kendall, 2012). Similarly to Streptococci, Gram-positive *S. aureus* infects host tissues, evades the immune system and scavenges nutrients and minerals from the host environment via a diverse number of secreted virulence factors, of which, SPase contributes to most of the secretome (Schallenberger *et al.*, 2012) and a similar dependence for S. suis protein translocation is highly likely. Located at the bacterial membrane surface, this protein represents a potential antimicrobial target (Paetzel, Dalbey and Strynadka, 2000). The broad-spectrum bacteriostatic drug arylomycin which targets Type I SPase, has been demonstrated to cause an insufficient flux of proteins through the secretion pathway and subsequent mislocalisation of proteins in *S. pneumoniae* (Kulanthaivel *et al.*, 2004; Smith and Romesberg, 2012) and may also have similar mechanisms of action in *S. suis*. Previous studies investigating the intracellular tropical parasite *Leishmania major* have demonstrated that a recombinant Type I SPase vaccine decreased the parasitic load by 81% in challenge studies via the Th-1 mediated immune

response (Rafati, Ghaemimanesh and Zahedifard, 2006). This indicates that whilst the Type I SPase is a membrane associated protein, there is also potential to investigate vaccination development based on the targeting of such pathways.

With invasive and systemic disease such as those caused by *S. suis,* the ability to diagnose infection and begin antibiotic therapy promptly is critical. There is an increasing demand for point-of-care diagnostics. Increasing numbers of rapid diagnostic assays such as lateral flow immunoassays which are currently used as alternative to PCR for diagnosis of Covid-19 (Chaimayo *et al.*, 2020). Immunochromatographic tests which identify *S. pneumoniae* antigens in the CFS of human patients with suspected meningitis have also been developed (Moïsi *et al.*, 2009). Lateral flow assays are highly desirable since they are associated with greater ease of use because they do not require laboratory investigation or trained individuals, they have a raid turnaround time, low cost, and are portable (Koczula & Gallotta, 2016). The development of a lateral flow assay which specifically identifies *S. suis* antigens may be especially desirable for the rapid diagnosis of *S. suis* in humans in developing countries, since they often have fewer resources to facilitate laboratory analysis of samples (Giri and Rana, 2020). Since Type I SPases are antigenic and located in the cell membrane, *S. suis* SSU0212 could potentially be a novel immunochromatographic target for the development of more rapid diagnostic assays. Never the less, the true function of SSU0212 should be elucidated since it does appear to have the potential to be an antibiotic, vaccine and immunochromatographic target.

Bacterial oligopeptide permease (Opp) transport systems are associated with nutrient acquisition and the internalisation of signal peptides which contribute to quorum-sensing pathways (Gardan *et al.*, 2009). The transport system belongs to an ATP-binding cassette transporter family and is encoded by a polycistronic operon which contains OppA, OppB, OppC, OppD and OppF (Wang *et al.*, 2005) and OppD was identified as essential for growth in the H_2O_2 phenotype (See Table 3.6). Opps have been highlighted to have a complex association with virulence capacity in Group A Streptococci and are associated with regulation of virulence-associated genes. A *S. suis* deletion mutant lacking OppA was demonstrated to have dual effects on gene regulation: responsible for the positive and negative regulation of pyrogenic exotoxins and the negative regulation of haemolysin (Wang *et al.*, 2005). Defective mutants were associated with an increase in the transcription of erythrogenic toxins *in vitro* and significantly lower mortality and epidermis damage compared to the wild-type strain in challenge studies and further supports the complex nature of Opps in bacterial virulence and pathogenesis.

The role of Opps have been implicated in quorum sensing, a process by which bacteria gain information about their environment and adjust gene expression accordingly (Rutherford and Bassler, 2012). In *Streptococcus thermophilus,* Opps have been demonstrated to be essential in the triggering bacterial competence (Gardan *et al.*, 2009), with high competency amongst Streptococci species, including *S. suis* associated with interspecies exchange of antibiotic resistance genes (Salvadori *et al.*, 2019). In addition, quorum sensing enhances the response of bacteria to oxidative stress. The transactivator OxyR*,* has been demonstrated to be responsive to H_2O_2 in *Pseudomonas aeruginosa,* and appears to also influence the expression of quorum sensing transcriptional regulators (Wei *et al.*, 2012). Furthermore, quorum sensing may enhance the stress response to H_2O_2 (García-Contreras *et al.*, 2014), therefore since Opps are associated with quorum sensing in Streptococci, the essential role of OppD in *S. suis* exposed to H_2O_2 requires further investigation because it could participate in the quorum sensing within host environments since Opps appear to be multifunctional within the cell, and the role of Opps in *S. suis* competence, quorum sensing and pathogenesis of infection should be investigated further.

4.3.2.3 *S. suis P1/7 Haemolysin-III-related protein and role in virulence*

Perhaps one of the most significant and well understood Streptococcal virulence factors is the secretory haemolytic exotoxins which exert cytolytic activity against host cells during pathogenesis (Rosa-Fraile, Dramsi and Spellerberg, 2014). Several haemolysins have been characterised, including Streptolysin S (SLS) and Streptolysin O (SLO). The SLS protein has been associated with damage to host soft tissues, resistance to phagocytosis, translocation of bacteria across epithelia, and iron acquisition through the lysis of host erythrocytes (Molloy *et al.*, 2011). The SLO protein has been implicated in the formation of pores in eukaryotic cells, suppression of neutrophil oxidative burst, neutrophil degranulation and disruption of the formation of extracellular traps (Uchiyama *et al.*, 2015). The *S. suis* haemolysin, Suilysin (SLY) is antigenically related to Group B Streptococcal SLO (He *et al.*, 2014)*,* and has been described to be related to virulence of *S. suis* in almost every stage of pathogenesis. Functions include but are not limited to increasing the permeability of tight junction proteins and translocation across epithelial cells in the primary stages of infection (Bercier et al., 2020), destruction of immune cells and perturbation of macrophages resulting in a proinflammatory cascade once bacteraemia has been established (Billington, Jost and Songer, 2000), and potentiation of inflammation of astrocytes and glial cells which increases the permeability of the blood-brain barrier (Vadeboncoeur *et al.*, 2003; Yau *et al.*, 2018).

The *S. suis* putative haemolysin-III (Hly-III) (SSU0854) was identified as being essential for growth in the presence of H_2O_2 (Table 3.6). Hly-III has been demonstrated to contribute to virulence in several ways. A mutant of the highly virulent Chinese strain ZY05719 was demonstrated to exhibit reduced cytolytic and haemolytic activity and growth in whole blood. Furthermore, pathogenicity was also reduced in a zebra fish challenge model where mortality rate decreased compared to the wild type strain. Critically, knockout mutants were associated with a low expression of SLY, indicating that Hly-III inactivation may result in the blockage of pathways associated with SLY secretion (Zheng et al., 2013). Recently, an attenuated *S. suis* live vaccine deficient in a number of virulence factors including SLY, was found to show reduced invasive capacity in the brain, lung and liver and had a weak ability to induce inflammation and stimulate streptococcal toxic shock-like syndrome in a murine infection model. The vaccine also triggered T-cell dependent immunity and conferred protection to inoculated animals during subsequent exposure (Li *et al.*, 2019). Given the putative Hly-III protein may also be responsible for haemolytic and cytolytic activity in different serotypes of *S. suis* and be associated with the SLY secretion pathway, SSU0854 should certainly be investigated to determine its specific role during porcine infection and may be a promising candidate for subsequent live-attenuated vaccine development.

4.4 *Future directions and comparative study links*

Recently, two other studies of Streptococci have been reported, investigating the role of genes using transposon mutagenesis for fitness using a number of different *in vitro* and *in vivo* based models of infection. *S. equi* subsp. *equi* is a significant equine pathogen which is the primary aetiological agent for strangles in horses. This manifests as an upper respiratory infection which results in the formation of abscesses in the submandibular and retropharyngeal lymph nodes which can lead to respiratory distress (Boyle, 2017). Current attenuated strain based vaccines for strangles have been associated with adverse reactions in horses, including bacterial replication at the vaccination site, and the need for safer alternatives has been recognised (Kemp-Symonds, Kemble and Waller, 2007). In an attempt to identify genes related to fitness during pathogenesis, *S. equi* subsp. *equi* bacterial mutants were created using the pG$^+$host::IS*S1* plasmid and comparative analysis undertaken using a TraDIS based approach. Mutants were grown in whole equine blood and blood with the addition of H_2O_2 (Charbonneau *et al.*, 2020). In equine blood with the addition of H_2O_2, 15 genes had significantly reduced fitness and included genes associated with energy production and conversion, replication, recombination, and repair. Although the genes identified in the *S. equi* study did not cross-over with genes identified in this study, there are functional similarities

between a number of genes associated with energy metabolism identified as important for fitness in both studies. Whilst the *S. suis* in the current study was grown in BHI media with the addition of H_2O_2, *S. equi* was grown in equine blood with the addition of H_2O_2, and therefore bacteria in blood were additionally exposed to cellular elements including erythrocytes and leukocytes in addition to crystalloids and other nutritional components (Basu and Kulkarni, 2014), making direct comparison between both studies difficult.

Using whole host blood as an *in vitro* model may provide a more representative environment for elucidating genes associated with the pathogenesis rather than H_2O_2 with bacterial media, given the presence of leukocytes, complement and the possibility of pre-existing antibodies from previous Streptococcal infections within blood. Especially considering the fact that the H_2O_2 environment only models one portion of the highly complex cascade of interactions within neutrophils and monocytes during an immune response to infection (Singel and Segal, 2016; Nguyen, Green and Mecsas, 2017). Future research should aim to investigate the fitness of *S. suis* mutant strains in a variety of whole porcine and human blood types to elucidate those genes associated with pathogenesis of infection in both pigs and humans, and also to identify whether these genes share homology, since a cross-over may indicate similarities or differences in the pathogenesis of systemic infections in different mammalian species.

In a further study analysis of an *S. suis* serotype 10 strain was performed using a Tn-Seq approach where mutants were recovered from blood, CFS and brain meninges of inoculated piglets in an intrathecal experimental infection model (Arenas et al., 2020). In total, 361 genes were identified as conditionally essential for infection *in vivo,* and comparative to this study, genes were primarily associated with metabolism, cell regulation and transport processes. Genes associated with ribosomal structure and biogenesis, transcription, and cell wall and membrane envelope biogenesis, stress defence and immune evasion were also identified as contributing to the infectivity capacity of *S. suis.* A total of 23 genes identified in the *in vivo* model of infection were also identified and discussed in further detail in the current project and are summarised in Table 4.1.

Table 4.1: Genes shared between the current study and those identified in an intrathecal experimental infection of *S. suis* serotype 10 mutants of piglets*.

Gene	Functional pathway
BHI	
pEP	Carbon metabolism
rpoB	Purine and pyrimidine metabolism
cysE	Biosynthesis of amino acids
thrB	Biosynthesis of amino acids
oppF	Transporters
potA	Transporters
cps2B	Cell envelope biosynthesis
ezrA	Transcriptional regulators
spxA	Transcriptional regulators
Shared (BHI and H_2O_2)	
hpt	Purine and pyrimidine metabolism
gmk	Purine and pyrimidine metabolism
ftsY	Cell envelope biosynthesis
grpE	Stress tolerance
dnaK	Stress tolerance
ccpA	Transcriptional regulators
H_2O_2	
fhs	Carbon metabolism
secE	Cell envelope biosynthesis
SSU0114	Transporters
SSU1675	Transporters
SSU0883	Transporters
SSU0018	Cell envelope biosynthesis
SSU0473	Membrane and secreted structures
SSU1608	Transcriptional regulators

*Genes are categorised based on the phenotype that they were identified in in the current study and compared with those found in the study by (Arenas *et al*., 2020).

The similarities in genes between the intrathecal experimental study and the current study provide validation for the PIMMS protocol to identify genes essential for survival and for survival in refined environments. Genes associated with fundamental cellular processes required for survival including carbon metabolism, the biosynthesis and metabolism of amino acids, transporters and transcriptional regulators were consistently found across both experiments. Additionally, genes associated with virulence which would be expected to be identified in the *in vivo* model of infection were also identified as being essential for survival in H_2O_2 in the current study. This suggests that this study using H_2O_2 as a model for phagocytic respiratory burst during *in vitro* experiments may be used to facilitate the collection of preliminary data on the pathogenesis of systemic diseases without the requirement for infecting live animals.

4.5 Conclusion

To conclude, this study was the first to identify essential genes for the growth of *S. suis* P1/7 in the presence of BHI and with the addition of H_2O_2 using the mutagen pG$^+$host::IS*S1* and the PIMMS bioinformatic pipeline. In total, the PIMMS analysis strategy successfully identified 160 genes essential for growth in BHI, 198 genes essential for growth in both BHI and H_2O_2 and 35 genes essential for growth in H_2O_2. An additional 17 truncated genes were identified in H_2O_2 which were categorised as important for *S. suis* P1/7 survival and potential virulence. Survival and growth of cells in BHI media was predominately associated with genes involved in genetic information processing, metabolism, and cellular signalling processes. Furthermore, genes essential for survival and growth in H_2O_2 were associated with genetic information processing, metabolism, environmental information processing and cellular processes. A switch from carbohydrate metabolism to general metabolism was observed in the transition between the BHI phenotype and H_2O_2 phenotype and the role of alternative metabolic pathways in the presence of oxidative stress including the pentose phosphate pathway and its association with the resistance to reactive oxygen species and amino acid synthesis were highlighted as requiring greater investigation.

Several genes were highlighted as potential therapeutic, vaccine and diagnostic targets. These included the FtsEX, complex associated with the cell division cycle, carbohydrate metabolism genes FtsY in the signal recognition particle pathway, Fhs, FolD and associated with central carbohydrate metabolism that were essential for growth in the BHI phenotype. In addition, OppD, identified in both the BHI and H_2O_2 phenotypes, and SecE in the H_2O_2 phenotype were associated with the expression and secretion of virulence factors. The potential role of putative signal peptidase I (SSU0212), and secretory virulence factor putative haemolysin-III (Hly-III) (SSU0854) identified in the H_2O_2 phenotypes were also discussed in greater detail. Although highlighted as having promising potential as novel targets, the genes require further investigation to further elucidate their function in survival and virulence during a phagocytic respiratory burst through the generation of knockout mutants and growth in whole porcine blood and in macrophage cell lines to validate current findings and continue to refine the *in vitro* model for *S. suis* P1/7 infection without the requirement for infecting animals. The PIMMS protocol provides an excellent framework for the identification of virulence-associated genes and alternative antimicrobials and vaccine targets in bacterial pathogens which are significant to veterinary and human medicine.

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Appendix 1

A summary of the credits gained between whilst completing the Master of Research qualification.

Date	Event	Source	Credits
07.05.20	Challenges in antibiotic resistance: Gram Negative bacteria: online course	Futurelearn	1
11.05.20	The role of vaccines in preventing infecious diseases and antimicrobial resistance: online course	Futurelearn	0.5
16.05.20	Bacterial genomes: from DNA to protein function using bioinformatics: online course	Futurelearn	1
17.06.20	Structured reviews of literature: Evidence synthesis: online seminar	UoN Graduate centre	1
20.05.20	Research integrity: online course	UoN Graduate centre	1
30.06.20	Excel basic course: online course	UoN Graduate centre	0.5
01.07.20	Presenting with power: online course	UoN Graduate centre	0.5
03.07.20	Presentation Design Masterclass: online course	Udemy	1
07.07.20	Emotional intelligence: online course	UoN Graduate centre	0.5
07.07.20	Word intermediate course: online course	UoN Graduate centre	0.5
07.07.20	Being assertive in the right way: online course	UoN Graduate centre	0.5
07.07.20	The 7 steps to the perfect telephone etiquette: online course	UoN Graduate centre	0.5
21.08.20	Antimicrobial resistance in the food chain: online course	Futurelearn	1
06.09.20	Ethical decision making in care: online course	Futurelearn	1
18.10.20	What makes an effective presentation?: online course	Futurelearn	0.5
24.11.20	Social Media For Policy Engagemen: online seminar	UoN Graduate centre	1
24.10.20	How to succeed at interviews: online course	Futurelearn	0.5
07.11.20	Systematic literature review: an introduction	Futurelearn	1
01.07.20	Spring Post Graduate Symposium: attendance and presentation	UoN Graduate centre	5
15.08.20-17.08.20	Healthcare Assistant clinical training course	Fn CPD	15
16.12.20	International Veterinary Vaccinology Network Virtual Symposium: attendance	IVVN	3
28.02.21	Research Portfolio Module (VETS4027)	SVMS, UoN	20
Total:			56.5

Appendix 2

Essential genes for growth in H_2O_2 and H_2O_2 and BHI phenotypes identified as having shared putative function with other bacterial species, the phenotype and functionality determined by DEG analysis.

S. suis gene	Essentiality in additional species	Associated phenotype	COG Pathway	q_Start	Q_End	% Homology
SSU0078 *rpmC*	*Streptococcus mutans* UA159	Blood agar	Ribosomal protein L29	1	68	94.1
	Streptococcus agalactiae A909	Rich medium	Ribosomal protein L29	1	68	92.6
SSU0854	*Streptococcus agalactiae* A909	Rich medium	Predicted membrane protein, hemolysin III-like protein; COG1272R	4	216	77.4
	Streptococcus pyogenes NZ131	Todd-Hewitt medium	Predicted membrane protein, hemolysin III-like protein; COG1272R	1	171	77.1
SSU0092 *rplO**	*Streptococcus agalactiae* A909	Rich medium	Ribosomal protein L15	1	146	89.7
	Streptococcus mutans UA159	Blood agar	Ribosomal protein L15	1	146	89.0
	Staphylococcus aureus subsp. aureus MRSA252	MHBII medium	Ribosomal protein L15	1	146	82.2
	Bacillus subtilis 168	Rich medium	Ribosomal protein L15	1	146	71.2
SSU0093*	*Streptococcus sanguinis*	Rich medium	Preprotein translocase subunit SecY	1	436	70.9
	Streptococcus agalactiae A909	Rich medium	Preprotein translocase subunit SecY	1	436	70.9
	Streptococcus pyogenes NZ131	Todd-Hewitt medium	Preprotein translocase subunit SecY	1	436	70.6
	Streptococcus mutans UA159	Blood Agar	Preprotein translocase subunit SecY	1	436	72.0

SSU0087*	*Streptococcus sanguinis*	Rich medium	Ribosomal protein L6P/L9E	1	178	87.0
	Streptococcus pyogenes NZ131	Todd-Hewitt medium	Ribosomal protein L6P/L9E	1	178	82.6
	Streptococcus agalactiae A909	Rich medium	Ribosomal protein L6P/L9E	1	178	83.7
SSU0703*	*Streptococcus pneumoniae*	Rich medium	Uncharacterised conserved protein, heparinase superfamily	3	437	88.3
	Streptococcus sanguinis	Rich medium	Uncharacterised conserved protein, heparinase superfamily	1	437	87.9
	Streptococcus pyogenes NZ131	Todd-Hewitt medium	Uncharacterised conserved protein, heparinase superfamily	3	437	85.6
	Streptococcus agalactiae A909	Rich medium	Uncharacterised conserved protein, heparinase superfamily	1	437	84.7
	Streptococcus mutans UA159	Blood Agar	Uncharacterised conserved protein, heparinase superfamily	1	437	84.7
SSU0951*	*Streptococcus mutans* UA159	Blood Agar	Flavoprotein (flavin reductase) subunit CysJ of sulfite and N-hydroxylaminopurine reductases	1	295	89.5
	Streptococcus sanguinis	Rich medium	Flavoprotein (flavin reductase) subunit CysJ of sulfite and N-hydroxylaminopurine reductases	1	294	88.4
	Streptococcus agalactiae A909	Rich medium	Flavoprotein (flavin reductase) subunit CysJ of sulfite and N-hydroxylaminopurine reductases	1	294	86.7
SSU0952 *pstC**	*Streptococcus agalactiae* A909	Rich medium	ABC-type phosphate transport system, permease component	1	305	87.2
	Streptococcus mutans UA159	Blood Agar	ABC-type phosphate transport system, permease component	1	305	85.5
SSU0953 *pstS**	*Streptococcus sanguinis*	Rich medium	ABC-type phosphate transport system, periplasmic component	1	289	78.2

	Streptococcus mutans UA159	Blood Agar	ABC-type phosphate transport system, periplasmic component	3	288	74.8
	Streptococcus agalactiae A909	Rich medium	ABC-type phosphate transport system, periplasmic component	1	288	73.2
SSU1014 *atpD**	*Streptococcus sanguinis*	Rich medium	FoF1-type ATP synthase, beta subunit	1	468	93.8
	Streptococcus pneumoniae	Rich medium	FoF1-type ATP synthase, beta subunit	1	468	93.6
	Streptococcus pyogenes NZ131	Todd-Hewitt medium	FoF1-type ATP synthase, beta subunit	1	468	91.8
	Streptococcus mutans UA159	Blood Agar	FoF1-type ATP synthase, beta subunit	1	466	90.6
	Streptococcus agalactiae A909	Rich medium	FoF1-type ATP synthase, beta subunit	1	468	91.7
	Bacillus thuringiensis BMB171	Rich medium	FoF1-type ATP synthase, beta subunit	1	466	78.2
	Staphylococcus aureus subsp. aureus MSSA476	MHBII medium	FoF1-type ATP synthase, beta subunit	1	466	77.9
	Mycoplasma pulmonis UAB CTIP	Rich medium	FoF1-type ATP synthase, beta subunit	4	463	70.3
SSU1028 *ftsY**	*Streptococcus pyogenes* NZ131	Todd-Hewitt medium	Signal reception particle GTPase FtsY	117	424	86.0
	Streptococcus sanguinis	Rich medium	Signal reception particle GTPase FtsY	116	424	85.8
	Streptococcus agalactiae A909	Rich medium	Signal reception particle GTPase FtsY	44	424	71.0
SSU1239 *ftsE**	*Streptococcus sanguinis*	Rich medium	Cell division protein FtsB	1	230	91.3
	Streptococcus pneumoniae	Rich medium	Cell division protein FtsB	1	230	88.2
SSU1517 *glr**	*Streptococcus sanguinis*	Rich medium	Glutamate racemase	1	264	81.1

	Streptococcus pneumoniae	Rich medium	Glutamate racemase	1	264	79.5
	Streptococcus agalactiae A909	Rich medium	Glutamate racemase	1	264	75.4
	Streptococcus pyogenes NZ131	Todd-Hewitt medium	Glutamate racemase	1	264	75.0
SSU1692 *rplM**	*Streptococcus sanguinis*	Rich medium	Ribosomal protein L13	1	148	96.6
	Streptococcus agalactiae A909	Rich medium	Ribosomal protein L13	1	148	95.9
	Streptococcus mutans UA159	Blood Agar	Ribosomal protein L13	1	148	93.2
SSU1952*	*Streptococcus mutans* UA159	Blood Agar	Ribosomal protein L13	1	280	77.1
	Streptococcus pyogenes NZ131	Todd-Hewitt medium	Energy-coupling factor transporter ATP-binding protein EcfA2	1	280	74.3
	Streptococcus agalactiae A909	Rich medium	Energy-coupling factor transporter ATP-binding protein EcfA2	1	280	71.8
	Streptococcus pneumoniae	Rich medium	Energy-coupling factor transporter ATP-binding protein EcfA2	1	273	74.7
SSU1954*	*Streptococcus sanguinis*	Rich medium	Phosphatidylglycerophosphate synthase	1	179	78.8
	Streptococcus pneumoniae	Rich medium	Phosphatidylglycerophosphate synthase	1	178	79.8
	Streptococcus agalactiae A909	Rich medium	Phosphatidylglycerophosphate synthase	2	179	80.3
	Streptococcus mutans UA159	Blood Agar	Phosphatidylglycerophosphate synthase	2	179	76.4
	Streptococcus pyogenes NZ131	Todd-Hewitt medium	Phosphatidylglycerophosphate synthase	1	179	72.1
Total	16 genes					

*essential in both H2O2 and BHI