PROTEOMIC INVESTIGATION OF THE GROUP B *STREPTOCOCCUS*

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Abstract

The Group B *Streptococcus* (GBS) is a Gram-positive opportunistic pathogen which is a leading cause of neonatal disease globally. In 2000-2001, the general incidence of neonatal GBS infection was 0.72 per 1000 live births in U.K. and the mortality rate is about 10%, because of which neonatal GBS disease is a significant burden on society.

GBS is part of the commensal flora, colonising the vagina and gastrointestinal tract of women. Vertical transmission is the main cause of early onset GBS disease. During the process of GBS neonatal disease, GBS must be able to survive in several very different host environments, including the vagina, amniotic fluid, the neonate's lung and blood. The vagina is normally acidic, low oxygen and with limited nutrients while the neonate's lung and blood are neutral, high oxygen and with abundant nutrient. Proteomic investigations of GBS protein expression under conditions representing those associated with benign maternal colonisation and foetal exposure may help us understand the molecular basis of GBS virulence.

GBS growth characteristics, long term survival, acid adaptation, viable but non-culturable state and biofilm formation were investigated to help us understand how GBS survives in different environments and also help us to develop an *in vitro* model to reflect *in vivo* conditions during GBS disease development.

An *in vitro* model of GBS growth under conditions reflecting maternal vaginal carriage (low pH, low oxygen, nutrient stress) and exposure to body fluids during invasive disease (neutral pH, aeration, nutrient sufficient) was established. Proteins expressed under each growth conditions were separated by two dimensional electrophoresis. Individual proteins were subjected to in-gel trypsin digestion and identified using liquid chromatography-mass spectrometry with peptide mass fingerprinting followed with bioinformatic research. A total of 76 proteins were identified and 16 of these were expressed differentially. The putative virulence factor C protein β antigen and proteins involved in responses to oxidative stress were up-regulated under the conditions reflecting neonatal exposure.

Another *in vitro* model of GBS growth on Todd Hewitt agar in the presence or absence of 10% human serum was established and followed by proteomic investigation of proteins differentially expressed under these two conditions, as this model reflects GBS neonatal septicaemia (exposure to serum). A total of 84 proteins were identified and 11 of which were expressed differentially. The putative virulence factor C protein β antigen, arginine deiminase, an ABC transporter substrate-binding protein and glyceraldehyde-3-phosphate dehydrogenase were up-regulated in the presence of human serum.

List of contents

List of Tables

List of Figures

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Declaration

I declare that the work contained in this thesis is all my own work. The data in Chapter 3 has not yet been submitted for publication. Chapter 4 was published as 'Yang, Q., Zhang, M., Harrington D.J., Black G.W. & Sutcliffe I.C. (2010) A proteomic investigation of S*treptococcus agalactiae* grown under conditions associated with neonatal exposure reveals the upregulation of the putative virulence factor C protein β antigen. *International Journal of Medical Microbiology*. 300, 331-337.' and Chapter 5 was submitted as 'Yang, Q., Zhang, M., Harrington D.J., Black G.W. & Sutcliffe I.C. A proteomic investigation of S*treptococcus agalactiae* reveals that human serum induces the C protein β antigen and arginine deiminase'.

Name:

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Chapter 1

Introduction

1.1. Epidemiology of Group B *Streptococcus* disease

1.1.1. Morbidity and mortality of neonatal Group B *Streptococcus* disease

Streptococcus agalactiae (also referred to as the Group B *Streptococcus,* GBS) is a Gram-positive opportunistic pathogen, which is a leading cause of neonatal disease, including pneumonia, septicaemia and meningitis (Doran and Nizet, 2004; Johri et al., 2006).

GBS was first described in 1887 and reported as a fatal human pathogen in 1938 (Heath and Schuchat, 2007). During the 1970s and 1980s, GBS was identified as the most common cause of neonatal infectious disease in Western countries. GBS neonatal disease can be divided into early onset disease (EOD) and late onset disease (LOD). Early onset neonatal disease refers to when the infant is infected by GBS in the first 6 days after birth. About 60-70% of neonatal infections are EOD. Late onset neonatal disease occurs at 7-90 days after birth (Heath and Schuchat, 2007; Johri et al., 2006). The mortality rate of GBS EOD was around 10% in the UK in 2000. Heath et al. (2004) showed that in the UK during 2000-2001, among EOD, 63% was sepsis and 26% was pneumonia. 43% of LOD was meningitis and 7% was focal infection (Heath et al., 2004). Neonatal sepsis and meningitis are often accompanied by neuronal developmental impairment. Half of GBS meningitis cases show neurological

deficiencies including blindness, deafness, partial sensory loss and profound mental retardation (Heath and Schuchat, 2007; Mullaney, 2001).

Strains of GBS have been classically divided into 9 serotypes (Ia, Ib, II-VIII) based on their capsular polysaccharide structure. Recently, a new GBS serotype IX has been defined by Slotved's group (Slotved et al., 2007). Serotype distributions are different with different geographical regions. Ia, II, III and V are the majority serotypes in North America and Western Europe. IV and VIII are the predominant serotypes in Japan (Brochet et al., 2009). In the U.K., the frequency of GBS serotypes were 48% serotype III, 27% serotype Ia, 10% serotype V, 6% serotype Ib, 4% serotype II, 1% serotype IV and 1% serotype VI (Weisner et al., 2004). In Denmark during 1984-2002, the majority of strains were serotype III (59%), Ia (16%), Ib (8%) and II (5%) (Ekelund et al., 2004). In Norway between 1996 and 2006, serotype III (53%) was the predominant strain in neonatal GBS disease; other serotypes were Ia (12.8%), V (11.9%) and Ib (10.1%) (Bergseng et al., 2008). In Malawi, serotype Ia and III were the predominant serotypes and responsible for 77% of GBS neonatal disease (Gary et al., 2007). About 90% of all infection cases are caused by serotypes Ia, Ib, II, III and V. Serotype III is the dominant cause of both EOD and LOD GBS disease and presents with a higher rate of meningitis (Shet and Ferrieri, 2003; Mullaney, 2001; Weisner et al., 2004). Ekelund et al. showed 63% of fatal cases in Denmark were infected by serotype III (Ekelund et al., 2004).

The morbidity and mortality cause the neonatal GBS disease is a significant burden on society. The incidence of neonatal GBS disease in developed countries ranges from 0.4-0.81 per 1000 live births (Table 1.1). The

Table 1.1. Morbidity and mortality of GBS neonatal disease worldwide.

Research Year	Country	EOD/1000	LOD/1000	Total/1000	Fatality Rate	Reference
1984-2002	Demark	0.3	0.1	0.4	8%	Ekelund et al., 2004
1991-2000	Jamaica	0.66	0.23	0.91	3.6%	Trotman & Bell., 2005
1996-2006	Norway	0.46		0.66	6.5%-20%	Bergseng et al., 2008
1997-2001	Netherlands	0.43	0.14	0.57	8%-5%	Trijbels-Smeulders et al., 2007
1997-2001	Sweden	0.4				Hankansson et al., 2006
1999-2005	USA	0.47-0.34	0.34	0.81-0.68	5%-9%	Phares et al., 2008
2000-2001	UK & Ireland	0.48	0.24	0.72	9.7%	Heath et al., 2004
2000-2003	USA	0.31-0.52	0.30-0.36			Apostol et al., 2004
2000-2004	USA (Alaska)	0.42				Castrodale et al., 2006
2003-2005	Italy	0.27	0.23	0.50		Berardi et al., 2007
2004-2005	Malawi (Africa)	0.92	0.89	1.81	33%	Gary et al., 2007

morbidity of neonatal GBS disease in developing countries is likely to be higher than in developed countries. The incidence of neonatal GBS disease was 0.91 per 1000 live births in Jamaica during 1991-2002 and 1.81 per 1000 live births in Malawi (Africa) during 2004-2005 (Trotman et al., 2006; Gary et al., 2007). The comparatively lower incidence of neonatal GBS disease in some developed countries may have been caused by the introduction of intrapartum prophylaxis (Johri et al., 2006). There is a difference of incidence between different geographical regions. The variation of GBS incidence may be caused by geography, economy and methodology differences (Heath et al., 2004).

EOD GBS disease accounted for about 50%-84% of all neonatal GBS disease (Trijbels-Smeulders et al., 2007; Phares et al., 2008; Heath et al., 2004). The mortality rate of EOD GBS disease ranged from 3.6% of India (Caribbean) to 33% of Malawi (Africa) (Trotman et al., 2006; Gary et al., 2007). The fatality rate in western countries ranged from 5% of USA to 20% of Netherland (Phares et al., 2008; Bergseng et al., 2008).

The health and social care cost for neonates with GBS disease is high, approximately two fold higher than neonates without GBS disease in the first 2 years. This means neonatal GBS disease is a significant burden on society. Finding cost-effective prevention and treatments of GBS disease will benefit both patients and society. (Schroeder et al., 2009).

1.1.2. Risk factors of GBS neonatal disease

Vertical transmission is the main cause of GBS EOD. Neonates acquire bacteria via aspiration of the vaginal contents or amniotic fluid infected by GBS. The risk factors of EOD GBS disease among newborns are: GBS bacteriuria during pregnancy; prolonged rupture of membranes more than 18 hours; preterm birth date less than 37 weeks and temperature higher than 38^{o}C (Heath et al., 2004; Hakansson et al., 2007).

GBS is a common part of the vaginal flora in women. The maternal GBS carriage rate varies among different countries ranging from 1.8% in Maputo (Mozambique, Africa) to 65% in USA (De Steenwinkel et al., 2008; Meyn et al., 2009). The maternal GBS carriage rates in representative countries are summarised in Table 1.2. Half of these studies showed the maternal GBS carriage rate was more than 20%. Some of these studies of GBS colonisation may be underestimates due to poor specimen collection and inadequate detection methods (Barcaite et al., 2008). Even in the same country, the maternal GBS carriage rate also varied between different race and ethnic groups (Barcaite et al., 2008). In the UK, the study by Jones et al. (2005) showed a maternal GBS carriage rate that was higher than that reported by Colbourn and Gilbert (2007). Colbourn and Gilbert (2007) showed that the colonisation rate was higher using vaginal and rectal swabs (18.1%) compared with only using a single vaginal swab (14%) (Colbourn and Gilbert, 2007). The reported differences of maternal GBS carriage rate may be affected by geographic, ethnic factors and culture techniques (Hansen et al., 2003).

Table 1.2. Maternal carriage rate and vertical transmission rate.

Research Year	Country	Carriage Rate	Transfer	Reference
1996-2006	Europe	6.5%-36%		Barcaite et al. 2008
1999-2003	UK	14%	36%	Colbourn & Gilbert., 2007
2001-2003	UK (Oxfordshire)	21.3%		Jones et al., 2006
2004-2006	Poland	17.2%-20%		Strus et al., 2008
2005	Sweden	25.4%	68%	Hakansson et al., 2008
2005-2006	Switzerland	21%		Rausch et al., 2009
2005-2006	Bangui (Central African Republic), Darkar (Senegal)	20.0%-17.5%		Brochet et al., 2008
2006	Maputo, Mozambique	1.8%		De Steenwinkel., 2008
2008	Uruguay	17.3%		Laufer et al., 2008
	USA	65%		Meyn et al., 2009

GBS maternal colonisation in the gastrointerstinal and genital tract is a primary condition for EOD. Maternal carriage of GBS is dynamic (Phares et al., 2008). Hansen et al. (2003) showed in Denmark during a 2 year period, the average GBS colonization rate was 36% and the highest colonization rate was 54% among pregnant women. 28% of the pregnant women were persistent carriers (defined as a GBS carriage time span over 12 weeks) and 19% of the pregnant women were intermittent carriers. The stable carriage of GBS by women without symptoms suggests there was a state of commensalism between GBS and the host (Hansen et al., 2003).

Vertical transmission is the main cause of EOD (Heath & Schuchat., 2007). Women colonised with GBS prenatally have a high opportunity to transmit infection to their babies (Barcaite et al., 2008). It has been reported that by vaginal delivery and without intrapartum antibiotics, the vertical transmission rate from GBS carriage mothers to their infants was 36.4% in the UK during 1999-2003 and 68% in Sweden in 2005 (Colbourn and Gilbert, 2007; Hakansson et al., 2007). The babies apparently acquired GBS from their mothers as the same strains were detected from both neonates and their mothers (Hansen et al., 2003). Carriage of GBS and the extent of GBS colonisation are linked to the risk of GBS disease (Hansen et al., 2003). Intensive maternal colonization leads to a higher rate of vertical transmission. 73% (32/44) of GBS heavy carrier mothers have ‘colonised babies’, which is higher than light carrier mother (35%, 6/17) (Ancona et al., 1980). The degree of GBS carriage by pregnant women plays a role in vertical transmission.

Pass et al. (1979) showed that 31% of GBS carriage babies were heavy

carriers. 8% of these heavy carriage babies developed early sepsis. This rate was higher than light carriers and those that were not carriers (Pass et al., 1979). Similar results were obtained by Gerards et al. (1984). GBS heavily colonised neonates had an increased risk of developing disease compared to lightly colonised neonates (Gerards et al., 1984). 23% of EOD neonates were born preterm with average gestational age of 31 weeks (Phares et al., 2008). The risk of development of GBS disease among premature neonates was two times higher than in term infants (Hakansson et al., 2006). Pregnant women with clinically identifiable risk factors are more likely to have baby with EOD and high mortality rate (Heath et al., 2004).

Phares et al. (2008) showed that among the EOD neonates, the GBS positive culture rate was 72% at 24 hours and 95% at 48 hours. All these samples came from blood (96%) or cerebrospinal fluid (4%) (Phares et al., 2008). EOD begins from a few hours to 6 days after birth and 90% of EOD occurs within 12 hours after birth. Without therapy with antibiotics, monkeys with GBS disease died within 10 hrs from sepsis (Rubens et al., 1991). The rapid onset of disease in neonates suggests that in many cases the infection must have been established in the uterus. GBS can grow rapidly in amniotic fluid both *in vivo* and *in vitro* experiments. Hemming et al. (1985) found that GBS had similar growth rates in amniotic fluid and Todd Hewitt broth media (Hemming et al., 1985). Abbasi et al. showed GBS grew in both second and third trimester amniotic fluid as well as in Todd Hewitt Broth media (Abbasi et al., 1986). In clear amniotic fluid, GBS cultures with a starter inoculation of 10^2-10^3 CFU/mL reached to 10^7-10^8 CFU/mL after 8 hours growth. This suggested

GBS proliferated rapidly in amniotic fluid (Abbasi et al., 1986, Hemming et al., 1985). Romero et al. (1991) found amniotic fluid culture rates of GBS were higher in meconium-stained amniotic fluid than in patient without staining. The risk that pregnant women with meconium-stained amniotic fluid will deliver a preterm neonate is higher than pregnant women with clear amniotic fluid (Romero et al., 1991). Amniotic fluid expressed limited bacteriostatic properties to GBS when compared with its ability to inhibit growth of *E. coli* (Eidelman et al., 2002). Meconium stained amniotic fluid can enhance GBS growth (Eidelman et al., 2002). The prolonged rupture of amniotic membranes can induce amniotic fluid staining with meconium, which may increase GBS colonisation of newborn babies (Eidelman et al., 2002).

The incidence of GBS neonatal disease ranges from 0.4 to 1.81 cases per 1000 live births world wide. The fatality rates of GBS neonatal disease are significant, ranging from 3.6% to 33% of cases (Table 1.1). The significant morbidity and serious mortality rates caused by GBS establish this bacterium as an important pathogen of neonates.

1.2. The pathogenesis of GBS disease

The development of GBS neonatal pneumonia, septicaemia and meningitis is a complex process. In the pathogenesis of these diseases, the bacterial need to adhere to and penetrate into different host cells including the vaginal epithelium, placental membranes, respiratory epithelium and blood-brain barrier endothelium (Figure 1.1). When GBS get into host blood and deeper tissues, it

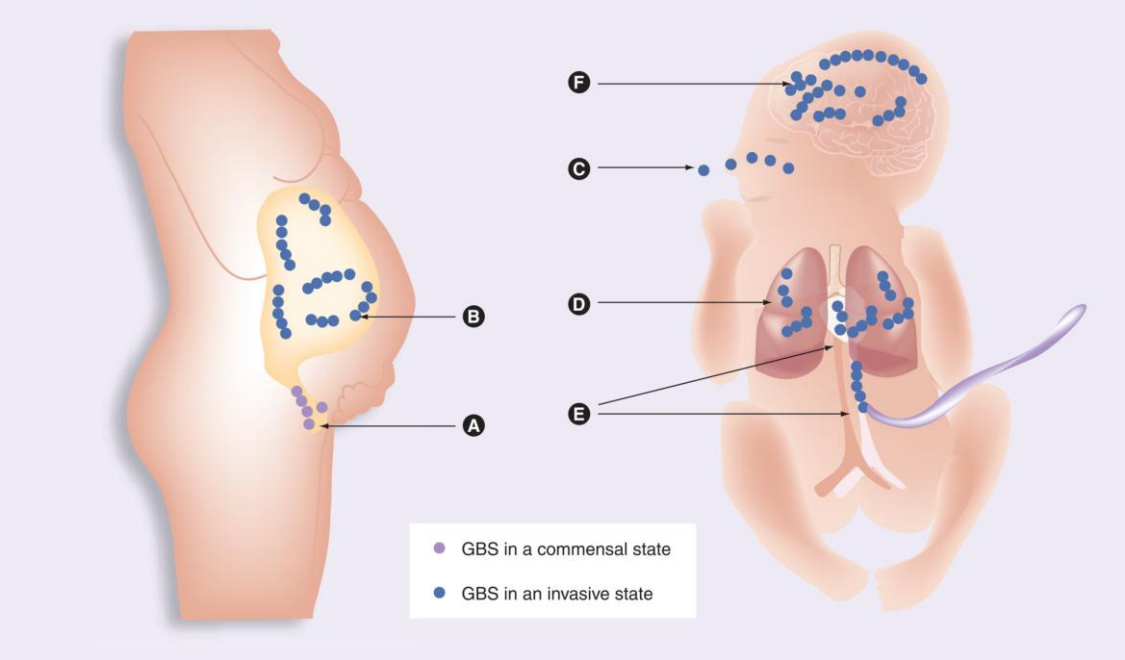


Figure 1.1. The development of GBS neonatal disease (taken from Rajagopal, 2009).

(A) GBS maternal colonization in vaginal and lower gastrointestinal tracts.

(B) GBS penetration of the intrauterine compartment.

(C) Neonatal aspiration of GBS during birth.

(D) GBS invade the neonatal lung causing pneumonia.

(E) GBS arrive in the neonatal bloodstream causing sepsis.

(F) GBS penetration of the blood-brain barrier causing meningitis.

will trigger the host immune responses to eradicate the bacteria. GBS possess virulence factors which can help the bacteria to avoid host immune clearance and activate host inflammatory responses (Doran and Nizet, 2004).

Animal experiments showed GBS was found in the lung, liver, spleen and brain in GBS infected neonatal primates (Rubens et al., 1991). Inflammatory reactions occurred as monocytes and macrophages were present at infected tissues with haemorrhaging. The symptoms of sepsis and neonatal pneumonia include hypoxemia, hypercapnia and apnea. After a few hours without treatment, the neonatal primates died with hypotension, metabolic acidosis and respiratory failure (Rubens et al., 1991). There are inflammatory reactions and focal lesions at the early stage of GBS meningitis. This is accompanied with cerebral blood flow changes, cerebral hypoxia, ischaemia and oedema. This can cause permanent neuronal damage as necrosis in the cortex and neuronal loss and apoptosis were found in animal models (Maisey et al., 2008; Spellerberg, 2000)

1.2.1. GBS virulence factors that affect adherence to epithelial surfaces

GBS is a part of the common flora colonizing at the vagina, rectum, urinary tract and gastrointestinal mucosa. GBS adherence to host cells is the initial step of GBS neonatal disease. Tamura et al. (1994) showed GBS can adhere to pulmonary epithelial cells under different conditions. The ability of GBS to adhere to epithelial cells was decreased when the bacteria were treated with trypsin. This indicated that bacterial surface proteins are involved in the

adherence to the host cells (Tamura et al., 1994). Surface proteins including pili, C5a peptidase, α-C protein, Lmb, FbsA and Rib are linked to the firm adherence of GBS to host cells. These cell wall components and surface proteins interact with host cell extracellular matrix (ECM) components including fibronectin (Fn), fibrinogen, laminin and integrins to mediate GBS adherence (Doran and Nizet, 2004; Lindahl et al., 2005). The GBS cell envelope component lipotechoic acid (LTA) is also involved in the adherence to the host cells. This adherence is relative weak and reversible (Doran and Nizet, 2004).

The GBS pilus is an important cell surface component which is involved in bacterial colonization and invasion of host cells (Rosini et al., 2006). The GBS pilus consists of three surface proteins: PilA (Gbs1478), PilB (Gbs1477), PilC (Gbs1474). Gbs1477 is the essential component which forms the backbone of pilus and distributes throughout the length of the pilus. Gbs1478 is a pilus associated adhesin and Gbs1474 is a minor pilus associated component. These two surface proteins distribute intermittently along the pilus (Dramsi et al., 2006; Rosini et al., 2006). In GBS strain NEM316, class C sortases, *srtC3-C4* were found to be involved in pilus assembly (Dramsi et al, 2006). A GBS *srtA* deletion strain showed a impaired adherence to epithelial cells and binding to extracellular matrix. Thus SrtC is involved in catalysing Gbs1477 polymerization and SrtA is involved in anchoring the pilus to the peptidoglycan cell wall (Rosini et al., 2006; Dramsi et al., 2006). 2 pilus islands (PI), PI-1 and PI-2 (including 2 alleles, PI-2a and PI-2b) are localized in 2 distinct genetic loci. A GBS Gbs1478 sortase deletion strain exhibited significantly reduced adherence to A549 epithelial cells compared with the wild type strain (Dramsi et

al., 2006). GBS COH1ΔBP-1 and COH1ΔBP-2a strains exhibited a lower translocation across ME180 cell monolayers compared with the wild type strain (Pezzicoli et al., 2008). This indicates that pilus structure is important for bacteria to pass through intercellular junctions. 3-Dimensional confocal microscopy experiments showed GBS pili located in the intercellular space protruding from translocating bacteria. Thus the pilus structure of GBS plays an important role of GBS adherence and invading host cells (Pezzicoli et al., 2008). GBS pili are well conserved among strains. All strains expressed at least 1 of the 3 pili substructures of surface protein. Margarit et al. (2009) suggested that if a vaccine included all pilus components it will be effective against GBS infection (Margarit et al., 2009). RogB positively regulates the expression of *gbs1477, gbs1478* and *srtC3-4*. A GBS 6313 *RogB* deletion strain showed a decreased adherence to human epithelial cells (Dramsi et al, 2006).

The surface proteins of GBS include α-C protein, β-C protein, α-like proteins (Alp1, Alp2 and Alp3) and Rib protein (Lindahl et al., 2005). The α-C protein varies between 62.5 and 167 kDa and is the prototype of a family of streptococcal surface proteins. These proteins share a common structure, including an N-terminal region, a series of tandem repeats of amino acids and a C-terminal region containing a LPXTG peptidoglycan-attachment motif (Bolduc et al., 2002; Persson et al., 2008). Experiments demonstrated that α-C protein can bind to the surface of ME180 human cervical epithelial cells. Deletion of the α-C protein gene attenuated the virulence of GBS seven fold (Bolduc et al., 2002). Experiments have suggested that the N-terminal region of the α-C proteins is involved in adherence to and invasion of cervical epithelial cells.

The N-terminal domain of α-C protein has been found to bind epithelial cell-associated glycosaminoglycan and the surface receptor α1 β1-integrin (Baron et al., 2004; Pannaraj et al., 2007; Bolduc et al., 2007). Rib protein, a member of the Alp family from type III strains can promote bacterial binding to epithelial cells (Doran and Nizet, 2004; Lindahl et al., 2005).

The surface anchored protein FbsA mediates GBS adhesion to host cells (Rajagopal, 2009). Purified FbsA protein can bind fibrinogen directly and GBS FbsA deletion strain showed a decreased binding to fibrinogen and impaired growth in human blood compared with wild type strain (Schubert et al., 2002). FbsA is positively regulated by RogB and negatively regulated by RovS and CovR/CovS (Rajagopal, 2009). Lra1 adhesin family proteins mediate GBS attachment to host cell laminin. Some experiments showed the Lra1 family member, laminin-binding protein Lmb mediated GBS binding to host placental laminin but other experiments showed that the ability of Lmb protein bind laminin was debatable (Lindahl et al., 2005).

The GBS C5a peptidase is a 120 kDa surface protein which has a function in adhesion to host cells (Liu and Nizet, 2004). C5a peptidase is encoded by the *scpB* gene and is composed of a protease domain, a protease-associated (PA) domain and 3 fibronectin type III (Fn) domains. The protease domain is located at the N-terminus and the 3 Fn domains are located at the C-terminus. An RGD sequence (Arg-Gly-Asp) is found in the protease domain and another RGD sequence is found between the F1 and F2 Fn domains. RGD sequences have been demonstrated to be able to bind integrins and Hep2 epithelial cells (Brown et al., 2005). Cheng et al. (2002) demonstrated that

purified ScpB binds directly to Hep2 and A549 cell lines (Cheng et al., 2002). ScpB also binds to the extracellular matrix protein immobilized Fn, but not to soluble Fn. The binding was concentration dependent. As C5a peptidase binding to Fn may be involved in attachment and invasion of eukaryotic cells, this may contribute to the pathogenesis of GBS disease (Beckmann et al., 2001). Hull et al. (2008) showed that ScpB binds to a point created by the association of multiple Fn molecules (Hull et al., 2008). This binding affinity is independent of the C5a peptidase activity of ScpB as the adherence was not affected by deletion of 4 amino acids of the peptidase motif (Tamura et al., 2006). However, GBS adherence was only partially affected by deletion of the *scpB* gene. This suggests that there are other cell components of GBS involved in adherence to host cells by binding to fibronectin (Cheng et al., 2002; Lindahl et al., 2005). RgfC/RgfA and possibly RgfB negatively regulate ScpB expression in a strain specific manner (Rajagopal, 2009). CovR/CovS also repress ScpB expression in a strain specific manner as this was not observed in strain A909 (Rajagopal, 2009).

1.2.2. GBS virulence factors affecting penetration of host cellular barriers

Animal experiments showed GBS was found in the lungs, liver, spleen and brain in GBS infected neonatal primates (Rubens et al., 1991). GBS penetration of host cells and/or tissues is the key step of GBS neonatal disease. In the GBS infection, bacteria can penetrate chorion cells and amnion cells and induce placental basement membrane rupture (Spellerberg, 2000). In GBS pneumonia and sepsis, bacterial invade the pulmonary epithelial cell layers, the basement

membrane and the vascular endothelial cell layer. GBS penetration of the blood-brain barrier can cause bacterial meningitis (Spellerberg, 2000). Electron microscopy showed that GBS can invade A549 epithelial cells when co-cultured for 2 hours (Rubens et al., 1992). The invasion capacity of GBS strains isolated from the blood of infected infants was higher than that of GBS strains isolated from the vaginas of asymptomatic women. This suggests the GBS invasion is related to bacterial virulence potential (Soriani et al., 2005).

GBS cross the lung epithelial barrier by a combination of three processes: intracellular invasion, direct cytolytic injury and damage induced by the inflammatory reaction of infected neonates (Soriani et al., 2005). Transmission electron microscopy and confocal imaging observed that there are both singular bacteria and bacterial chains present between ME180 cells and Caco2 cells. Both of these cell lines expressed tight junctions. This suggested that GBS can form an intimate association with intercellular junctions and has the capacity to disrupt epithelial cells by a paracellular mechanism (Soriani et al., 2005). Pezzicoli et al. (2008) found GBS pili located in the intercellular space protruding from translocating bacteria. This suggested GBS pilus is involved in a paracellular invasion of host tissues (Pezzicoli et al., 2008). It has been reported that GBS can enter host cells by endocytotic uptake and survive in these cells (Soriani et al., 2005). In *in vivo* experiments, GBS was found in the vacuoles of respiratory epithelial cells, macrophages and fibroblasts in infected infant primates. Multiple bacteria in one vacuole suggested that there was bacterial replication (Rubens et al., 1991).

Hyaluronate lyase is secreted by the GBS and may contribute to inflammation. This surface accessible protein is encoded by the gene *hylB* which is expressed as a 110 kDa protein (Liu and Nizet, 2004; Spellerberg, 2000). Hyaluronate lyases can degrade hyaluronan and this may help GBS spread as hyaluronan is one of the main polysaccharide components of connective tissues and the nervous system. The placenta and lung have a high concentration of hyaluronan and so this tissue damage maybe important early in the pathogenesis of GBS disease (Liu and Nizet, 2004). GBS hyaluronate lyase can break down the substrate chain and release disaccharides (Liu and Nizet, 2004). It has been reported that the hyaluronate lyase was expressed at a higher level in GBS strains from infected neonates than GBS strains from asymptomatic neonates (Liu and Nizet, 2004; Spellerberg. 2000). However, the role of *hylB* in the course of GBS infection has not been confirmed (Liu and Nizet, 2004; Spellerberg. 2000).

Surface-anchored protein Spb1, the fibronectin-binding C5a peptidase ScpB and alpha C protein can increase GBS epithelial cell invasion. Bolduc et al. (2002) showed that ME180 epithelial cells internalisation of a GBS wild type strain with a 9-repeat alpha C protein was nearly threefold higher than an alpha C-deficient mutant. GBS wild type translocation across polarized ME180 membranes was five-times higher than that of the alpha C-deficient mutant. These data suggest that alpha C protein is involved in both GBS internalization and translocation across human cervical epithelial cells (Bolduc et al, 2002). Both anti-ScpB serum treatment and a *scpB* deletion mutant showed a decreased invasion of lung epithelial A549 cells and Hep2 cells by GBS. This

suggested that ScpB plays a role in GBS invasion of human cells (Cheng et al., 2002). FbsB can promote GBS entry into host cells as a GBS FbsB deletion strain showed a severely impaired invasion into lung epithelial cells compared with the wild type strain (Gutekunst et al., 2004).

Beta-hemolysin is regarded as an important virulence factor of GBS and a key phenotypic feature of GBS is the presence of beta-haemolysis surrounding colonies on blood-agar plates (Liu and Nizet, 2004). Hemolysin synthesis has been linked to the *cyl* gene cluster (Gottschalk et al., 2006). The *cylA* and *cylB* genes are located in the *cyl* gene cluster. These genes encode the ATP binding and transmembrane domains of an ABC transport system. *CylA* is the ATP-binding domain and *cylB* is the transmembrane portein (Gottschalk et al., 2006). *cylE* encodes a 78 kDa predicted protein product and GBS with CylE were shown to damage lung epithelial cells, brain epithelial cells and macrophages (Nizet et al., 1996). GBS beta-hemolysin damages eukaryotic cells in various ways. A microtiter plate lactate dehydrogenase (LDH) release assay showed LDH was released from monolayers of A549 alveolar epithelial cells when A549 cells were exposed to log-phase hyperhemolytic GBS strains and to purified hemolysin. LDH release from lung epithelial cells induced by beta-hemolysin was inhibited by dipalmitoyl phosphatidylcholine (DPPC), a known hemolysin inhibitor and a major phospholipid component of human pulmonary surfactant (Nizet et al., 1996). Trypan blue assay showed 35-100% of cell nuclei were positively stained by trypan blue when A549 cells were incubated with hyperhemolytic GBS strains. Trypan blue is a vital dye, which can stain cell nuclei when cells have lost the membrane function to pump it out.

Investigation of GBS induced lung epithelial cell injury by electron microscopy showed that beta-hemolysin is a pore-forming toxin. The injury included discrete cytoplasmic membrane disruption, cell swelling, organelles and chromatin changes, water influx into the cell and hypo-osmotic damage (Nizet et al., 1996). All these experiments suggest that the expression of beta-haemolysin by GBS contributes directly to injury of human lung epithelial cells (Nizet et al., 1996). Two component systems are involved in regulation of beta-haemolysin expression. The CovS/CovR system represses beta-haemolysin expression while the serine/theonine kinase Stk1 relieves CovR repression on beta-haemolysin. The transcriptional regulator RovS activates beta-haemolysin expression, which is independent of CovS/CovR and Stk1 (Rajagopal, 2009).

The CAMP factor is a 23.5 kD extracellular protein, which is named after its original descriptors: Christie, Atkins and Much-Peterson (Liu and Nizet, 2004). In 1944, Christie found that coculture of *Staphylococcus aureus* and GBS on sheep blood agar plates produced an increased zone of haemolysis around GBS colonies near to those of *S. aureus* (Liu and Nizet, 2004; Spellerberg, 2000). This is because CAMP factor damages the membranes of sheep red blood cells which have been pretreated by the beta-hemolysin of *S. aureus*. Pretreatment with the *S. aureus* sphingomyelinase increases the binding of CAMP protein to the sheep red cell membrane and then pore formation and cell lysis occurs (Liu and Nizet, 2004; Spellerberg, 2000). Osmotic protection experiments show that CAMP factor forms discrete transmembrane pores more than 1.6 nm and up to 12-16 nm in diameter when observed by electron microscopy. These transmembrane channels allowed

small molecules (e.g. ions) to pass through but not large molecules (e.g. haemoglobin). The consequence was increased intracellular osmotic pressure, water infusion into the cell and cell lysis (Lang and Palmer, 2003). Intravenous injection of purified CAMP factor into rabbits and mice can cause rapid death of these animals (Skelka and Smola, 1981). A GBS CovR deletion strain did not express CAMP factor as CovR binding and activation were necessary for CAMP factor expression. CAMP factor expression is positively regulated by CovR/S and negatively regulated by Stk1 (Rajagopal, 2009).

The amphiphilic molecule LTA is a component of GBS cell envelope. LTA is anchored to the cell membrane via its glycolipid anchor, which carries a polyglycerophosphate chain extending into the cell wall (Doran et al., 2005). LTA anchoring to the cell membrane is a key aspect of GBS blood-brain barrier invasion and the pathogenesis of GBS meningitis. It has been shown that the GBS strains isolated from infected neonates have a higher quantity of LTA than strains isolated from asymptomatic infants (Doran et al., 2005). Deletion of the Δ*iagA* gene resulted in a failure to synthesise the glycolipid anchor of LTA and increased LTA release into the supernatant (Doran et al., 2005). The GBS *iagA* deletion mutant showed a decreased invasion of epithelial cells compared with GBS wild type (Doran et al., 2005).

1.2.3. GBS virulence factors that help avoidance of immunological clearance

GBS escape from host immune clearance is an important step of GBS neonatal disease. When GBS get into the host's deeper tissues and circulatory system, the host immune system will respond to clear it (Spellerberg, 2000). Bacterial counts in the lung were much higher than in the liver and spleen which suggested that macrophages may be involved in the immune response (Rubens et al., 1991). Phagocytic cells including neutrophils and macrophages clear bacteria following the production of specific antibodies or complement (Spellerberg, 2000). *In vivo* experiments showed both complement-dependent opsonic activity and complement-independent opsonic activity was increased in GBS infected primates (Rubens et al., 1991). Neonates are more easily infected by GBS due to a deficiency of antibodies and complement. However, GBS possess virulence factors which can help them to avoid host immune clearance and activate host inflammatory responses (Doran and Nizet, 2004).

The surface polysaccharide capsule (CPS) can inhibit complement factor C3b deposition and prevent the activation of the alternative pathway of complement. Classically, GBS was divided into nine serotypes (Ia, Ib, II-VIII) according to distinct capsular polysaccharides and serotype IX was identified in 2007 (Slotved et al., 2007). The specific capsular polysaccharides are composed of different arrangements of glucose, galactose, N-acetylglucosamine and sialic acid (Doran and Nizet, 2004; Spellerberg, 2000; Cieslewicz et al., 2005). GBS capsular polysaccharide plays an important role in resistance to complement mediated opsonophagocytic killing by host

leukocytes (Doran and Nizet, 2004; Spellerberg, 2000). C3-binding assays showed the binding capacity of a GBS capsule deficient strain to C3 molecules was eight times higher than wild-type strain (Marques et al., 1992). GBS capsule deficient mutants were more readily killed compared with wild-type strain when cells were incubated with leukocytes. The ability of neuraminidase-treated GBS to bind to C3 was two to three times higher than untreated cells. Neuraminidase treatment of GBS can remove bacteria capsular sialic acid residues (Marques et al., 1992). This suggested GBS capsular sialic acid residues are important for resisting complement binding. Complement C3 deposits on the GBS surface and is then degraded to the active factor C3bi. CR3 receptors of neutrophils recognise C3bi which contributes to neutrophil attachment and ingestion of the bacteria (Campbell et al., 1991). The capsular polysaccharide can inhibit the activated complement factor C3 binding to the surface of GBS, preventing the activation of the alternative complement pathway (Doran and Nizet, 2004; Spellerberg, 2000). The capsular polysaccharide may help GBS avoid host clearance by decreasing immune recognition (Mullaney, 2001). A transcriptional regulator, RogB, negatively regulates CPS expression in a strain specific manner (as GBS strains A909 and COH1 do not express RogB). The CovR/CovS system positively regulates CPS expression in strain NEM316 (Rajagopal, 2009).

C5a peptidase contributes to GBS pathogenesis in many ways (Liu and Nizet, 2004), including adhesion (see above). C5a peptidase can attenuate the recruitment of polymorphonuclear leukocytes *in vitro*. Active C5a-peptidase can be released from the bacterial cell wall and specifically cleaves the complement

chemotaxin C5a. C5a, a 74 amino acid protein, normally recruits polymorphonuclear leukocytes leading to inflammatory reactions. C5a peptidase inactivates C5a to avoid clearance by polymorphonuclear leukocytes (Cheng et al., 2002; Cleary et al., 2004). A ScpB deletion strain was cleared more rapidly in the adult mouse lung compared with the wild-type strain. Immunization of mice with ScpB can increase clearance of GBS from lung. This suggested that antibody generated by immunization played a protective function (Cheng et al., 2002).

Alpha C protein may help avoid phagocyte killing by antigenic variation (Spellerberg, 2000). The β-C protein is a 130 kDa surface protein, which can bind to the Fc part of IgA. This non-specific binding to IgA may interfere with opsonophagocytosis of GBS (Spellerberg, 2000). CAMP protein also binds to the Fc portion of human IgG and IgM (Spellerberg, 2000).

GBS beta-haemolysin can cause cytolytic injury to macrophages and monocytes and induce apoptosis in these cells (Fettucciari, 2000; Liu et al, 2004). GBS induced macrophage apoptosis was confirmed by several techniques based on morphological changes and DNA fragmentation (Fettucciari, 2000). GBS induced macrophage cytolysis and apoptosis when macrophages were incubated with a GBS hypohemolytic strain at high concentration. A GBS haemolysin deficient strain was cleared by macrophages and neutrophils. GBS survival was associated with the production of the orange carotenoid pigment, which is known as a free radical scavenger and provides protection against the H_2O_2 and superoxide produced by phagocytes during their oxidative burst. Inhibition of GBS beta-haemolysin activity can inhibit

macrophage apoptosis (Fettucciari, 2000). This suggests the beta-haemolysin contributes to GBS resistance to host innate immune mechanisms (Liu et al, 2004).

GBS Mn-cofactored superoxide dismutase (SodA) catalyzes conversion of superoxide to hydrogen peroxide which is further metabolised to molecular oxygen and water in the presence of peroxidases. SodA enables bacteria to defend against oxidative stress during infection. A GBS SodA deletion strain showed sensitivity to oxidative stress *in vitro* and enhanced clearance by macrophages in a mouse infection model (Poyart et al., 2001). RovS positively regulates SodA as SodA expression was decreased in a RovS deletion strain (Rajagopal, 2009).

A GBS surface associated penicillin-binding protein (PBP1a), encoded by *ponA*, also contributes to bacterial resistance against phagocyte clearance. A GBS PBP1a deletion stain was more sensitive to phagocyte clearance compared with the wide type strain (Hamilton et al., 2006). However, the *ponA* mutant strain showed a similar ability in blocking complement activation and was as sensitive to oxidative killing as the wild type strain. Experiments demonstrated the ponA mutant was more sensitive to killing by cationic antimicrobial peptides (Hamilton et al., 2006). This may explain why the *ponA* mutant was sensitive to phagocytosis, but since there was no change on the surface of the mutant strain, the mechanisms by which PBP1a affects resistance to antimicrobial peptides are still not clear (Rajagopal et al., 2009).

1.2.4. GBS virulence factors that activate inflammatory reactions

When GBS get into the deeper tissues, they can induce host inflammatory reactions. Animal models of neonatal GBS infection showed there was a huge accumulation of GBS and intensive inflammatory infiltration in the neonatal lung (Henneke et al., 2005). Experiments also showed that neonatal GBS infection is associated with increased production of proinflammatory cytokines. Production of tumour necrosis factor alpha, interleukin (IL)-8 and IL-10 by GBS infected human respiratory epithelial cell line A549 was significantly higher than for uninfected cells. There was a significant difference in the production of these cytokines among different GBS strains (Mikamo et al., 2004). This suggested that there was inflammatory reaction of phagocytes to GBS (Henneke et al., 2005).

GBS beta-hemolysin acts synergistically with cell wall components to induce macrophage production of nitric oxide synthase (iNOS) and generation of nitric oxide. This contributes to neonatal sepsis. Experiments have shown that expression of iNOS and the amount of nitric oxide released is 4-fold more when macrophages were exposed to GBS beta-hemolysin positive strains compared to GBS beta-hemolysin negative mutants (Ring et al., 2002). Beta-hemolysin exhibited proinflammatory properties in the sepsis cascade as the production of macrophage iNOS induced by beta-hemolysin was via intracellular pathways, which is similar to lipopolysaccharide incuced iNOS production (Ring et al., 2002). Pathologic samples from lung have shown there are markedly damaged epithelial cells, beta hemolysin production and deposition of complement component C3, IgG, and fibrin in the infected lung

(Doran et al., 2002). Septic shock has been associated with increased amount of inducible nitric oxide production in animal models (Ring et al., 2002). It has also been shown that GBS beta-hemolysin can cause host cells to release neutrophil chemoattractant IL-8 in a dose and time dependent fashion. A *cylE* deficient GBS mutant induced less IL-8 release. IL-8 is a chemoattractant which can recruit neutrophils to kill bacteria and is a measure of the local inflammation reaction. IL-8 also can induce inflammatory cells to release proteases and reactive oxygen. A high level of IL-8 has been found in respiratory secretions of neonates with lung disease (Doran et al., 2002).

GBS LTA has been shown to have the ability to induce inflammatory reactions. LTA can stimulate mononuclear cells to release TNF-alpha, IL-1, IL-6 and iNOS. Animal models showed the consequences of releasing these inflammatory cytokines are the provocation of septic shock, circulatory collapse and systemic infection (Doran et al., 2005; Hunolstein et al., 1997).

1.3. Environmental factors affect GBS growth and virulence factor expression

1.3.1. GBS survive in different host environments

The rapid development of early onset GBS disease (often <24 hours after birth) suggests that vertical transmission of GBS from mother to infant occurs either during or immediately prior to birth. During the process of GBS neonatal disease, GBS colonising the mother's vagina are thought to be aspirated by the neonate during delivery. GBS may also access the neonate following entry into

the amniotic fluid (Hemming et al., 1985). The GBS then colonise the neonate's pulmonary epithelium, invade the epithelial cells, enter the infant lung, spread to the blood and can eventually penetrate the blood-brain barrier to get to the cerebral tissue. Therefore it is interesting that GBS must be able to adapt to and survive in several very different host environments (Rajogopal, 2009).

The pregnant woman's vagina is very acidic (pH less than 4.5) whereas the neonate's lung and blood are approximately neutral (Tamura et al., 1994; Yamamoto et al., 2006). The vagina of pregnant women is a low oxygen environment with pO_2 of 48 mmHg whereas the new born baby's arterial blood has a high oxygen condition with pO_2 of 60 to 100 mmHg (Johri et al., 2003). Other factors such as ion concentrations and nutrient availability are also likely to be different between these environments. The concentration of sodium chloride in blood (150 mM) is relatively high compared to the vagina (30 nM). Calcium and magnesium concentrations are also higher in blood than in the vagina (Tamura et al., 1994).

As noted earlier (Section 1.1.2) GBS can grow rapidly in amniotic fluid in both *in vivo* and *in vitro* experiments. Hemming et al. (1985) found that GBS had similar growth rates in amniotic fluid and Todd Hewitt broth media (Hemming et al., 1985). Abbasi et al. (1986) showed GBS grew in both second and third trimester amniotic fluid. The amniotic fluid was analysed and found that the components and quantity (including total protein, albumin, zinc, inorganic phosphorus, transferrin, lysozyme activity and immunoglobulin levels) were similar to those in amniotic fluid specimens reported to inhibit bacterial growth (Abbasi et al., 1986). Amniotic fluid stained by meconium can enhance

the growth of GBS (Eidelman et al., 2002). A transcriptome experiment showed that GBS strain NEM316 grew rapidly in human amniotic fluid with logarithmic phase starting at 35 mins (Sitkiewicz et al., 2009). Transcripts of genes related to amino acid, carbohydrate and nucleotide metabolism of GBS strain NEM316 were expressed highly in amniotic fluid compared with in Todd Hewitt-Yeast extract (THY) medium. Some virulence genes including capsule, hemolysin and interleukin-8 proteinase were up-regulated when GBS was cultured in amniotic fluid. The transcripts of genes expressed variably during different phases of GBS growth. The finding that GBS strain NEM316 remodels its transcriptome in response to culture media relevant to pathogenesis confirms it has the ability to adapt to changing environments (Sitkiewicz et al., 2009).

When GBS was incubated in human blood for 30 minutes, a large proportion of its genes were down-regulated compared with growth in THY. However, GBS genes encoding proteins related to bacterial-host interactions, including the host coagulation/fibrinolysis system, were upregulated in human blood compared with in THY. This indicates that GBS can adapt to exposure to human blood immediately after invading which may help cause host infection in a short time (Mereghetti et al., 2008b). Moreover, the inhibitory effects of cerulenin and triclosan (type II fatty acid synthesis pathway inhibitors) on GBS growth were alleviated when media were supplementted with human serum *in vitro*. This is important as evidence that human serum can provide unsaturated fatty acids to act as nutrients (Brinster et al., 2009). Confirming this, GBS *fabF* (fatty acid elongation) and *fabM* (unsaturated fatty acid synthesis) deletion strains grew better in serum than in Todd Hewitt medium (Brinster et al., 2009).

It has been reported that at least 10% of GBS genes were expressed differently in response to changes in the environmental temperature (Mereghetti et al., 2008a). In stationary phase, GBS genes related to sugar metabolism, ion uptake and cell envelope synthesis were upregulated at 30^{o}C compared with at 40^{o}C. Virulence factors including hemolysin and surface-anchored (LPXTG motif) proteins were upregulated at 40^{o}C compared with growth at 30^{o}C. GBS growth at 40^{o}C was considered to reflect bacteria colonising in a host with a severe infection and high fever condition and GBS growth at 30^{o}C condition was considered to reflect bacterial persistence in an asymptomic host or bovine milk.

GBS transcriptome experiments revealed that gene transcript levels were expressed differentially throughout the cells' growth phases *in vitro* (Sitkiewicz & Musser, 2009). Genes encoding stress response proteins (e.g. *hrcA*, *grpE*, *dnaK*), and those involved in energy production (eg. ATP synthase subunits) and nutrient utilisation (carbohydrate utilisation and transport) were expressed at a higher level in stationary phase. Genes encoding regulators and signal transduction systems were expressed at high transcript levels in log phase. Among GBS virulence factors, 6 genes were upregulated and 11 genes (for cell wall anchored proteins including C5a peptidase precursor, putative fibronectin binding protein, laminin binding protein, hemolysin, sortase, capsule synthesis and putative group B antigen biosynthesis) were down regulated in stationary phase (Sitkiewicz & Musser, 2009).

Together, these studies show that GBS can survive in a wide-range of conditions and has the ability to remodel its genes in response to environmental

changes including temperature, pH, oxygen level and nutrition (Mereghetti et al., 2008b). These changes in bacterial gene expression reflect the biological process of metabolism, adaptive responses and changes in virulence factor expression. GBS uses multiple mechanisms to mediate its gene expression in response to environment changes (Rajogopal, 2009). These processes are important for GBS to survive in multiple host environments and cause disease to the host (Sitkiewicz et al., 2009; Rajogopal, 2009).

1.3.2. Oxygen affects GBS metabolism and virulence factor expression

GBS is known to be a fermentative and acid-producing bacterium. Mickelson found that GBS undergoes fermentative metabolism in anaerobic conditions and undergoes respiration metabolism in aerobic conditions (Mickelson, 1971). The aerobic conditions were created by culturing GBS in large vessels with shaking. The anaerobic condition was created by culturing GBS in small vessels without oxygen (Mickelson, 1971). GBS can aerobically respire using externally derived haem and quinones (Yamamoto et al., 2005; Rezaïki et al., 2008), suggesting these may be derived from the host and/or other vaginal bacteria (Rezaïki et al., 2008). Haem and quinone serve as acceptors in the electron transport chain reactions. A series of respiration related genes, including genes for NADH dehydrogenase and the cytochrome bd subunits CydA and CydB have been found in GBS genome sequences (Yamamoto et al., 2005). However the biosynthetic pathways for quinone and haem synthesis were not found in GBS. Respiration occurred when GBS cultured in an aerobic condition with externally supplied quinone and haem (Yamamoto et al., 2005).

GBS was cultured in M17 medium, with horse blood and menaquinone added as exogenous sources of haem and quinone. The respiration metabolism caused an increased GBS growth, with a twofold increase in biomass production. The respiration metabolism also produced a higher final culture pH and increased survival after 24 hours (Yamamoto et al., 2005).

Johri et al. (2005) showed that GBS growth rate and environmental oxygen influence GBS adherence and invasion to host tissue. GBS adherence to human alveolar epithelial carcinoma A549 cells at fast growth rate (t_d=1.8h) was significantly higher than at slow growth rate (t_d=11h). In the presence of oxygen, the capability of GBS to adhere to A549 cells was significantly higher than in the absence of oxygen (Johri et al., 2005). For an encapsulated GBS strain, the invasion ability into host cells at a fast mass doubling time (t_d=1.8h) was 20 to 700 fold higher than at a slow growth time (t_d=11h) (Malin and Paoletti, 2001). The environmental oxygen level can also affect GBS invasion of host cells. In an investigation of GBS invasion of PFSK-1 human neuroepithelial cells, CFU/mL recovered under 5% oxygen conditions was 71-fold higher than with anaerobic conditions (Johri et al., 2003). Also, GBS invasion into A549 epithelial cells in the presence of 20% oxygen was significantly higher than without oxygen (Johri et al., 2003). GBS adherence and invasion to A549 epithelial cells reached a peak at fast growth rate with 15% oxygen compared with slow growth rate or absence of oxygen (Johri et al., 2005). One dimensional sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) electrophoresis analysis showed that there were significant differences in GBS (type V strain 2603V/R) cell component expression when GBS was grown at different growth rates and environments (Johri et al., 2007). A GBS

gene microarray experiment found that there were 43 genes upregulated when GBS was grown at a fast growth rate with 12% oxygen (high invasive conditions) compared with slow growth rate without oxygen (Johri et al., 2007). This combined genomic and proteomic research showed that there were 36 genes and proteins expressed when GBS grew at fast growth rates with oxygen, which included 8 surface proteins (Johri et al., 2007). Protein SAG0765, SAG0823, SAG1007, SAG1350, and putative lipoprotein SAG0971 were upregulated when GBS grew at a fast growth rate. Iron transporter-binding protein SAG1007 and glutamine-binding protein SAG1466 expression were modulated by both growth rate and oxygen level. Penicillin-binding protein family members including SAG0519, SAG0287, SAG0765 and SAG2066 were found to be expressed during GBS invasion of host cells (Johri et al., 2007). GBS production of capsular polysaccharide, C proteins, CAMP factor and beta-haemolysin at fast growth rate (t_d=1.4h) were significantly higher than at low growth rate of t_d=11h. However, the production of alkaline phosphatase decreased when the GBS growth rate was raised (Ross et al., 1999). GBS adherence and invasion to host tissue may be mediated by these components and their expression relates to differences in metabolism and environment (Mikamo et al., 2004). In contrast to the findings of Johri et al. (2007), Rubens et al. (1992) reported that GBS at stationary phase and lag-phase (slower growth) were more readily able to invade host cells than at log-phase (faster growth). Active protein, DNA and RNA synthesis were necessary for GBS invasion of host cells. The GBS invasion into A549 cells was significantly inhibited when GBS was treated with minocycline (a protein synthesis inhibitor), rifampin (an RNA synthesis inhibitor) and ciprofloxacin (Rubens et al., 1992).

1.3.3. pH affects GBS adherence

The capability of GBS to attach to epithelial cells increased in acidic conditions (Tamura et al., 1994). GBS adherence to A549 pulmonary epithelial cells and vaginal epithelial cells at pH 4 was 10 to 20 fold higher than at neutral pH. There was no difference in GBS adherence to A549 pulmonary epithelial cells between log and stationary phase cells. Transcription experiments showed there were 317 genes up-regulated and 61 genes down-regulated when GBS was incubated in pH 5 media compared with pH 7 medium (Santi et al., 2009). In GBS wild type strains, CsrRS (a two component regulatory system, also known as capsule synthesis regulator, regulator and sensor components) regulated virulence genes including *cylE* (encoding beta hemolysin) and *scpB* (encoding C5a peptidase) were up regulated in pH 7 media comparing with in pH 5 media. These changes were not shown in a ΔcsrRS strain. These results revealed CsrRS is involved in GBS responses to environmental pH change (Santi et al., 2009). The majority of genes involved in response to environment pH change include genes regulation transport, metabolism, stress response and virulence (Santi et al., 2009).

1.4. Theories of GBS long term survival in relation to colonisation

The healthy vagina is normally very acidic (pH less than 4.5). This low pH environment is a natural barrier employed by the human body to protect against microorganisms (Koutsoumanis & Sofos, 2004). GBS long term carriage by women might be explained by acid tolerance/adaptation theory or alternative adaptations to the acid vaginal environment. These are reviewed below.

1.4.1. Acid tolerance responses

Bacterial resistance to acid killing in normally lethal acid conditions after they were transiently exposed to a mild sublethal acidic condition has been defined as an acid tolerance response (ATR) (Svensater et al., 2001, Nascimento et al., 2004, Martin-Galiano et al., 2005, Papadimitriou et al., 2007). However, there is no research as to whether GBS has an ATR. Research on other bacteria showed ATR is a complicated biological mechanism since different systems are involved in this process (Koutsoumanis & Sofos, 2004). In the ATR, there are several physiological changes associated with the protection of the bacteria from the lethal acid environment which include *de novo* protein synthesis and changes in the fatty acid composition of the cell membrane (Koutsoumanis & Sofos, 2004).

Streptococcus mutans can survive in a very acid environment and this ability has been well investigated. *S. mutans* could survive at pH 4.0 after preacidification (Takahashi & Yamada 1999). Two-dimensional gel electrophoresis results showed there were 64 proteins up-regulated and 49 proteins down-regulated in *S. mutans* grown in pH 7 medium compared to pH 5 medium (Svensater et al., 2001). Proteomic analysis of *S. mutans* also revealed the differentially expressed proteins between pH 5 and pH 7 were associated with stress response pathways and involved in DNA replication, transcription, translation, protein folding and proteolysis (Len et al., 2004).

Experiments have shown that non-mutans streptococci also exhibited acid tolerance. After preacidification, non-mutans streptococci can survive at pH 4.0 for 60 mins (Takahashi & Yamada 1999). *Streptococcus sobrinus*

showed an increased survival rate in pH 5 medium and had the ability to decrease the medium pH value though glycolysis (Nascimento et al., 2004). Two-dimensional gels showed there were more than 9 proteins up-regulated and 22 proteins down-regulated when S. *sobrinus* was grown in pH 5 medium compared to pH 7 medium (Nascimento et al., 2004). Global expression analysis of *Streptococcus pneumoniae* showed there were 126 genes expressed differentially in acid shock experiments (Martin-Galiano et al., 2005). These experiments suggested that certain sublethal acid conditions may allow activation of physiological mechanisms which can protect the bacterial against lethal acid conditions (Koutsoumanis & Sofos, 2004). Koutsoumanis and Sofos's research found *Listeria monocytogenes*, *Escherichia coli* O157:H7 and *Salmonella typhimurium* had different pH ranges of acid resistance and different pH values of maximum acid tolerance (Koutsoumanis & Sofos, 2004).

F_1F_0-ATPase is involved in Gram positive bacterial resistance to killing in acid environments (Cotter et al., 2003). F_1F_0-ATPase activity increased two-fold when *S. mutans* UA159 was grown in pH 5 medium compared with in pH 7 medium (Nascimento et al., 2004). Gene transcriptional analysis of S. *mutans* revealed all the genes encoding the eight subunits of F_1F_0-ATPase were up-regulated in pH 5.5 medium (Gong et al., 2009). *Streptococcus macedonicus* showed a logarithmic-phase ATR when cells were exposed to sublethal acidic conditions. The survival rate of S. *macedonicus* was decreased when cells were treated with N, N'-dicyclohexylcarbodimide (DCCD), a F_1F_0-ATPase inhibitor, after exposure to sublethal acid conditions (Papadimitriou et al., 2007). Two-dimensional gel results showed the β subunit of *L. monocytogenes*

ATPase is induced by acid stress (Cotter et al., 2003). F_1F_0-ATPase has the ability to hydrolyse ATP to generate a proton gradient for different transport processes and allows protons to be removed from the cell cytoplasm, resulting in a drop in environmental pH (Martin-Galiano et al., 2005). The activity of bacterial F_1F_0-ATPase was up-regulated when the environment pH decreased and this contributed to the bacterial ATR (Nascimento et al., 2004, Martin-Galiano et al., 2005).

The genome sequence of *S. mutans* showed it is impossible for these streptococci to undergo aerobic respiratory metabolism as there are no genes for the aerobic electron transport chain. The carbohydrate fermentation pathway is the main source of energy for *S. mutans*. The end products of complete glycolysis and fermentation include lactic acid, acetate, formate, ethanol, CO_2 and ATP. This process provides ATP for the F_1F_0-ATPase to translocate protons out of the cell and to maintain the cytoplasm at neutral pH (Ajdić et al., 2002). Sheng and Marquis found malolactic fermentation by *S. mutans* can protect bacteria against acid killing and contribute to their aciduricity (Sheng & Marquis, 2007). Malolactic fermentation produces L-lactate, carbon dioxide and ATP. The production of ATP is used by the ATPase to extrude protons across the membrane (Sheng & Marquis, 2007; Lemme et al., 2010).

The arginine deiminase system (ADS) of *L. monocytogenes* contributes to both growth and protection of bacteria against acid killing (Ryan et al., 2008). RT-PCR analysis revealed the ADS gene was upregulated in both acid and anaerobic environments with the addition of arginine. Arginine deiminase is

involved in conversion of arginine to ornithine, CO_2, ammonia and the production of ATP. Again, ATP is used to extrude protons across the membrane and maintain pH homeostasis. Also, the production of ammonia can neutralize acid and protect bacteria from lethal acidic enviroments (Ryan et al., 2009). An arginine deiminase deficient bacterium, S. *mutans,* possesses the agmatine deiminase system, which has a similar function as the ADS (Griswold et al., 2005).

The phosphoenolpyruvate:sugar phosphotransferase system (PTS) is a major sugar transport system of Gram positive bacteria (Lemos & Burne, 2008). The PTS is composed of components including enzyme I (EI), the heat-stable phosphocarrier protein HPr and several sugar-specific permeases known as EII complexes. The PTS has the ability to transport carbohydrates and contribute to acid adaptation (Nascimento et al., 2004; Ajdic et al., 2002). Glucose PTS activity was increased when *S. sobrinus* was grown in pH 5 medium compared with pH 7 medium. However, there was no significant difference in *S. sobrinus* F_1F_0-ATPase levels under these conditions (Nascimento et al., 2004). This suggested that the glucose-specific PTS plays an important role in the S. *sobrinus* acid tolerance response. Both F_1F_0-ATPase and glucose specific PTS activities increased at log-phase when *S. macedonicus* was transiently exposed to sublethal acid medium, suggesting these components contribute to S. *macedonicus* resistance to acid killing (Papadimitriou et al., 2007).

Bacterial two-component systems are signal transduction systems, which are composed of a sensor kinase and a response regulator (Kawada-Matsuo et al., 2009). The sensor kinase is a transmembrane signal receptor that

recognizes environmental changes and phosphorylates a specific histidine residue. The response regulator accepts the phosphoryl group transferred from the sensor kinase and modulates expression of target genes (Biswas et al., 2007). Two-component signal transduction systems detect environmental changes including oxygen, nutrition and osmotic potential and regulate gene expression to allow adaptation to these environmental changes (Biswas et al., 2007). Global transcriptional analysis of S. *mutans* revealed there are about 14% of the genes expressed differentially between pH 5.5 medium and pH 7.5 medium (Gong et al., 2009). Both gene transcriptional analysis and real-time qRT-PCR showed that genes encoding multiple two-component systems, including CiaHR, LevSR, LiaSR, ScnKR, Hk1037/Rr1038 and ComDE, were up-regulated in pH 5.5 medium compared with pH 7.5 medium (Gong et al., 2009). S *mutans* strains with deletion of histidine kinase genes revealed a retarded growth and impaired ATR (Gong et al., 2009).

There is link between chaperones and acid stress as these proteins respond to acid stress in some Gram-positive bacteria (Cotter et al., 2003). Both acid shock experiments and proteome analysis of S. *mutans* revealed the heat shock proteins GroEL and DnaK were up-regulated in acid conditions (Nascimento et al., 2004; Len et al., 2004). GroEL and DnaK are members of the class I chaperonins. These proteins are able to regulate signal transduction and are involved in protein folding, translocation and aggregation (Cotter et al., 2003; Len et al., 2004). Chloramphenicol and actinomycin D partially inhibited acid tolerance of S. *macedonicus*, indicating ATR is related to protein synthesis (Papadimitriou et al., 2007). Some bacterial cell wall synthesis inhibitors

including vancomycin and penicillin can inhibit the acid tolerance of S. *macedonicus*, which suggest cell wall synthesis is necessary for the ATR (Papadimitriou et al., 2006). Cell membrane components including monounsaturated fatty acids and longer chain fatty acids increased when S. *mutans* was grown in pH 5 medium compared with in pH 7 medium (Cotter et al., 2003). Bacterial signal recognition particle (SRP) pathway is composed of SRP54 protein (Ffh), 4.5 or 7S small cytoplasmic RNA (scRNA) and SRP receptor (FtsY). The SRP complex is involved in cotranslational translocation and secretion of specific proteins through the plasma membrane (Hasona et al., 2005). S. *mutans* with deletion of genes endoding Ffh, scRNA and FtsY cannot survive under stress conditions, including at pH 5, in 3.5% NaCl and 0.3 mM H_2O_2. This suggests the SRP pathway contributes to bacterial resistance to environmental stress changes (Hasona et al., 2005). S. *mutans* SRP deletion mutants showed a decreased membrane ATPase activity in both neutral and acid conditions. This is a partial explanation for the susceptibility to acid stress of SRP deletion mutants (Hasona et al., 2006).

The above examples demonstrate that the mechanism of the ATR in bacteria can be complicated. F_1F_0-ATPase, glucose-specific PTS and two-component systems all are likely to play important roles in bacterial ATR. Changes in the composition of the cell envelope, production of heat shock proteins and chaperones are also contribute to protection of bacteria from acid killing (Cotter et al., 2003).

1.4.2. Biofilm formation contributes to acid tolerance

A bacterial biofilm is composed of groups of bacteria surrounded by an extracellular polysaccharide matrix (Kaur et al., 2009; Hall-Stoodley and Stoodley, 2009). The extracellular matrix is composed of water, polysaccharides, proteins, lipids, extracellular DNA, membrane particles and ions (Karatan and Watnick, 2009). This three dimensional complex is a coordinated community and allows bacteria to adapt to and survive in host environments (Hall-Stoodley and Stoodley, 2009). Bacteria in biofilms detect environmental changes and respond to it in order to survive in diverse and stressful conditions (Hall-Stoodley and Stoodley, 2009). Biofilms generate resistance to antibiotics by decreasing the antibiotic's penetration rate and mediating bacterial gene expression. Transmission electron micrographs reveal biofilms protect bacteria against phagocytes (Hall-Stoodley and Stoodley, 2009). There are different proteins expressed between biofilm cells of S. *mutans* and planktonic cells (Welin-Neilands and Svensäter, 2007). S. *mutans* biofilm cells expressed significant greater acid tolerance ability compared with planktonic cells both at 3 hours and 3 day time points. These experiments showed that the survival rate of biofilm-grown *S. mutans* in pH 3.5 medium after transient exposure to pH 5.5 medium was 820 to 70,000 fold more than for planktonic cells (Welin-Neilans and Svensäter, 2007). Bacterial acid tolerance is increased with increased biofilm density (Welin-Neilands and Svensäter, 2007).

Bacterial biofilm formation is regulated by different environment signals including mechanical, nutritional, metabolic and host-derived signals,

secondary messenger and protein transcriptional regulators (Karatan and Watnick, 2009). Recent *in vitro* experiments have shown that GBS is able to form biofilms and the maximum biofilm formation time was 48 hrs (Kaur et al., 2009). This biofilm formation was influenced by environmental changes including pH, carbohydrate and osmolarity. GBS biofilm formation was favoured without sugar supplementation of the medium and decreased at pH below 6 (Kaur et al., 2009). The GBS pilus is an important cell surface component which is involved in biofilm formation (Konto-Ghiorghi et al., 2009; Rinaudo et al., 2010). The PilA and PilB pilus proteins are essential for biofilm formation in GBS strain NEM316. There was an impaired biofilm formation in GBS NEM316 pilus deletion mutants (Konto-Ghiorghi et al., 2009). Similarly, in *Streptococcus pyogenes*, wild-type strain formation of biofilms on polystyrene was five to six fold more than that by pilus deletion strains (Manetti et al., 2007).

These recent studies suggest that formation of biofilms by some GBS strains may be an important feature of colonisation *in vivo*.

1.4.3. Long term survival in stationary phase by streptococci and other reference organisms

Colonisation by GBS *in vivo* may depend on the organisms ability to withstand conditions of low nutrient availability. Studies with other bacteria suggest survival in stationary phase may therefore be a relevant *in vitro* model. In neutral Todd-Hewitt broth media, *S. pyogenes* showed long term survival for more than 4 weeks in stationary-phase and remained culturable for more than 1

year. However, the pH of the culture medium can affect the survival of bacteria. *S. pyogenes* survived less than 1 week in medium when the pH was below 5.6 (Wood et al., 2005). Based on the phenomenon of long-term survival in stationary phase, the bacterial life cycle was expanded to five phases: lag phase, exponential phase, stationary phase, death phase and long-term stationary phase (Finkel, 2006). In death phase most of the cells lose viability. *S. pyogenes* lost more than 99% of viability after 4 days culture (Trainor et al., 1999). This may be attributed to high density cell cultures exhausting the limited nutrients available and inducing bacterial 'apoptosis' (Finkel, 2006). Surviving cells can then use the material of dead cells as their nutrition. After death phase, remaining viable cells enter into long-term stationary phase. In this phase, bacterial show lower rates of division and keep a balanced rate of 'birth' and 'death' (Finkel 2006). After 4 weeks of stationary phase *S. pyogenes* produced two kinds of colonies: atypical large colonies and microcolonies (Wood et al., 2005). Proteomic analysis of these two colony types showed they possessed both common and unique characters compared with each other (Wood et al., 2005). This suggested there was an accumulation of mutants surviving in long-term stationary phase and these expressed differences in more than metabolism (Wood et al., 2009). *S. pyogenes* exhibited a starvation resistance response in carbon or phosphorous limited medium, with a constant cell number for more than 4 weeks. However cell numbers decreased dramatically when S. *pyogenes* was cultured in nitrogen limited medium (Trainor et al., 1999). S. *pyogenes* lost culturability in less than 72 hours when cells at long-term stationary phase were transferred from glucose depleted rich medium to phosphate buffered saline or by adding antibiotics. This suggested

bacteria at long-term stationary phase remain metabolically active (Wood et al., 2009). Bacterial metabolic pathways were detected by measuring the medium components before S. *pyogenes* entry into stationary phase and at 12 weeks. Results showed there was an increase in formate and acetate concentrations and decrease in lactate level (Wood et al., 2009). Bacteria use the pyruvate pathway to convert lactate to acetate and produce ATP. There were other intermediate products such as amino acid catabolic products that could enter the pyruvate pathway and it was shown that there was more acetate production than lactate consumption (Wood et al., 2009). Thus it appears that the pyruvate pathway and amino acid catabolic pathway are the main metabolic pathways of bacteria surviving during long-term stationary phase (Wood et al., 2009).

These studies suggest that stationary phase survival may provide novel insights into the ability of GBS to survive low nutrient conditions.

1.4.4. Viable but non-culturable hypothesis

Stress environments such as nutrient starvation, ranges of culture temperature, high or low pH, osmotic and oxygen concentration can be lethal to bacteria. Bacteria can sense these environmental changes and activate survival mechanisms to enter into a viable but nonculturable (VBNC) state (Oliver, 2009). In the VBNC state, bacteria are unable to grow on standard medium and form colonies but are still alive (Bogosian & Bourneuf, 2001; Oliver, 2004). Bacteria in the VBNC state are considered to be in a dormant phase, accompanied by very low levels of metabolic activity, macromolecular synthesis

and respiration, but ATP levels and membrane potential are still high. VBNC cells can be resuscitated in favourable environments such as by changing temperature or incubation with other organisms including the protozoan *Acanthamoeba polyphaga*, yolk sacs of embryonated eggs and *in vivo* animal hosts (Oliver 2009). However, whether the cells enter VBNC state as a physiological phenomenon to survive in stress environments or this is a degenerative process leading to death is still in debate (Mula et al., 2008).

Bacterial entrance into a VBNC state is accompanied by metabolic changes including nutrient transport, respiration rates and macromolecular synthesis (Oliver 2004). Proteomic analysis of *E. faecalis* in growing, starvation and VBNC states revealed there are different metabolic levels including glycolysis, oxidative phosphorylation and phospholipid biosynthesis among these states. This suggests VBNC cells need to change their metabolic pathways to survive in harsh environments (Heim et al., 2002). Muela et al. (2009) constructed stress environments of *E. coli* starvation, growth in seawater or under visible light. They found there were outer membrane subproteome changes under these conditions (Muela et al., 2009). *E. coli* also underwent biochemical changes in the cell wall when undergoing the transition from a growth state to the VBNC state. HPLC results showed there was an increase in peptidoglycan cross-links and muropeptides with tripeptide moieties and a decrease in the length of glycan strands of peptidoglycan in VBNC cells compared with growing cells (Signoretto et al., 2001). Since peptidoglycan plays an important role in maintaining cell shape in growth stages, the change

in the peptidoglycan may be explained as a cellular adaptation to the new environment (Signoretto et al., 2001).

Cumulatively, these studies suggest that entry in a VBNC state may provide an alternative mechanism that might promote GBS survival *in vivo*.

1.5. Proteomics

Proteomics is defined as the investigation of the total proteins that are expressed by an organism at a certain time under a certain condition (Liebler, 2002). The classical proteomics method is two-dimensional (2D) gel based proteomics which is composed of three key techniques: 2D gel electrophoresis, mass spectrometry (MS) and bioinformatics (Beranova-Giorgianni, 2003).

Appropriate preparation of protein samples is essential for good 2D gel electrophoresis results. Cells are firstly disrupted and then proteins are extracted with lysis solution containing chaotropes, detergents, reductants and solubilising agent (Beranova-Giorgianni, 2003). Commonly, 8M urea is used as a denaturant which can solubilise and unfold most proteins. 2-4% CHAPS is also effective for solubilising samples. 20 to 100 mM dithiothreitol (DTT) is usually used as reductant to break any difulfide bonds and maintain proteins in a reduced state. Immobilized pH gradient (IPG) buffer is used to prevent protein aggregation. Processing protein samples should be carried out at low temperatures to avoid protein degradation. After extraction of protein samples, some contaminating substances, such as nucleic acids, salts, lipids or detergents may remain in the sample and interfere with the 2D results. These

substances can be removed by washing with a 2-D Clean-up Kit, acetone, ammonium sulphate or trichloroacetic acid (Dunn & Görg, 2001).

2D electrophoresis is a powerful protein separation technique, which can separate thousands of proteins on a single 2D gel. Proteins are separated in the first and second dimensions according to two independent properties. The first dimension of 2D electrophoresis is isoelectric focusing (IEF), which can separate proteins according to their isoelectric point (pI). Proteins carry either positive, negative or zero charge depending on their amino acid content. Under an electric field and in a pH gradient, a protein will move to the position, its isoelectric point, at which it obtains zero charge (Liebler, 2002; Twyman, 2004). The second dimension of 2D is sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), which separates proteins according their molecular weights. The negatively charged SDS detergent molecule binds to proteins proportionally to their molecular weight. Under an electric field, the protein-SDS complexes migrate through the cross-linked polyacrylamide gel to the positive electrode (Dunn & Görg, 2001; Liebler, 2002). There is an approximately linear relationship between the migration distance and the logarithm of the molecular weight. After separation, 2D gels are commonly stained by Coomassie blue stain or silver stain methods. Proteins can be visualized as spots with different sizes, intensities and position on the gel (Beranova-Giorgianni, 2003). However there are still some limitations of 2D based proteomics, as only a portion of all the proteins that are present can be visualised on 2D gels. Low-abundance proteins, very small or very large,

alkaline proteins and hydrophobic proteins are difficult to analyze by 2D (Beranova-Giorgianni, 2003).

After 2D electrophoresis, the protein of interest can be digested into small peptides by proteases in preparation for MS analysis (Liebler, 2002). Trypsin is the most widely used protease, which cleaves proteins at the position C-terminal to lysine and arginine residues (Liebler, 2002).

MS has played an important role in proteomics as it allows the analysis of proteins separated by 2D with great accuracy, rapidly and sensitivity. A typical mass spectrometer is composed of: an ionization source, to produce ions from sample; a mass analyzer, to separate the ions based on their mass-to-charge ratio; a detector system, to detect the ions; and a computer, to record the data and analyse data (Patterson, 2001; Liebler, 2002).

Electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) are the most commonly used ion source techniques. Samples ionized by MALDI are dry and co-crystallized with a matrix, which is used to analyse simple peptide mixtures. Samples ionized by ESI are transferred into gaseous ions at atmospheric pressure and delivered to the mass analyser through a capillary tube which experiences an electric field. ESI combined with LC-MS is used to analyse more complex samples (Lane, 2005; Glish and Vachet, 2003). Accuracy and precision are two important standards for any mass analyzer (Glish and Vachet, 2003). Ion trap, time-of-flight (TOF), quadrupole and Fourier transform ion cyclotron resonance (FT-ICR) are the common types of mass analyzer (Lane, 2005).

Peptide fragment fingerprinting (PFF) is a protein identification method, in which the peptide MS/MS spectra are measured by MS and matched to corresponding peptides predicted from protein sequence databases. Mascot and Sequest are the commonly used search algorithms. In a Mascot search, the experimentally measured MS/MS fragment masses are compared with peptide sequences databases, and a match probability score is calculated (Lane, 2005).

The fast growth of gene and protein sequence databases provides a means to identify proteins expressed in organisms. Bioinformatics tools make it easier to access these databases to search, match and identify a protein component (Beranova-Giorgianni, 2003; Liebler, 2002). Uni-PROT, trEMBL and NCBInr are the widely used protein sequence databases.

Biological systems are complex and highly dynamic. The proteome will reflect the changes in the biological system, including individual proteins of a cell, regulatory mechanisms in the cell and environmental factors. Proteomics is therefore a highly sensitive and high throughput technique to analyse proteins (Liebler, 2002). Thus 2D gel based proteomics is valuable tool to compare the changes in a biological system. Recently, multi-dimensional protein identification technology (MudPIT) has been applied to proteomics. Although MudPIT can identify more proteins than 2D based proteomics, it cannot compare the changes in the biological system.

1.6. Aims of the present study

The aims of the present study were to investigate the pathogenesis of GBS by developing different *in vitro* models to explore the GBS growth characteristics in conditions associated with maternal colonization, neonatal exposure and invasion. This *in vitro* work would be followed by proteomic investigation under these conditions. Specifically, this study aimed to

1. investigate long term survival of GBS under conditions of nutrient limitation and acid exposure, including planktonic and biofilm modes of growth.
2. Develop an *in vitro* model of GBS growth reflecting bacterial *in vivo* progression from maternal colonisation to neonatal exposure and to use proteomics to explore GBS adaptation under these conditions.
3. Identify and functionally analyse differentially expressed proteins of GBS under the conditions reflecting maternal colonisation and neonatal exposure.
4. Establish an *in vitro* model of GBS growth with human serum to reflect GBS invasion into blood.
5. Use proteomics to explore GBS adaptation under conditions relevant to host invasion i.e. exposure to human serum.
6. Identify and functionally analyse differentially expressed proteins of GBS grown in the presence and absence of human serum.

Chapter 2

Materials and Methods

2.1. GBS growth experiments

2.1.1. Bacterial strains, media and microbiology techniques

2.1.1.1. Bacterial strains

S. agalactiae strain A909 was the principal strain used in the project. Strain A909 is a serotype 1a strain which was originally collected by Rebecca Lancefield and which has been genome sequenced (Tettelin et al., 2005). *S. agalactiae* strain A909 was obtained from Dr Dean Harrington (University of Bradford). *S. agalactiae* strain NEM316 is a serotype III strain that has been genome sequenced (Glaser et al., 2001) and was kindly provided by Dr Shaynoor Dramsi (Institut Pasteur, Paris, France). GBS strains NCTC 8181^{T}, the type strain of *S. agalactiae*, NCTC 8184 and NCTC 9993 were from the collection of Professor Iain Sutcliffe (University of Northumbria) and were originally obtained from the National Collection of Type Cultures. GBS strain CT1 is a clinical isolate that was obtained from Dr Roland Koerner (Sunderland Royal Hospital).

2.1.1.2. Media

All media and solutions were made up with 18.2 MΩ H_2O purified by a MILLIPORE Direct-Q H_2O purification system. All media and solutions were

sterilised by autoclaving (BioCote) at 121^{o}C, with a pressure of 1.05 par for 20 mins. The pH of the media were adjusted using HCl additions and a pH Meter 3310 (JENWAY) before autoclaving. Liquid media were stored at room temperature and solid media were stored at 4^{o}C.

2.1.1.2.1. Todd Hewitt Broth medium.

Todd Hewitt broth (THB) contained Todd Hewitt Broth base (Oxoid), 3.64 g per 100 ml. The pH of THB medium was adjusted to 4.3, 5, 6 and 7 with HCl. Todd Hewitt Agar (THA) was made from THB supplemented with 1.5 g agar per 100 ml. After autoclaving approximately 20 ml medium was poured into each petri dish once the medium had cooled to about 50^{o}C.

2.1.1.2.2. LB broth.

LB broth contained the following per 100 ml: tryptone, 1 g; yeast extract, 0.5 g; and sodium chloride, 1 g.

2.1.1.2.3. Vagina Simulative Medium

Vagina simulative medium (Table 2.1) was prepared as described by Owen and Katz (1999). To add glucose, a 50% w/v glucose stock was prepared and sterilised separately. To prepare 100 ml, all ingredients were combined into a volume of 90 ml. After pH adjustment to pH 4.2, the medium was adjusted to 99ml. After autoclaving, 1 mL glucose stock was added to the medium.

Table 2.1 Vagina simulative medium as defined by Owen and Katz (1999).

Component	100ml
Sodium chloride	350 mg
Potassium hydroxide	140 mg
Calcium hydroxide	22 mg
Bovine serum albumin	1.8 mg
Lactic acid	200 mg
Acetic acid, glacial	100 mg
Glycerol	16 mg
Urea	40 mg
Glucose	500 mg

2.1.1.2.4. GBS growth in human serum.

100 µl GBS A909 overnight starter culture was inoculated into 5 ml THB and 5 ml human serum (Sigma H4522 - 100mL) and incubated at 37^{o}C with shaking. Colony forming units (CFU)/mL were determined at 2, 4 and 6 hours (section 2.1.2.2). Human serum from human male AB plasma was stored at -20^{o}C and thawed immediately before use. The experiment was performed in a Class II Microbiological Safety Cabinet (Envair) sterilised before use with 70% ethanol.

2.1.1.2.5. Comparison of GBS A909 growth in pH 5 THB with and without 10% human serum.

20 ml pH 5 THB with 10% human serum medium was made by adding 3.64g THB to 80ml 18.2 MΩ H_2O and adjusting pH to 5. The medium was adjusted to 90ml and divided into 5 aliquots of 18 ml in Universals and autoclaved. 2 ml of human serum was added to each universal before inoculation.

200 µl GBS A909 overnight starter culture was inoculated into 20 ml pH 5 THB with and without human serum, and incubated at 37^{o}C. OD and CFU/mL were determined at 0, 2, 4, 6, 8 and 24 hours time points (Section 2.1.2.2).

2.1.1.2.6. M17 agar and M17 with heme/menaquinone supplementation

M17 agar medium (Oxoid, 10 g per 100 ml) was autoclaved and cooled to 50^{o}C. 400 µl of a 50% w/v glucose stock was added to the M17 agar medium to give a final concentration of 0.2%. Approximately 20 ml of medium was poured into each Petri dish.

M17 agar plates were supplemented with menaquinone and heme (to allow respiration metabolism, M17R medium) as described by Yamamoto et al. (2005). A 20 mM menaquinone stock (Vitamin K12, Sigma) was prepared containing 8.88 mg/ml menaquinone in 100% ethanol. The menaquinone stock was preserved at -20^{o}C.

Alkaline water (0.05M NaOH) was prepared containing 2 mg/ml NaOH. Heme stock (20 mM, Sigma) was prepared containing 13.0 mg/ml heme diluted in alkaline water. The heme stock was preserved at -20^{o}C and filter sterilized before use.

To prepare M17R agar plates with menaquinone and heme, M17 agar medium was autoclaved and cooled to 50^{o}C. 400 µl of 50% w/v glucose stock, 50 µl menaquinone stock (final concentration of 10 µM) and 50 µl of heme stock (final concentration of 10 µM) were added to the M17 agar medium. Approximately 20 ml of medium was poured into each Petri dish.

GBS strains A909 and 8181 were inoculated from THA plates using a sterile wire loop to a 5 ml THB pH 7.4 starter culture and shaken overnight at 37^{o}C. 100 µl of each GBS culture was serially diluted and 100 µl samples from the 10^{6} dilution transferred onto replicate agar plates (six M17 and six M17R). The inoculum was spread using sterile Q tips. All plates were wrapped in parafilm to stop them drying out. After incubating at 37^{o}C for 24 hours (3 plates, each medium) and one week (3 plates, each medium), the CFU/mL were counted.

2.1.1.3. Microbiology techniques

2.1.1.3.1. Bacterial stocks and storage

Bacterial strains were stored in a final concentration of 15% w/v glycerol. 500 µl 30% (w/v) glycerol was mixed with 500 µl bacterial broth culture and stored at -80^{o}C for long term use and -20^{o}C for short term use.

For short term storage, GBS strains were streaked to yield single colonies on THA plates. Prior to streaking, THA plates were dried by placing them open, face down at 65^{o}C for 10 minutes. One loop of GBS starter culture was streaked on the surface of the agar using a sterile wire loop. The plate was incubated face down in an incubator at 37^{o}C overnight. GBS strains were stored for up to 1 month on THA plates at 4^{o}C.

2.1.1.3.2. Gram staining of GBS

A clean microscope slide was prepared by quickly passing through a Bunsen flame. Bacteria from a single colony were mixed with a droplet of sterile 18.2 MΩ water using a sterile wire loop and spread over the central part of the slide to produce a thin film. Alternatively a small loopful of broth culture was applied to the slide. The film of bacteria was air-dried at room temperature and then passed through the Bunsen flame to fix it. The film was cooled before staining.

Firstly, the film was flooded with methyl violet for 30-40 seconds and rinsed with tap water. Secondly, the film was flooded with Gram's iodine for 30-40 seconds and rinsed with tap water. Then the film was decolourised by flooding with acetone for 2-3 seconds and rising with tap water immediately.

Finally, the film was counterstained with safranin for 15-20 seconds. After rinsing with tap water, the film was gently blotted dry with a paper towel.

Gram-stains were examined directly by microscopy using the oil immersion (×100) lens. Gram-positive bacteria were shown as deep purple and Gram-negative bacteria were shown as pale pink.

2.1.1.3.3. Lancefield typing of streptococci

A Prolex^{TM} streptococcal grouping latex kit (Pro-Lab Diagnostics) was used for Lancefield typing of streptococci. Following the manufacturer's instructions, one drop of Extraction Reagent 1, one loop of GBS culture (or a few colonies from a THA plate) and one drop of Extraction reagent 2 were added to an eppendoff tube serially and mixed together for 5-10 seconds. Five drops of extraction reagent 3 were added to the tube and the contents mixed together.

One drop of 'Blue latex suspension Group B' was dispensed onto a circle on the test card. One drop of extract from the GBS tube was pipetted onto the test card and mixed with the Blue latex. The card was gently rocked for one minute and the agglutination observed. GBS strains showed aggregation. In some cases, a negative control showing no aggregation was performing by testing the cell extracts with 'Blue latex suspension Group A' which only aggregates *S. pyogenes*.

GBS cultures were checked by Gram stain and Lancefield typing before every experiment to make sure the cultures were pure.

2.1.1.3.4. Phosphate buffered saline (PBS)

PBS solution was made as shown in Table 2.2, and the pH was adjusted to 7.4 and autoclaved. PBS stock was stored at room temperature. PBS was used to wash harvested cells and wash biofilms.

2.1.2. GBS growth.

2.1.2.1. GBS growth curves measured by microtiter plate reader

GBS strains were inoculated from THA agar plates using a sterile wire loop to a 5ml pH 7 THB starter culture and incubated at 37^{o}C shaking overnight. A 48-well tissue culture plate was used for this assay. 1 ml THB medium (pH 4.3, 5, 6 and 7) was added into quadriplicate wells. 25 µl GBS overnight starter culture was inoculated into 3 replicate wells (1:40 dilution) and one blank well (without inoculum) was left as a control. Growth curves were measured as increase in optical density (OD) at 570 nm using a BIO-TEC ultra microplate reader ELx808. Plates were incubated at 37^{o}C. Optical density was measured every 20 min. following automatic shaking of the microtitre plate. The experiment was repeated for three times for each strain tested and the data were analysed using Excel.

2.1.2.2. GBS growth quantification at pH 4 by measured by colony forming unit formation

200 µl GBS A909 overnight starter culture was inoculated into 20 ml (1:100

Table 2.2 Phosphate buffered saline

Components	1 Litre
NaCl	8 g
KCl	0.2 g
KH_2PO_4	0.2 g
$Na_2HPO_4.12H_2O$	1.44 g

dilution) THB media with different pH values (pH 4.3 and pH 7) and incubated at 37^{o}C. At different time points (t=0, 2, 4, 6, 24 and 48 hrs), the GBS culture was sampled by adding 100 µl culture into 900 µl THB. GBS was serially diluted and 100 µl of culture was spread onto THA plates using a sterile Q tip (Figure 2.1). After incubating at 37^{o}C for 24 hours, the colony forming units (CFU) were counted and CFU/mL in the original culture were calculated. The experiment was repeated for three times and a replicate with a different strain (GBS strain 8181) was carried out.

2.1.3. GBS Long term survival experiments

2.1.3.1. GBS long term survival experiments

200 µl GBS overnight starter cultures were inoculated into 20 ml (1:100 dilution) THB media with different initial pH values (pH 5 and pH 7) and incubated at 37^{o}C. GBS long term survival experiments were carried out by taking samples at 1 day, 2 day, 3 day, 1 week, 2 week, 3 week, 4 week and 8 week time points. 100 µL samples were serially diluted and cultured for CFU/mL determination (as in Section 2.1.2.2). In some experiments, 5 µL samples were also taken for Live-Dead staining (Section 2.1.4).

2.1.3.2. Measuring survival of multiple GBS strains in stationary phase using a spot plate method

200 µL each of overnight starter cultures of GBS strains A909, 8181, 8184,

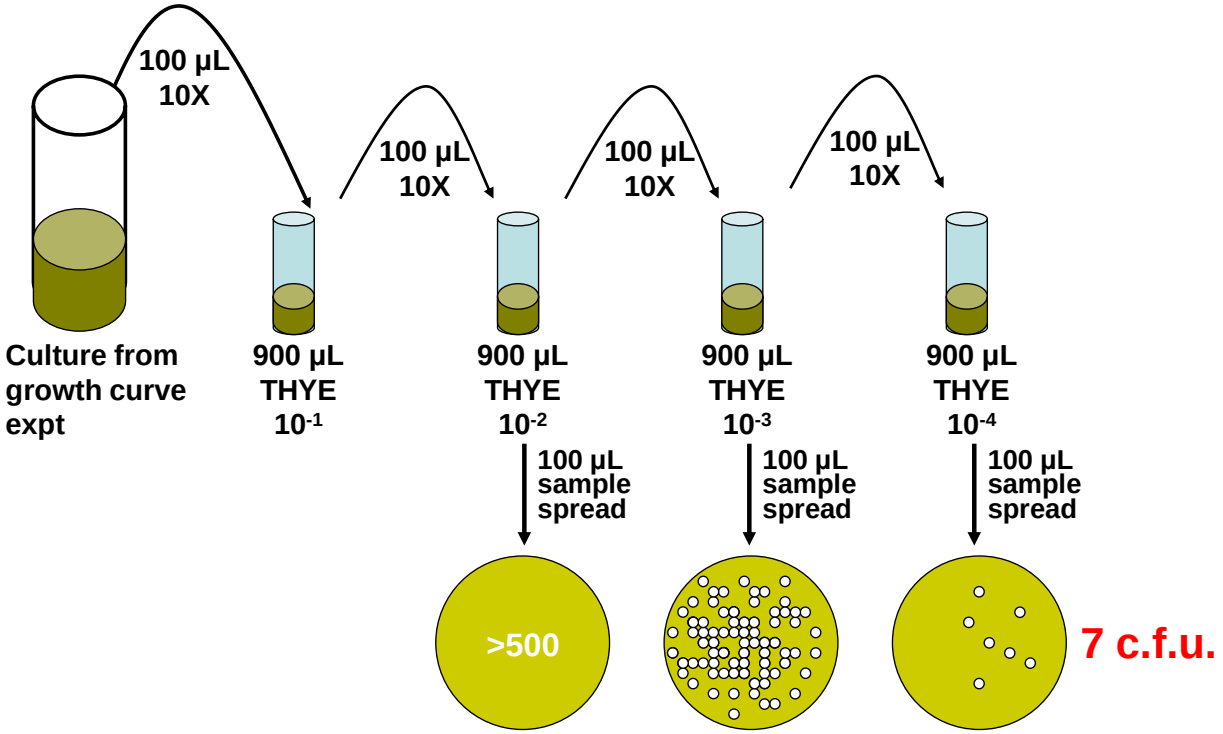


Figure 2.1 Sampling and serial dilution strategy for determining CFU/mL during GBS growth. Sample calculation: for a sample diluted for 4 10-fold dilutions with final colony count of 7 CFU per 100 µL is $7\times10\times10^4=7\times10^5$ CFU/mL.

9993 and CT1 were inoculated into 20 ml THB medium with different initial pH (4, 5 and 7) at 37^{o}C. 5 μL from each culture were spotted directly onto THA plates (Figure 2.2). Samples were taken at day 1, 2, 3, and week 1, 2, 3, 4 time points. Broth cultures were shaken to resuspend cells that had settled out under gravity before spotting onto the THA plates. Fresh (overnight) cultures of GBS strain A909 were spotted in duplicate onto each plate as positive controls (Figure 2.2). Sampling 5 μL directly onto plates gives a lower limit of detection of 200 CFU/mL (= 1 CFU/5 μL).

2.1.4. GBS Live-Dead staining

Live/Dead staining was performed with ethidium bromide/acridine orange solutions (kindly provided by Dr. Lisa Lee-Jones, University of Northumbria). ethidium bromide/acridine orange solution contained per ml:

Ethidium bromide 10mg/ml stock	100 μl
Acridine orange 10mg/ml stock	30 μl
Phosphate buffered saline	870 μl

Solutions were mixed well and diluted 1:100 in phosphate buffered saline to make a working solution containing final concentrations of 10 μg/ml ethidium bromide and 3 μg/ml acridine orange. Working solution was stored at -20^{o}C for extended periods and 4°C for up to 1 month.

GBS was broth cultured in 5 ml THB (pH 5 and pH 7) and incubated at 37^{o}C. Samples were taken at 6, 24, 48, 72 hrs and 1 week timepoints. 5 μl of

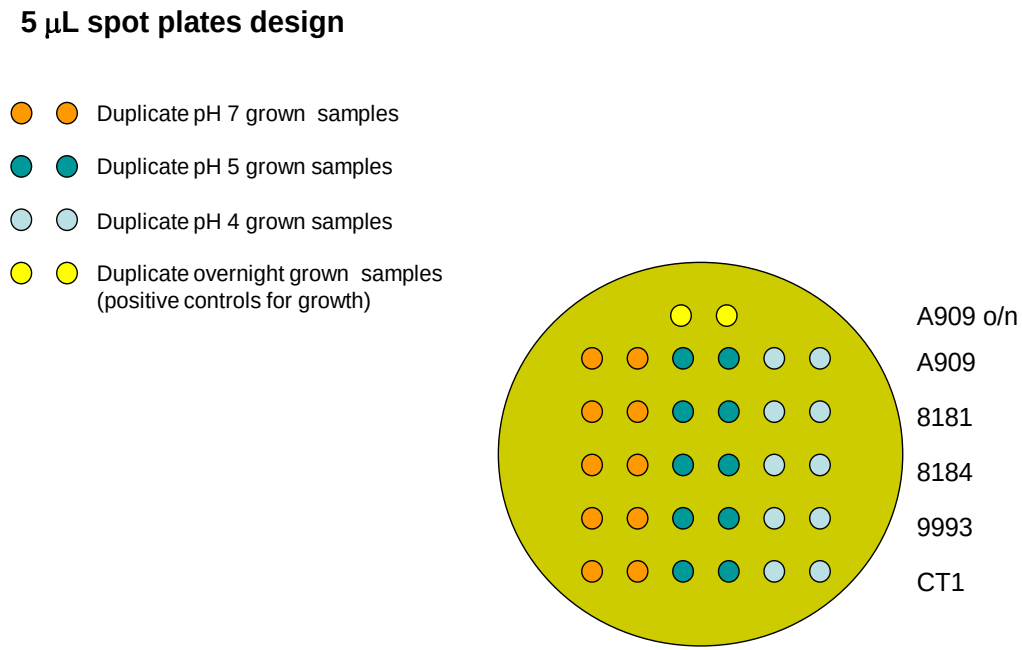


Figure 2.2 Long term survival measured by the spot plate method.

culture and 5 μl of working solution were mixed gently on a microscope slide and covered with a coverslip. The slide was sealed with clear nail varnish to stop the dye escaping.

Slides were immediately observed using a Simens Olympus BX40 microscope in fluorescence mode with a reading filter of 515 nm. The cells were observed under the microscope using visible light at 100X magnification. Keeping the visible light on, the microscope was switched to fluorescence mode to observe the cells. Live cells are observed to fluoresce green and dead cells fluoresce red. Live and dead cell numbers were counted for 400 cells in total.

2.1.5. Regrowth of extended stationary phase cells

GBS A909 was broth cultured in 20 ml of pH 5 and pH 7 THB and incubated at 37^{o}C to establish replicate stationary phase growths. 1 ml of each GBS A909 culture was inoculated into 20 ml of THB (pH 7) at 24 hrs, 1 week, 2 week and 4 week time points. OD and CFU/mL of regrown culture were measured at 0 hrs, 6 hrs and 24 hrs. OD was determined by taking 1 ml of sample into a cuvette and measurement at 600nm using a CE 2030 Spectrophotometer (CECIL). CFU/mL was determined essentially as described in Sections 2.1.2.2., with aerobic and anaerobic plate incubation conditions. Duplicate 100 µl samples serially diluted culture were spread onto pH 5 and pH 7 THA plates. One pH 5 and one pH 7 THA plate was incubated at 37^{o}C in a candle jar (with GasPak CO_2 generator strips; anaerobic condition) for 24 hrs. One pH 5 and

one pH 7 TH agar plate was incubated 37^{o}C in an incubator (aerobic condition) for 24 hrs. The experiments were repeated for 3 times. All samples were checked by Gram staining and Lancefield typing to confirm GBS purity.

2.1.6. Acid tolerance experiments

2.1.6.1. Auto acidification measurements

200 µl overnight starter culture of GBS A909 was inoculated into 20ml THB (pH 4.2, 5, 6, 7.4) and incubated at 37^{o}C. OD and medium pH were measured at 0 hrs, 6 hrs, 1 day and 7 days growth. To measure medium pH, cells were harvested by centrifugation at 4000 rpm (2320*g*) for 15 min at 4^{o}C. The supernatants were removed and autoclaved. The pH of the supernatants was measured using a pH meter. The experiments were repeated 3 times.

200 µl overnight starter culture of GBS strains (A909, 8181, 8184, 9993 and CT1) was inoculated into 20ml THB (pH 4.0, 5 and 7.4) and incubated at 37^{o}C. After incubating for 4 weeks, cells were harvested by centrifugation at 4000 rpm (2320*g*) for 15 min at 4^{o}C. The supernatants were removed and autoclaved. The pH of the supernatants was measured using a pH meter.

2.1.6.2. Adaptation experiments - strategy for exposure to acid

200 µL overnight THB (pH 7.4) starter culture of GBS A909 was inoculated into 20 ml pH 7 THB and incubated at 37^{o}C. After incubation for 3 hrs, 200µL of the pH 7 culture was 1:100 diluted into 20ml pH 6 THB and incubated at 37^{o}C

overnight. 200μL of the pH 6 culture was then 1:100 inoculated into 20ml pH 5 broth and pH 6 broth. CFU/mL were measured at 6 hrs (to give values set to 100%), 24 hrs, 48 hrs, 72 hrs, 1 week and two week time points.

2.1.7. Biofilm experiments

2.1.7.1. Survival of biofilm grown GBS

GBS A909 biofilm cultures were established by adding 2 ml pH 5 THB into each well of columns 1-3 of a 24 well plate and adding 2 ml of pH 7 THB into each well of columns 4-6. After checking the purity, 50 µL of a GBS strain A909 starter culture was inoculated into each well. The plate was put into a Tupperware box with wet tissue paper (soaked with sterile distilled water, to prevent drying out) and incubated at 37°C.

After incubation for 24 hours, the medium in each well of row 1 was removed carefully without disturbing the cells that had settled into a biofilm. The surface was gently washed with 1 ml of PBS twice. Each well in row 1 was processed as follows: 1 ml of pH 7 THB was added to the wells in columns 1 and 4. Bacteria were resuspended by agitation, repeatedly being drawn up and expelled from an automatic pipette. CFU/mL counts for the resuspended bacteria were determined by serial dilution and spreading onto THA plates. For wells in columns 2 and 5, 1 ml of PBS was added into each and the cells were resuspended as above. Bacteria resuspended in PBS were Live-Dead stained (see Section 2.1.4). Biofilm formation by GBS in columns 3 and 6 were assayed by crystal violet staining (see Section 2.1.7.2 below).

After sampling, the plate was returned to the incubator at 37°C. The CFU/mL counting, Live-Dead staining and Crystal violet staining assays were repeated at 4 day, 7 day and 14 day timepoints for the GBS A909 cultures in rows 2, 3 and 4 (Figure 2.3).

2.1.7.2. Crystal violet staining assay for biofilm formation

The crystal violet staining method was adapted from Marinetti et al. (2007). The medium above the biofilm grown GBS was gently removed by pipette and the cells PBS washed as described in Section 2.1.7.1. 1 ml 0.2% w/v crystal violet was added to each well. After staining for 10 minutes at room temperature, the crystal violet was removed from each well and cells washed with 1 ml distilled water 3 times. 1 ml of 1% sodium dodecyl sulphate (SDS) was added and left for 10 min to extract bound crystal violet. The extracted crystal violet was removed and the absorbance read at 540 nm.

2.1.7.3. Assay for improving biofilm formation by coating plates with extracellular matrix molecules (hyaluronic acid and heparin)

Assays to improve biofilm formation were carried out using 48 well tissue culture plates. To prepare the plates, 50 µL of sterile water was added to each well of the first row. 50 µL heparin stock (1 mg/mL in PBS; Sigma) was added to each well of the second row. 50 µL hyaluronic acid stock (1 mg/mL in PBS; Sigma) was added to each well of the third row. 50 µL heparin stock and 50 µL hyaluronic acid stock were added to each well of the fourth row. The plate was left at room temperature for 1 hour to allow binding of the polymers to the

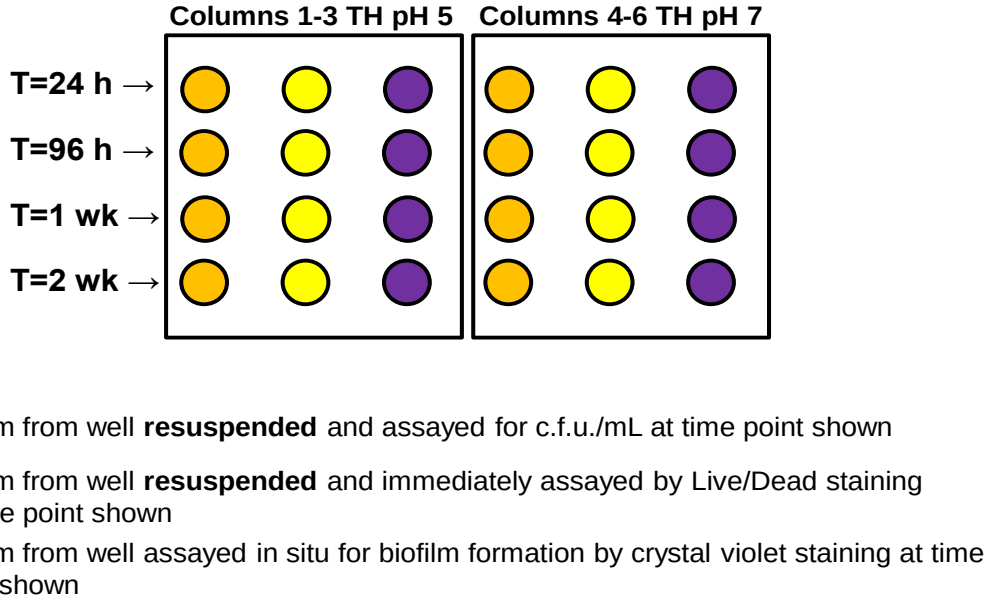


Figure 2.3. Long term survival of biofilm grown GBS assessed using three different assays.

bottom of the wells before careful removal of the excess liquid and air drying for 30 mins.

After coating, 2 mL of THB pH 7 was added to each well and inoculated with 100 µL overnight starter culture of either GBS strain A909 (columns 1-3) or NEM316 (columns 4-6). The microtitre plate was incubated at 37°C for 24 hrs. The supernatant medium was then removed and the biofilm cells washed with 1 ml sterile water. 1 ml THB was then added and the cells in each well resuspended for colony counting.

2.1.7.4. Alternative models for biofilm formation

An overnight starter culture of GBS A909 was 1:25 inoculated into four different pH 5 THB cultures (A, B, C and D), each containing 25 mL medium. All culture samples were incubated at 37^{o}C and CFU/mL were measured at 24 hrs and 1 week timepoints.

Culture A (planktonic culture, control) was set up in 50 ml Universal tube and incubated as a standing culture. CFU/mL were measured at 24 hrs and 1 week timepoints. Culture B (early biofilm formation) was set up in a 250 mL CELLSTAR® tissue culture flat flask and incubated for 24 hrs. The supernatant medium was discarded and the biofilm surface was gently washed with 10ml sterile PBS. Biofilm cells were resuspended with 25ml sterile PBS and CFU/mL were measured. Culture C (an established biofilm, fed with THB) was set up in a tissue culture flat flask. Cells were fed on days 2, 4 and 6 by replacing spent medium with 25ml fresh 1/10th strength THB. After 1 week, the biofilm surface

was gently washed with 10ml sterile PBS. The biofilm cells were resuspended with 25ml sterile PBS and CFU/mL were measured.

Culture D (an established human serum fed biofilm) was set up in a tissue culture flat flask and incubated. Cells were fed with 25ml 1/10th strength human serum medium at days 2, 4 and 6. After 1 week, the biofilm surface was gently washed with 10ml sterile PBS. The biofilm cells were resuspended with 25ml sterile PBS and CFU/mL were measured.

2.1.8 Statistic methods:

GBS growth experiments and proteomic experiments were analyzed statistically by using the SPSS Independent-sample t-test. Experimental data were set as groups and entered into SPSS16.0 data set. Data were analyzed by Compare Mean- Independent-Sample T Test. There was considered to be a statistically significant difference in the mean scores for the two groups where the p value was < 0.05.

2.2. Methods of proteomics

The strategy for the proteomics analysis is summarised in Figure 2.4.

2.2.1. GBS A909 biomass culture

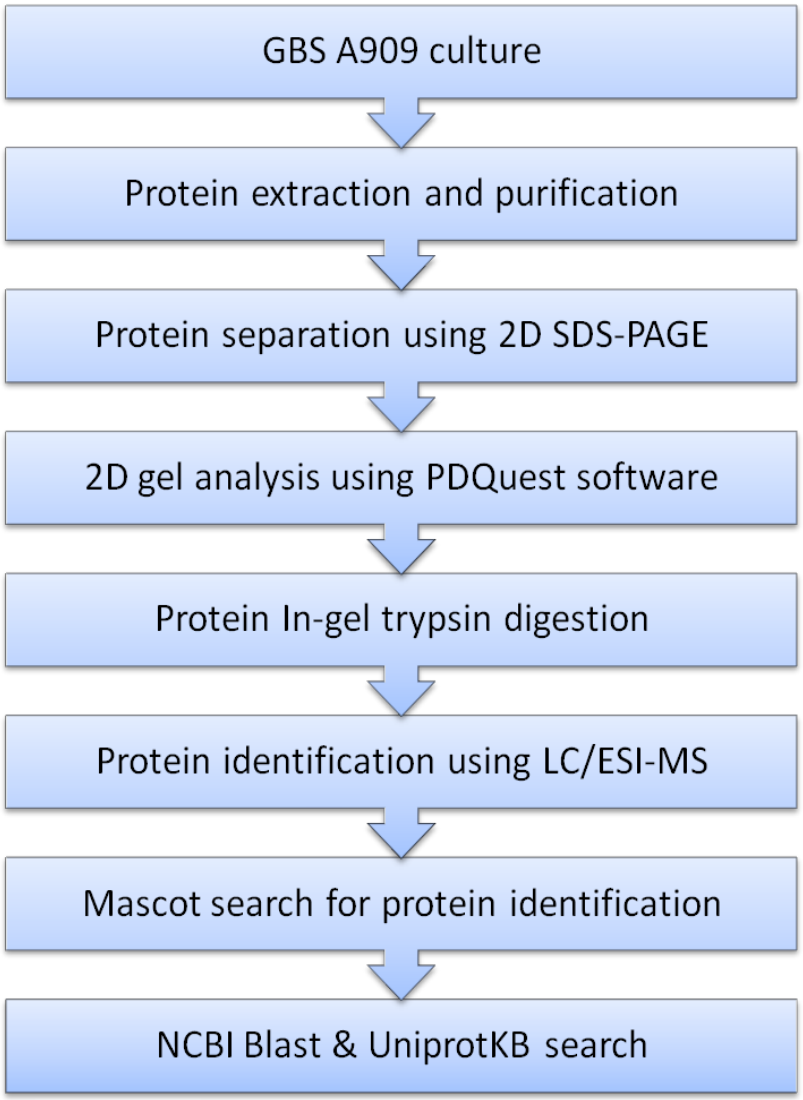


Figure 2.4. General steps of proteomics.

2.2.1.1. Biomass for proteomic investigation GBS grown under conditions associated with neonatal exposure

Acidic micro-aerobic conditions were induced by culturing GBS A909 in 100 ml medi-flat flasks with 100 ml pH 5 THB. Flasks were incubated at 37^{o}C as standing cultures. Cells were harvested after culturing for 6 hours. In this time the cells reached early stationary phase with OD_{600} of about 0.6. Subsequent neutral aerobic conditions were achieved by diluting a sample of the standing culture 1:100 into a 500 ml conical flask containing 100 ml pH 7.2 THB. Flasks were incubated at 37^{o}C with shaking at 100 rpm to provide aeration. Cells were harvested after culturing for 3 hours. In this time the cells reached mid-exponential phase with OD_{600} about 0.6. The strategy for growing biomass for 2D electrophoresis analysis is summarised in Figure 2.5. Cultures from conditions P1 and P2 were harvested by centrifugation at 4000 rpm for 30 mins at 4^{o}C. The cell pellets were washed with 10 ml ice cold PBS and centrifuged again at 4000 rpm for 30 mins at 4^{o}C. This PBS wash was carried out 3 times in total. The washed cell pellets were preserved at -20^{o}C for future use. 50 ml of original culture was used as one sample for proteomics.

2.2.1.2. Biomass for proteomic investigation of GBS exposure to human serum

Square polystyrene plates (Greiner, 12×12 = 144cm^{2}) were sterilised with ultraviolet radiation overnight before use. 100 ml THB agar with 10% human serum plates (TH-HS) were made by adding 3.64 g THB and 2 g agar to 80 ml

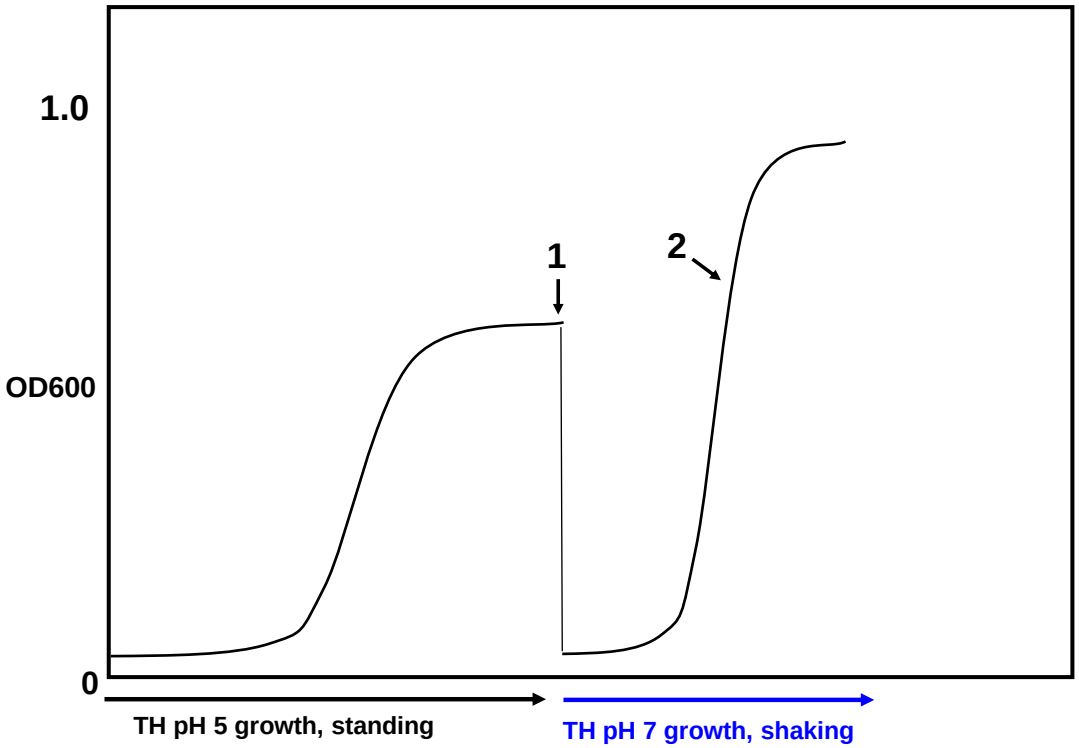


Figure 2.5. Comparing early stationary phase, pH 5 microaerophilic grown cells with mid-log pH 7 aerobic grown cells. Cells for proteomic analysis were harvested at time points indicated by 1 and 2.

18.2 MΩ H_2O and the pH adjusted to 6. The medium was adjusted to a final volume of 90 ml and autoclaved. When the medium cooled down to 55^{o}C, 10 ml of human serum was added, mixed well and the medium poured into the square plates.

1 ml GBS A909 overnight starter culture was spread onto THA and THA-HS square plates. The plates were incubated at 37^{o}C for 48 hours. After 48 hours culture, one plug of culture was removed into a microcentrifuge tube and the CFU/mL determined (section 2.1.2.2). Cultures from the surface of the square plates were harvested and washed three times with 10 ml ice cold PBS and spun down (section 2.2.1.1). The cell pellets were preserved at -20^{o}C for future use. 25 ml of resuspended culture (25% of bacteria from the surface of 1 plate) was used as one sample for proteomics.

2.2.2. Protein extraction and purification

2.2.2.1. Protein extraction

Lysis solution was made of 4.8 g urea and 0.4 g CHAPS in 10 ml distilled water. Lysis solution was divided into small volumes (e.g. 500 µl) and preserved at -20 ^{o}C.

100 µl lysis solution with 2 µl IPG buffer (Amersham Bioscience, UK, added into lysis solution immediately prior to use) was added to the cell pellet (one sample for proteomics) and the cells resuspended. The sample solution was transferred into a 1.5 ml microcentrifuge tube and sonicated. Each sample was sonicated at an amplitude of 14 microns for a total of 30 seconds, with 10

second intervals between each 10 seconds of sonication. The microcentrifuge tube was left on ice throughout the whole sonication process to avoid protein degradation. After sonication, the sample was spun down at 12,000*xg* at 4^{o}C for 45 minutes. Then the ca. 100 µl supernatant, containing the extracted proteins, was transferred to a new microcentrifuge tube and the pellet was discarded.

2.2.2.2. Protein purification.

2.2.2.2.1. Protein purification using a 2D clean up kit

The supernatant was processed with a 2D clean up kit (Amersham UK). 300 µl precipitant was added to the protein sample and mixed by vortexing. After incubation on ice for 15 minutes, 300 µl of co-precipitant was added to the mixture and mixed by vortexing briefly. The sample was centrifuged at 12,000*xg* for 5 min at 4^{o}C. The supernatant was carefully removed by micropipette so as not to disturb the protein pellet. 40 µl of co-precipitant was added on top of the pellet and incubated on ice for 5 minutes. The sample was centrifuged at 12,000*xg* for 5 min at 4^{o}C and the supernatant was carefully removed and discarded. The protein pellet was dispersed by adding 25 µl distilled water and vortexing for 5-10 seconds. 1 ml pre-chilled (-20 ^{o}C for at least 1 hour) wash buffer and 5 µl wash additive were added. The mixture was incubated on ice for 30 minutes and vortexed for 20-30 seconds every 10 minutes. The sample was centrifuged at 12,000*xg* for 5 min at 4^{o}C and the supernatant was carefully removed and discarded. The protein pellet was

briefly air dried and resuspended with rehydration solution.

2.2.2.2.2. Protein purification using acetone precipitation methods

To precipitate proteins with acetone, four sample volumes of pre-chilled (-20^{o}C) acetone were added to the protein suspension. The sample was mixed by vortexing and incubated at -20^{o}C for more than an hour. After centrifugation at 13,000xg for 10 minutes, the supernatant was decanted. The protein pellet was washed with 4 sample volumes of pre-chilled 80% acetone and centrifuged at 13,000xg for 10 minutes. After removing the supernatant, the pellet was briefly air dried (about 2 minutes) at room temperature and resuspended with rehydration solution.

2.2.3. Protein separation using 2D SDS-PAGE

2.2.3.1. Loading proteins on Immobiline DryStrip gels

Protein samples were dissolved in 350 µl rehydration solution (lysis solution supplemented with 4 mg/ml dithiothreitol (DTT), 7 µl IPG buffer (pH 4-7) and 3.5 µl 1% bromophenol blue solution). The sample was sonicated and centrifuged briefly to remove undissolved protein.

Before loading a protein sample onto the gel strip, the Immobiline DryStrip reswelling tray was levelled by adjusting the bubble in the centre of the spirit level. The full 350 µl protein sample was pipetted into one channel of the reswelling tray. The Immobiline Drystrip (pH 4-7, 18 cm; GE Heathcare) was

taken from its protective cover and placed onto the protein sample in the channel with gel side down and the anodic (+) end toward the tray end. The Immobiline DryStrip gel was moved gently to remove any bubbles between the gel strip and the reswelling tray. 4ml of DryStrip Cover Fluid (GE Healthcare) was pipetted on each of gel strips to prevent evaporation and urea crystallization. The DryStrip gel was rehydrated at room temperature overnight.

2.2.3.2. Isoelectric focusing (IEF)

IEF (i.e. the first dimension) was carried out by using a Multiphor II electrophoresis system (Amersham Bioscience) and the Immobiline DryStrip Kit. Before putting the immobiline DryStrip tray onto the cooling plate, 10 ml of DryStrip Cover Fluid was poured on top of the cooling plate. Larger air bubbles were removed to ensure a good thermal contact between the cooling plate and the tray. The anode and cathode electrode leads on the tray were connected to the Multiphor II unit. After pouring 10 ml of DryStrip Cover Fluid onto the Immobiline DryStrip tray, the immobiline DryStrip aligner was placed on the surface of DryStrip tray with the groove side up.

The rehydrated DryStrip gel was removed from the reswelling tray to the channel of DryStrip tray with the gel side up. The gel was placed with the anodic (+) ends toward the anodic side of Multiphor II unit. Two 11 mm electrode contact strips were cut, soaked with 0.5 ml distilled water and placed across the anodic and cathode end of the DryStrip gels. The electrodes were then placed on the top of electrode contact strips and pressed. 20 ml of

DryStrip Cover Fluid were poured onto the DryStrip gels. The lid was placed on the unit and the power supply was connected to the unit.

The IEF cooling system (Techne Circulator C-100 with TECAM heat exchanger 1000) was set up at 20^{o}C. The IEF was programmed according to Table 2.3. After running the IEF dimension, the Immobiline DryStrip gels were moved into a petri dish and preserved at -80^{o}C prior to the second dimension electrophoresis.

2.2.3.3. Equilibration

Equilibration buffer (1.5 M Tris-HCl, pH 8.8) contained Tris base 181.7 g per litre. The pH of the equilibration buffer was adjusted to 8.8 with HCl. The buffer was autoclaved and stored at room temperature. Equilibration buffer was used to prepare SDS equilibration buffer stock (Table 2.4), which stock was stored at -20 ^{o}C. Prior to the second dimension, the Immobiline Drystrip gels were equilibrated at room temperature. Each DryStrip gel was soaked in 10 ml SDS equilibration buffer stock with 100 mg DTT (1% w/v) added and shaken at room temperature for 15 minutes. The solution was discarded and the gel was further soaked in 10 ml of SDS equilibration buffer stock with 450 mg iodoacetamide (IAA, 4.5% w/v) added for 15 minutes. Then the Immobiline DryStrip gel was soaked with 18.2 MΩ H_2O for 10 minutes.

2.2.3.4. Resolving gel and stacking gel preparation

SDS resolving gels and stacking gels were prepared according to the recipes in Table 2.5 and Table 2.6. 10% SDS stock solution was made by adding 10 g SDS in 100 ml 18.2 MΩ H_2O. The solution was stored at room temperature. 10% w/v ammonium persulphate (APS; 100 mg/mL) was prepared either fresh in 18.2 MQ/cm H_2O or from a freshly defrosted aliquot stored at -20°C.

2.2.3.5. Casting 2D gels

Two clean glass plates (one of 20×20 cm and another 20×22 cm) were separated by 1.5 mm spacers and assembled by clamps on both sides. The glass plate sandwich was set standing on the rubber gasket of a plate holder. 18.2 MQ H_2O was poured between the two plates and left for 20 minutes to check for leakage, then decanted and the space dried with filter paper.

The ingredients of the resolving gel solution (Table 2.5) were mixed gently and pipetted into the space between the plates. The solution was pipetted to about 2 cm below the top of the short plate and with care taken to avoid the formation of air bubbles. Then 18.2 MΩ H_2O was added to the top of resolving gel to produce a straight top edge. The resolving gel was left for 50 minutes to polymerise. After the gel had polymerised, the 18.2 MΩ H_2O was removed from the top of gel with clean absorbent paper. A 1.5 mm thick comb with a large DryStrip gel well and a small marker well was inserted between two the plates at the top. The ingredients of the stacking gel solution (Table 2.6) were mixed and pipetted into the space between the comb and the resolving gel. The

Table 2.3. 2D SDS-PAGE IEF conditions.

Step	Voltage	mA	W	Time (h)
1	500	2	5	0.1
2	500-3500	2	5	1.5
3	3500	2	5	7

Table 2.4. SDS equilibration buffer solution stock.

Contents	200 ml includes
Equilibration buffer	10 ml
Urea	72 g
Glycerol	69 ml
SDS	2 g
Water	Added up to final volume of 200 ml

Table 2.5. 14% (w/v) acrylamide resolving gel recipe.

18.2 MΩ H_2O	58 ml
40% acrylamide	52.2 ml
1.5 M Tris-HCl, pH 8.8	37.5 ml
10% SDS stock	1.5 ml
10% APS	750 µl
TEMED	75 µl

Table 2.6. 4% (w/v)acrylamide stacking gel recipe.

18.2 MΩ H_2O	18 ml
0.5 M Tris-HCl, pH 6.8	7.5 ml
40% acrylamide	2.95 ml
10% SDS stock	300 µl
10% APS	150 µl
TEMED	30 µl

stacking gel was left for 40 minutes to polymerise. The comb was removed when the stacking gel was set.

2.2.3.6. Running the second electrophoresis dimension

One litre of 10X SDS-PAGE running buffer stock contained Tris base 30 g, glycine 144 g and SDS 10 g. The 10X running buffer stock was diluted 1:10 before use.

10 ml of 1% bromophenol blue contained 0.1 g bromophenol blue. The bromophenol blue stock was stored in a sterile universal at room temperature.

20 ml agarose sealing solution was made of 19 ml 1X running buffer supplemented with 0.1 g agarose and 40 µl of 1% (v/v) bromophenol blue in a 200 ml beaker. The agarose sealing solution was heated until the agarose dissolved and pipetted into the large well to seal the DryStrip gel. Prior to adding the agarose sealing solution to the large well, the equilibrated Immobiline DryStrip gel was placed in the large well of the stacking gel with the pH 4 side close to the marker well.

Two gel sandwiches were assembled on the sides of the cooling core. 200 ml 1X running buffer was poured into the space between the sandwich plates and the cooling core and left for 10 minutes to make sure there was no leakage. 7 µl protein standards (Biorad, All Blue) was loaded in the small marker well of each gel. The cooling core with the sandwich glass plates were carefully moved into the buffer tank. 2 litre of 1X running buffer was gently poured into the tank, avoiding the production of air bubbles.

The Techne Circulator C-100 and TECAM heat exchanger 1000 cooling system was set up at 10^{o}C. The tank was covered with its lid and connected to the power supply, which was switched on and the gels run at 80 mA (100-400 V) for 4-5 hrs. The second dimension electrophoresis was stopped when the bromophenol blue dye was observed to be running out from the bottom of the gels. The gels were removed from the glass plates gently and each transferred into a gel container. Each gel was fixed by adding 200 ml of fixing solution (Table 2.7) and shaking for more than one hour.

2.2.3.7. Staining gels with Colloidal Coomassie blue

One litre Colloidal Coomassie blue stock was made by dissolving 100 g ammonium sulphate in 20 ml phosphoric acid and 200 ml 18.2 MQ H_2O and dissolving 1 g coomassie blue G 250 in 100 ml 18.2 MQ H_2O. These two solutions were mixed together and 18.2 MQ H_2O was added to give a final volume of 1 litre. Colloidal Coomassie blue stock was made at least 24 hours prior to use and stored at room temperature.

Coomassie blue staining solution was made by mixing four parts Coomassie blue stock with one part methanol. Electrophoresis gels were stained in 200 ml of Coomassie blue staining solution with shaking overnight. The stained proteins were revealed by washing the gel with 18.2 MQ H_2O to destain the gel background.

Table 2.7 Fixing solution.

Total volume	1 Litre
Methanol	500 ml
Glacial acetic acid	120 ml

2.2.3.8. 2D gel documentation.

Gels were digitally photographed using a Bio-Rad GB-710 calibrated imaging densitometer. All gel images were acquired with the same size specifications: each gel image was scanned starting at the top left with 17cm length and 19cm width such that all visible spots were present in the gel images.

2.2.4. Analysis 2D gel using PDQuest software

Gels were analysed using PDQuest V8.0 advanced software. A new experiment folder was set up and 6 pairs of original gel images were surveyed. The 12 gels were auto assigned into 2 groups based on the replicate images. The faint, small and the largest spot cluster parameters were selected. The Spot Detection Wizard automatically processed the selected spot detection parameters and produced spot centres according to these parameters. Streaks in the gel images were removed by selecting the streak removal checkbox under 'Optional Controls'. The original gel images were filtered and smoothed to clarify the spots, and then three-dimensional Gaussian spots were created during the spots detected step. The final result was three separated images: the original unaltered image, the filtered and processed image and a synthetic Gaussian image. All spot matching and analysis in PDQuest were performed on the Gaussian images. Any spots that were not detected in the image could be added to the Gaussian image using the 'Edit spots' toolbar. Any incorrectly positioned or misidentified spots were removed from the images using the 'Remove Spot' toolbar from the Spot menu. Protein spots from the different gels

were matched to each other and synthesized into a MatchSet image. Each spot was matched using the 'Spot match' toolbar and unique spots were added to the gel. Those spot images expressed more strongly in one condition of 6 gels than in another condition of 6 gels were selected as spots of interest.

2.2.5. Protein in-gel trypsin digestion

2.2.5.1. Stock solutions

100 mM ammonium bicarbonate (NH_4HCO_3) contained NH_4HCO_3 79 mg in 10 ml. 50 mM ammonium bicarbonate was made by 1:2 dilution of 100 mM NH_4HCO_3 in 18.2 MQ H_2O. 10 ml of 50% (v/v) acetonitrile (ACN) was made by 1:2 dilution of ACN in 18.2 MQ H_2O.

10 ml of 50% (v/v) ACN/5% (v/v) formic acid (FA) was made by combining 5 ml of ACN and 500 µl of FA, with the addition of 18.2 MQ H_2O to give a final volume of 10 ml. 10 ml of 83% (v/v) ACN/0.2% (v/v) FA was made by combining 8.3 ml of ACN and 20 µl of FA, with the addition of 18.2 MQ H_2O to give a final volume of 10 ml.

0.1 µg/µl Trypsin solution was made by dissolving modified trypsin (New England BioLabs) in 1 ml of 100 mM ammonium bicarbonate and standing on ice.

2.2.5.2. Protein in-gel trypsin digestion

Protein spots were manually cut into 1 mm×1 mm gel slices and transferred

into 1 ml clean silicanised microcentrifuge tubes. 100 µl of 100 mM NH_4HCO_3 and 60 µl of 50% (v/v) ACN were added to each tube and shaken for 30 minutes to wash off the Colloidal Coomassie blue stain. The wash liquid was discarded and the wash was repeated for three times. The gel slices were dehydrated by adding 60 µl of ACN and incubated at room temperature for 5 minutes. The liquid was discarded and the dehydration was repeated for twice. The gel slices were then dried in a centrifugal evaporator for 15 minutes. 25 µl of 0.1 µg/ µl trypsin solution was added to each tube of gel slices and incubated on ice for 30 minutes. Then 30 µl of 100 mM NH_4HCO_3 was added into each tube and incubated at 25^{o}C overnight. The digested proteins were collected by adding 30 µl of 50% (v/v) ACN with 5% (v/v) FA and shaking at room temperature for 30 minutes. The peptides solution was transferred into a 1.5 ml microcentrifuge tube. Further digested peptides were collected by adding 30 µl of 83% (v/v) ACN with 0.2% (v/v) FA into the gel slice tube and shaking for 30 minutes. The extracted peptides were pooled with those intially extracted. The digested peptides solutions were either freeze dried directly or preserved at -80^{o}C for future use.

2.2.5.3. Freeze drying protein samples

Protein freeze drying was carried out by using an ALPHA 1-2 LD Freeze dryer (Chris). Each microcentrifuge tube containing frozen digested peptides was pierced with 3 holes into the lid with a needle. The microcentrifuge tubes were sat on a rack and placed in the ALPHA 1-2 LD Freezer dryer. After covering with the lid, the pump was switched on. The freeze dryer steps included a

pump warm up for 20 minutes and main drying, followed by a final drying at -54^{o}C overnight until crystal formation was observed.

2.2.6. Protein identification using liquid chromatography/electrospray ionization- mass spectrometer (LC/ESI-MS)

Buffer A contained 50 ml ACN and 1 ml FA per litre. Buffer B contained 950 ml ACN and 1 ml FA per litre.

Protein samples were analysed by liquid chromatography combined with electrospray ionization mass spectrometry (LC/ESI-MS). LC was performed using an Ultimate^{TM} 158 3000 system (Dionex, UK) comprising a WPS-3000 well-plate micro autosampler, a FLM-3200 flow manager and column compartment, and an LPG-3600 dual-gradient micropump. Protein samples were dissolved in 7 µl of buffer A and loaded into a well of the well plate. The peptides were separated on a monolithic column (200 mm i.d., 5 cm, LCPackings). The column was heated to 60°C in order to reduce backpressure and run at 3 ml/min. The initial solvent was Buffer A (5% v/v ACN and 0.1% v/v FA). Peptides were eluted by the application of a 22 min. linear gradient (0%-50%) of Buffer B (95% v/v ACN and 0.1% v/v FA). The LC system was directly coupled to an HCTUltra mass spectrometer (Bruker Daltonics) via a microspray source. Instruments were controlled using HyStar ^{TM} 3.2 (Bruker Daltonics) software and the experimental conditions for the analysis are summarized in Table 2.8.

Table 2.8 ESI-MS working parameters.

MS parameter	Range
Micro pump pressure	0-350 bar
Capillary voltage	4000 V
Current on capillary	30-40 nA
Full scan range	350-1800 m/z
MS/MS scan range	50-3000 m/z
Max. Ion trap accumulate time	200.00 ms
Average scans	4
Number of precursor ions	3
Nebulizer of ionization source	10.0 psi
Dry gas of ionization source	5.0 L/min
Dry temperature of ionization source	300 ºC

2.2.7 Mascot search for protein identification

Raw chromatography data were processed and Mascot compatible files created using DataAnalysis™ V 3.2 software (Bruker Daltonics) with the following parameters: compounds (autoMS) threshold 1000, number of compounds 250. Searches were performed using the online Mascot MS/MS ions search function (http://www.matrixscience.com/cgi/search_form.pl?FORMVER=2&SEARCH=MIS) and the NCBI non-redundant (nr) database (http://www.ncbi.nlm.nih.gov/). The searches were carried out with a peptide mass tolerance of ±1Da; fragment ion mass tolerance of ±0.5Da; peptide charge of 2+ and 3+; semitryptic specificity allowing for up to two missed cleavages; fixed modification with carboxymethyl of cysteine, and variable modification with oxidation of methionine. The search taxonomy was set as 'Firmicutes' and enzyme was specified as 'trypsin'. To ensure the highest quality of identification, the following criteria were applied: 1) the protein hits (positive peptides) were from Group B *Streptococcus*; 2) only peptides for which the MS/MS spectra exceeded Mascot's significance level (mascot score >40) were included; 3) the query matched peptides ≥2.

2.2.8 NCBI Blast & UniprotKB search

Protein sequences for bioinformatic analyses were retrieved from the UniProtKB database (http://www.uniprot.org/; Uniprot Consortium, 2009) and homology searches performed using BLAST (Altschul *et al*., 1997) accessed via the NCBI server (http://www.ncbi.nlm.nih.gov/BLAST/). Unfiltered BlastP

searches were performed with an E cut-off of 0.001. Protein domain analysis was performed using both the UniProtKB entry and the Conserved Domain Detection facility of BlastP. Genomic context was explored via the BioCyc annotation (Caspi et al., 2008) accessed via the UniProtKB entries for each protein.

2.3. Methods for one dimensional SDS-PAGE and Western blotting

2.3.1. Preparation of protein samples for one dimensional SDS-PAGE

GBS A909 cells from 50 ml THA with and without 10% human serum were harvested by centrifugation and washed with PBS. Protein extraction and purification was performed as described in Section 2.2.2. 40 µl of resuspended protein pellet was dissolved in 10 µl 1X SDS-PAGE loading buffer (Table 2.9).

SDS-PAGE 5X loading buffer was prepared according Table 2.10. 10 ml of 2M Tris-HCl pH 6.8 contained 2.42 g Tris base. The pH of the Tris base solution was adjusted to 6.8 using HCl. A total volume of 13.2 ml of 50% glycerol contained 6.6 g glycerol in water. The SDS-PAGE 5X loading buffer was stored in a foil wrapped universal or in a brown glass bottle at 4^{o}C.

12% 1D SDS-PAGE resolving gels were prepared as described in Table 2.10. 100 ml 1 M Tris-HCl pH 6.8 contained 12.1 g Tris base and the pH was adjusted to 6.8 using HCl. 5% 1D SDS-PAGE stacking gels were prepared as

described in Table 2.11. 100 ml 2 M Tris-HCl pH 8.8 contained 24.2 g Tris base and the pH was adjusted to 8.8 using HCl.

2.3.2. One dimensional SDS-PAGE

1D SDS-PAGE was performed with a Biorad MiniProtean 3 system. A short glass plate was placed on top of the spacer plate and assembled using the manufacturers casting frame. The gel cassette assembly was placed and fixed on the casting stand gasket with the short plate facing the front.

The resolving gel components (Table 2.10) were mixed gently and pipetted into the space between the plates, until the solution was 1.5 cm below the top of the small plate. 18.2 MQ H_2O was added above the resolving gel to produce a straight top edge. The gel was left for a minimum of 20 minutes to polymerise and then the H_2O layer was removed. The components of the stacking gel (Table 2.11) were mixed gently and pipetted above the resolving gel. A comb was inserted into the space between the plates. After the stacking gel had polymerised, the comb was removed. The gel cassette sandwich was then removed from the casting frame and placed into the electrode assembly chamber with the short plate facing inwards. The assembled gel cassette sandwiches and electrode chamber were put into the mini-Protean tank. 5 µl of protein marker and 10 µl protein samples were loaded in the wells of the stacking gel. SDS-PAGE 10X running buffer (Section 2.2.3.4.3) was diluted (1:10) and added into the tank. The lid was placed on the top of tank and connected with the power supply. SDS-PAGE gels were run using a Bio-Rad

Table 2.9 SDS-PAGE 5X loading buffer.

Total volume	10 ml
2M Tris-HCl pH 6.8	0.3 ml
50% glycerol	5 ml
10% SDS	2 ml
18.2 MΩ H_2O	2.7 ml
2-mercaptoethanol	0.5 ml (this reagent was added working in the fume hood)

Table 2.10 12% resolving gel for 1D SDS-PAGE.

Resolving gel	
Acrylamide (40%)	3.0 ml
18.2 MΩ H_2O	4.5 ml
2M Tris resolving gel buffer pH 6.8	2.5 ml
APS (10%)	50 µl
TEMED	10 µl

mini-Protean 3 system at 200V for 45 minutes.

2.3.3. Staining and photographing gels

After electrophoresis, one gel was stained with Coomassie Brilliant blue (Table 2.12) as a control. The gel was removed from the cassette and stained with 20 ml Coomassie Brilliant blue staining solution for 20 minutes. Then gels were destained with destain solution (Table 2.13) overnight. Gel images were scanned using a Bio-Rad GS-800 calibrated densitometer and photographed using Quantity One software (Bio-Rad).

2.3.4. Western blotting

PBS-Tween was made from PBS (Table 2.2) supplemented with 0.05 g of polyoxyethylenesorbitan (Tween 80) per litre. PBS-Tween-skimmed milk was made by dissolving 5 g skimmed milk powder (‘Marvel’ brand) in 100 ml PBS-Tween. PBS-Tween-skimmed milk was made freshly and stored at 4^{o}C overnight.

After electrophoresis, gels for Western blotting were soaked in Transfer buffer (Table 2.14) for 15 minutes. Proteins were transferred onto nitrocellulose membranes using a TRANS-BLOT® semi-dry transfer cell (Biorad). The Western blotting transfer sandwich was prepared on the surface of the transfer apparatus as follows, from the bottom to the top: two layers of thick filter paper soaked with transfer buffer, nitrocellulose membrane, the 1D electrophoresis

Table 2.11 5% Stacking gel for 1D SDS-PAGE.

Stacking gel	
Acrylamide (40%)	0.5 ml
18.2 MΩ H_2O	2.5 ml
2M Tris stacking gel buffer pH 8.8	1.0 ml
APS (10%)	30 µl
TEMED	10 µl

Table 2.12 Coomassie Brilliant blue stain solution.

Total volume	1 litre
Coomassie brilliant blue R250	1 g
Methanol	450 ml
Glacial acetic acid	100 ml
18.2 MΩ H_2O	450 ml

Table 2.13 Destain solution.

Total volume	1 litre
Methanol	100 ml
Glacial acetic acid	100 ml
18.2 MΩ H_2O	800 ml

Table 2.14 Western blotting transfer buffer.

Total volume	1 Litre
Tris base	5.82 g
Glycine	2.93 g
Methanol	200 ml
18.2 MΩ H2O	800 ml

gel, two layers of thick filter paper soaked with transfer buffer. The lid was placed and connected with the power supply. Blotting was carried out at 0.6 A for 90 minutes for 1 gel.

After protein transfer, the membrane was blocked in 20 ml PBS-Tween-skimmed milk at room temperature overnight. Membranes were then incubated for 2 hours in the primary antibody diluted in 10 ml PBS-Tween-skimmed milk and then washed 5 times with PBS-Tween (ca. 10 min and 15 mL buffer per wash). Membranes were then incubated for 2 hours in the alkaline phosphatase conjugated secondary antibody diluted in 10 ml PBS-Tween-skimmed milk for 2 hours and then washed 5 times with PBS-Tween (ca. 10 min and 15 mL buffer per wash). Each membrane was incubated in 10ml 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT, Sigma) reagent at 37^{o}C and until the colour developed and then the membrane was washed with distilled water. The membrane was documented using Quantity One software.

The primary and secondary antibodies available for use in Western blotting are summarised in Table 2.16. Anti-Bac was kindly supplied by Professor Gunnar Lindahl (Lund University, Sweden). Anti-Sip (which detects a reference GBS protein, Brodeur at al., 2000) was kindly supplied to Prof. Sutcliffe by Dr Clément R. Rioux (Université Laval, Canada). Anti-HPr, for detection of a prominent streptococcal antigen (Dubreuil et al., 2000), was kindly supplied to Prof. Sutcliffe by Dr Christian Vadeboncoeur (Université Laval, Canada).

Table 2.15 Primary and secondary antibody combinations used for Western blotting.

Primary antibody	Dilution	Secondary antibody (Sigma)	Dilution
Anti-Bac	1/1,000	Anti-rabbit	1/30,000
Anti-Sip	1/500	Anti-mouse	1/30,000
Anti-HPr	1/1,000	Anti-rabbit	1/30,000

Chapter 3

GBS growth characteristics *in vitro*

3.1. Background

GBS is opportunistic pathogen, which commensally colonises the vagina and gastrointestinal tract of pregnant women and causes serious neonatal disease (Heath, 2004). The rapid development of early onset GBS disease (often <24 hours after birth) suggests that vertical transmission of GBS from mother to infant occurs either during or immediately prior to birth. During the process of GBS neonatal disease, GBS must be able to survive in several very different host environments, including the women's vagina, amniotic fluid, neonate's lung and blood (Rajogopal, 2009). The vagina is normally acidic, low oxygen and with limited nutrients while the neonate's lung and blood are neutral, high oxygen and with relatively abundant nutrients, as suggested by rapid bacterial growth (Tamura et al., 1994; Yamamoto et al., 2006; Johri et al., 2003).

It is interesting to know that what kind of mechanisms help bacteria to be able to survive in these very different environments. It is reported that some mechanisms such as acid adaptation, biofilm formation and a viable but non-culturable state may help bacterial survival in stress environments (Cotter & Hill, 2003; Bogosian & Bourneuf, 2001; Hall-Stoodley & Stoodley, 2009). GBS growth characteristics, long term survival, acid adaptation, viable but non-culturable state and biofilm formation were investigated to contribute to knowledge of GBS survival in different environments and also help to develop an *in vitro* model to reflect *in vivo* condition of GBS disease development.

3.2. Results of GBS growth characteristics

3.2.1. GBS growth in different media

3.2.1.1. GBS growth in pH 4.3, 5, 6 and 7 THB medium measured by microtitre plate reader

GBS A909 growth in pH 4.3, 5, 6 and 7 THB was measured by microtiter plate reader at an optical density of 570 nm. GBS A909 growth in pH 7 Todd-Hewitt broth medium reached mid-late exponential (OD 0.5) at 150 minutes (Figure 3.1). GBS reached the onset of stationary at 240 minutes and established stationary phase at 320 minutes. GBS A909 exhibited a similar growth curve in pH 7 and pH 6 THB. GBS A909 growth in pH 5 THB reached mid-late exponential (OD 0.35) at 260 minutes, with onset of stationary phase at 300 minutes and established stationary phase at 360 minutes. Unexpectedly, GBS A909 grew very poorly in pH 4.3 THB (Figure 3.1). GBS strains 8181, 8184, 9993 and CT1 all showed similar growth characteristics in THB as GBS A909 (Figure 3.2).

The stationary phase OD of GBS A909 in THB of different pH were 0.1 (pH 4.3), 0.45 (pH 5), 0.8 (pH 6) and 0.8 (pH 7). Thus comparison of growth in unbuffered THB at pH 4.3, 5, 6 and 7 shows significantly reduced growths at pH 4.3 and 5 over a 0-24 hr time period, with both A909 and four other GBS strains (Figs. 3.1 and 3.2). GBS growth at pH 4.3 appears very poor as measured by OD and the pH of the culture medium is clearly an important factor for GBS growth.

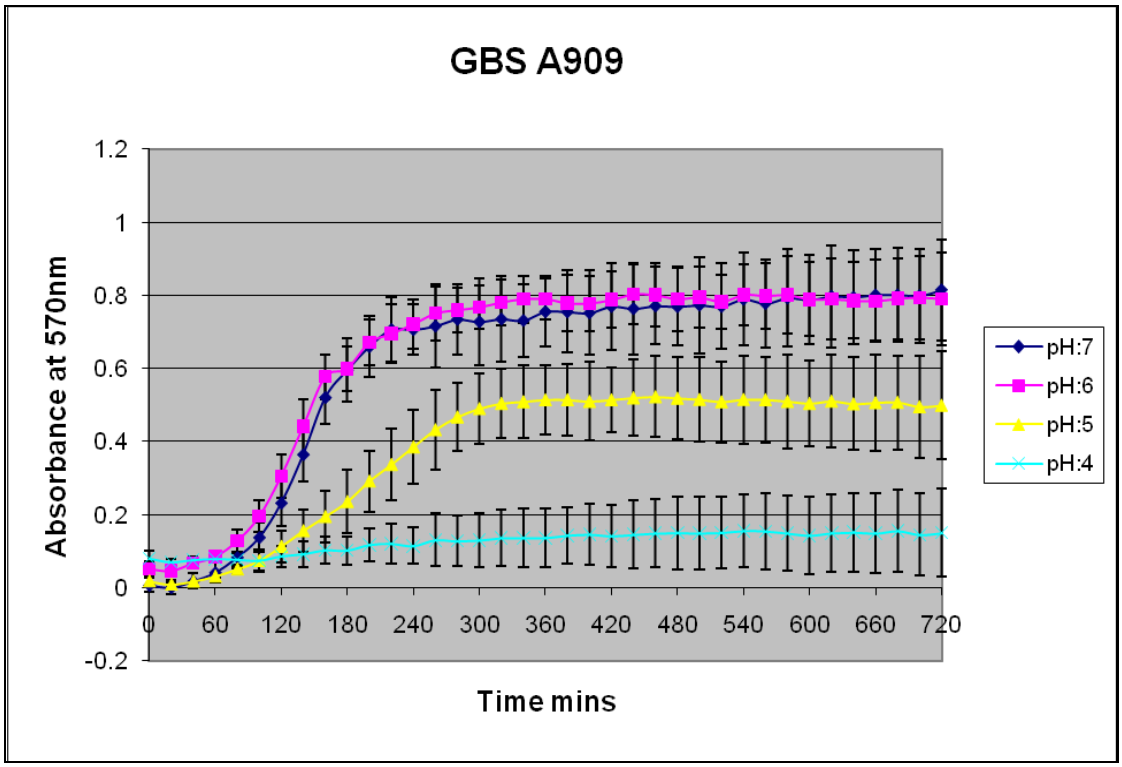
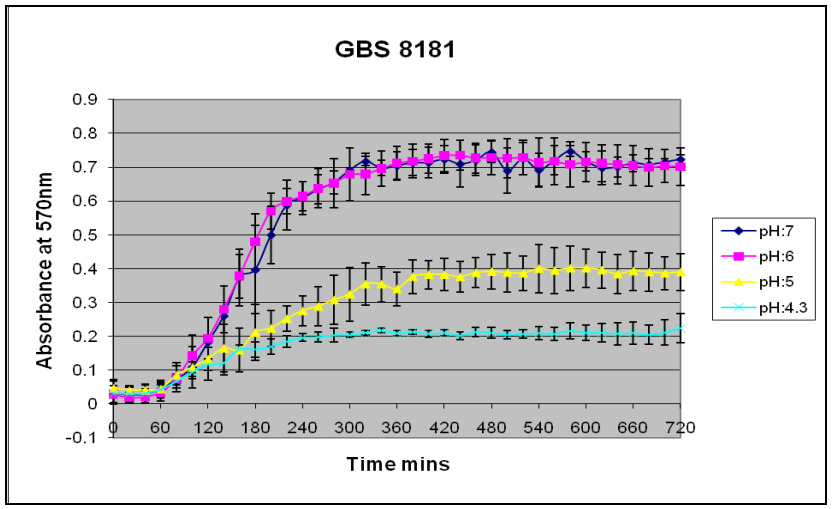
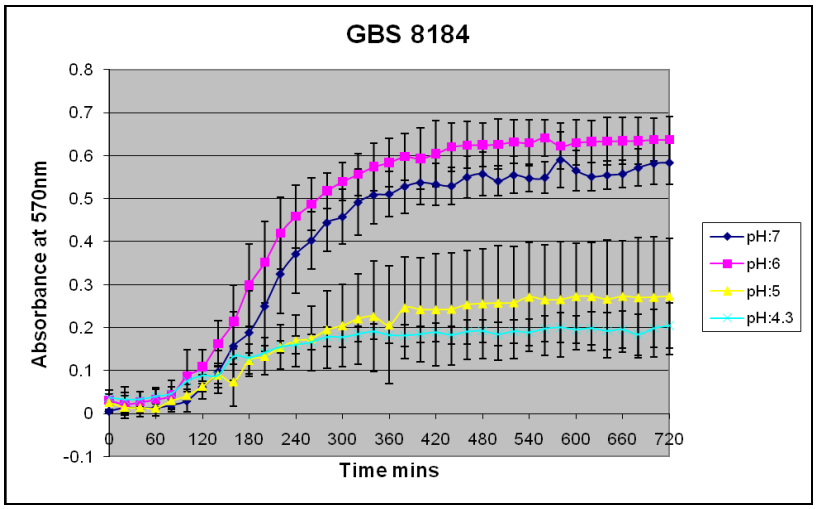
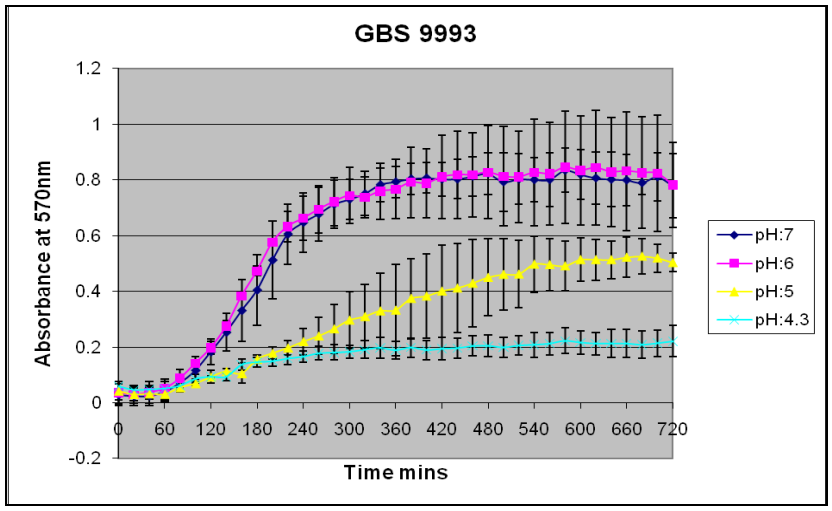


Figure 3.1 GBS A909 growth curves in pH 4.3, 5, 6 and 7 THB medium. Results show mean and SD from triplicate experiments.


GBS 8181
Absorbance at 570nm
Time mins
pH:7
pH:6
pH:5
pH:4.3


GBS 8184
Absorbance at 570nm
Time mins
pH:7
pH:6
pH:5
pH:4.3


GBS 9993
Absorbance at 570nm
Time mins
pH:7
pH:6
pH:5
pH:4.3

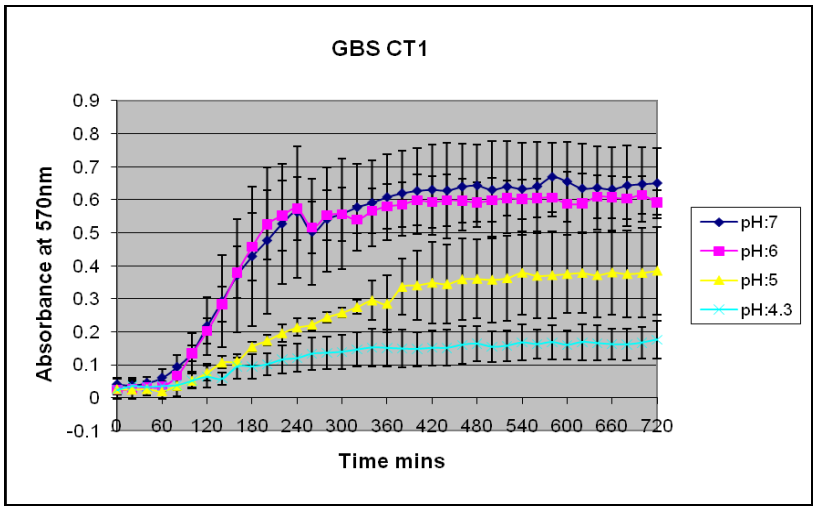

GBS CT1
Absorbance at 570nm
Time mins
pH:7
pH:6
pH:5
pH:4.3

Figure 3.2. GBS 8181, 8184, 9993 and CT1 growth curves in pH 4.3, 5, 6 and 7 THB medium. Results show mean and SD from triplicate experiments.

3.2.1.2. Quantification of GBS growth at different pH by measurement of colony forming units

GBS A909 growth was quantified by measuring the number of colony forming units obtained at different pH. In pH 6 THB, the GBS CFU/mL increased with time and reached a peak of 7.7×10^8 CFU/mL at 6 hours culture (Table 3.1). After 6 hours culture, GBS CFU/mL gradually dropped, but still had a high value of 1.17×10^7 after 48 hours which was 10 times the level of the CFU/mL at inoculation. GBS A909 inoculated into pH 4.3 TH medium had a similar CFU/mL (1.30×10^6) as in pH 6 (1.17×10^6) immediately after inoculation. However, at pH 4.3 the CFU/mL of GBS A909 decreased rapidly and dropped to 4.86×10^3 at the 6 hr time point, which is 0.37% of the CFU inoculated. After 48 hours culture at pH 4.3, CFU/mL dropped to 107, which is only 0.008% of the inoculated CFU (Table 3.1)

GBS 8181 growth in pH 6 THB showed a very similar pattern with a maximum CFU/mL at 6 hours culture, after which GBS CFU/mL gradually declined but still had a value of 6 times of the inoculated CFU at 48 hr (Table 3.2). Likewise, the CFU/mL of GBS 8181 inoculated into pH 4.3 THB decreased rapidly and after 48 hrs had dropped to 90 CFU/mL (0.005% of the inoculation, Table 3.2). These data confirmed the absence of growth at pH 4.3, as indicated previously by optical density measurement. The data also suggest that GBS strains survive less well at lower pH.

3.2.1.3. Comparing GBS growth in TH/YE and human serum

GBS A909 was grown in different culture media, Todd Hewitt with yeast extract

Table 3.1 GBS A909 growth quantification in THB by colony counting. Data show the mean from triplicate experiments.

Time (hrs)	CFU/mL (pH 4.3)	CFU/mL (pH 6)
0	1.3×10^{6} (100%)	1.17×10^{6}(100%)
2	2.11×10^{4} (1.62%)	1.02×10^{7} (872%)
4	9.82×10^{3} (0.76%)	1.42×10^{8} (12137%)
6	4.86×10^{3} (0.37%)	7.7×10^{8} (65812%)
24	463 (0.04%)	1.7×10^{8} (14530%)
48	107 (0.008%)	1.17×10^{7} (1000%)

Table 3.2 GBS 8181 growth quantification in THB by colony counting. Data show the mean from triplicate experiments.

Time (hrs)	CFU/mL (pH 4.3)	CFU/mL (pH 6)
0	1.73×10^{6} (100%)	2.1×10^{6} (100%)
2	8.53×10^{4} (4.93%)	3.7×10^{6} (176%)
4	8.07×10^{3} (0.47%)	1.17×10^{8} (5571%)
6	3.01×10^{3} (0.17%)	6.8×10^{8} (32381%)
24	313 (0.02%)	3.3×10^{8} (15714%)
48	90 (0.005%)	1.27×10^{7} (605%)

(TH/YE, pH 7) and commercial pooled human serum (HS, pH 7). GBS A909 grown in TH/YE reached early stationary phase after 4 hours culture with between 10^9 and 10^{10} CFU/mL. Although there was a small drop of CFU/mL after 6 hours culture, the CFU/mL count stayed at approximately 1×10^9 until up to 24 hours culture. GBS A909 grown in HS had a similar growth curve as in TH/YE (Figure 3.3).

3.2.1.4. GBS A909 growth in pH 5 THB supplemented with 10% human serum

GBS A909 growth in 20 ml pH 5 THB with and without 10% human serum added were measured by optical density (O.D.) at 600 nm. GBS A909 grown in pH 5 THB medium had a long lag phase (O.D. <0.1) of 3 hours and reached mid-late exponential (O.D. 0.4) at 5 hours culture, consistent with previous data (Fig. 3.1). GBS A909 reached early stationary phase (O.D. 0.62) at 6 hours and established stationary phase (O.D. 0.65) at 7 hours. GBS A909 grown in pH 5 THB with 10% human serum added had a similar growth curve as in pH 5 TH medium (Figure 3.4).

GBS A909 growth in 20 ml pH 5 THB with and without 10% human serum added were measured by determining the CFU/mL. GBS A909 grown in pH 5 THB medium reached a peak at 6 hours culture with CFU/mL of 5×10^7 and there was gradual decline in culturable cells after 6 hours. After 24 hours culture, CFU/mL dropped to 1×10^6. GBS A909 grown in pH 5 THB with 10% human serum medium had a similar growth curve as in pH 5 THB medium

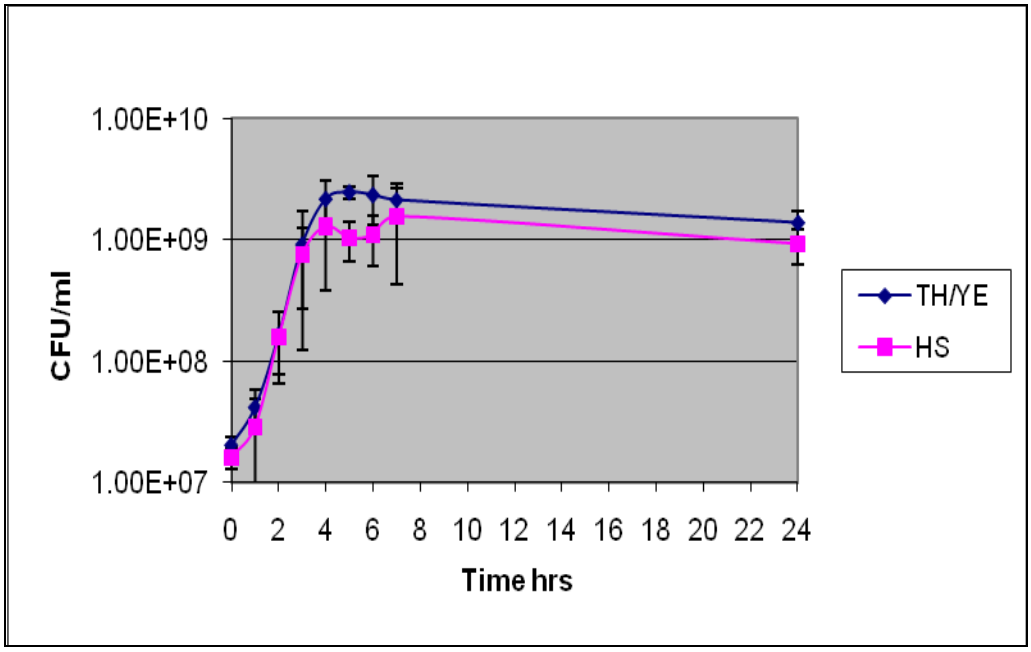


Figure 3.3. GBS A909 growth in TH/YE and human serum medium measured by CFU quantitation. Growth was measured by determining CFU/mL. Results show mean and SD from triplicate experiments.

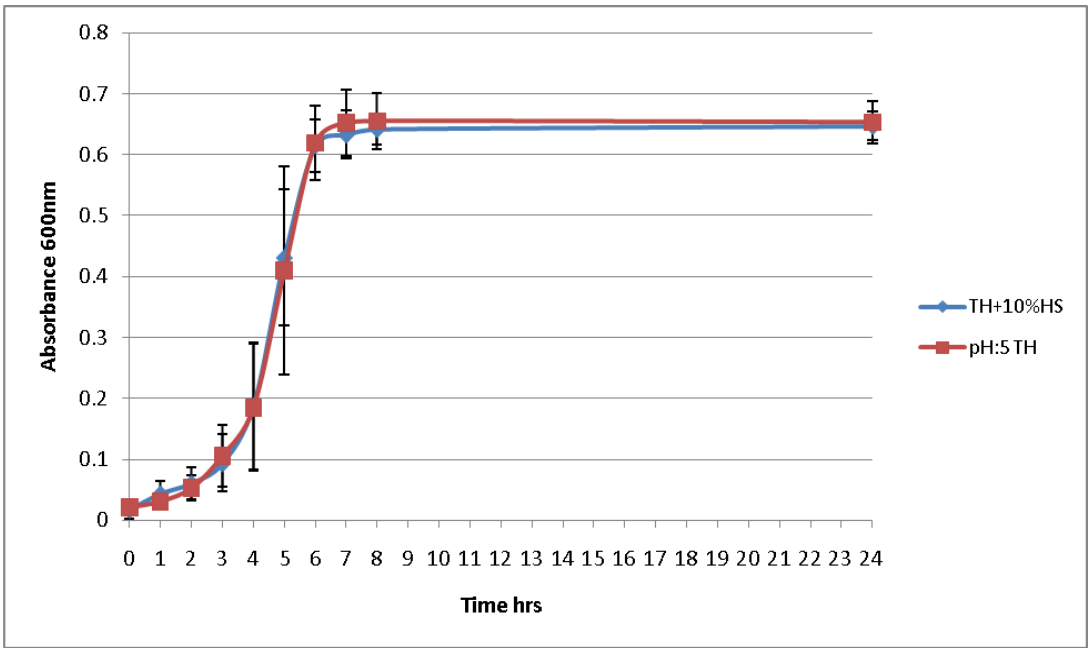


Figure 3.4. GBS A909 growth curve in pH 5 THB with and without 10% human serum medium. Data shown are representative of 3 experiments.

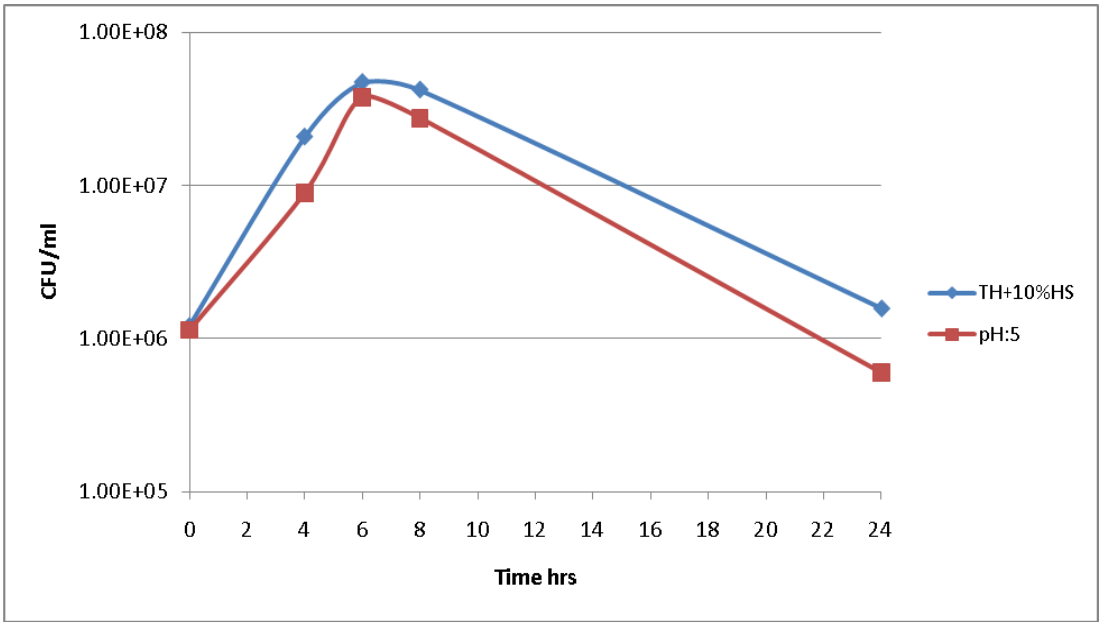


Figure 3.5. CFU/mL of GBS A909 growth in pH 5 THB with and without 10% human serum medium. Data shown are representative of 3 experiments.

(Figure 3.5). Comparing GBS A909 growth measured by colony form units and optical density, the optical density at 600 nm remained constant at around 0.64 whereas CFU/mL had declined after 6 hr. This suggested that more than 90% of the cells had lost their ability to form a colony on TH agar plates.

3.2.2. Long term survival of GBS

3.2.2.1. GBS survival in extended stationary phase measured by plate counting

GBS A909 long term survival in pH 7 and pH 5 THB were measured by determining CFU/mL for standing cultures over time. In pH 7 THB, GBS A909 growth peaked at 6 hours, with the cell numbers of nearly 10^9 CFU/mL and survived in stationary phase to 2 days with cell numbers between ca. 10^9 and 10^8 CFU/mL (Figure 3.6). After 2 days, cells entered the 'death phase' and cell numbers decreased from 10^8 to ca. 10^6 CFU/mL. After 2 weeks, the cells had entered the long-term stationary phase. Cells numbers were maintained at 10^5-10^6 CFU/mL for more than 8 weeks. In contrast, in pH 5 Todd-Hewitt broth medium, GBS A909 growth peaked at 6 hours with cell numbers between 10^8 and 10^9 CFU/mL and then entered death phase. Cells could not be recovered at 1 week. The limit of detection in these experiments was 10^5 CFU/mL. These data comparing survival at pH 7 versus pH 5 again suggested that GBS cells survived poorly at the lower pH, as observed when survival at pH 6 versus pH 4 was measured over 48 hrs (Section 3.2.1.2).

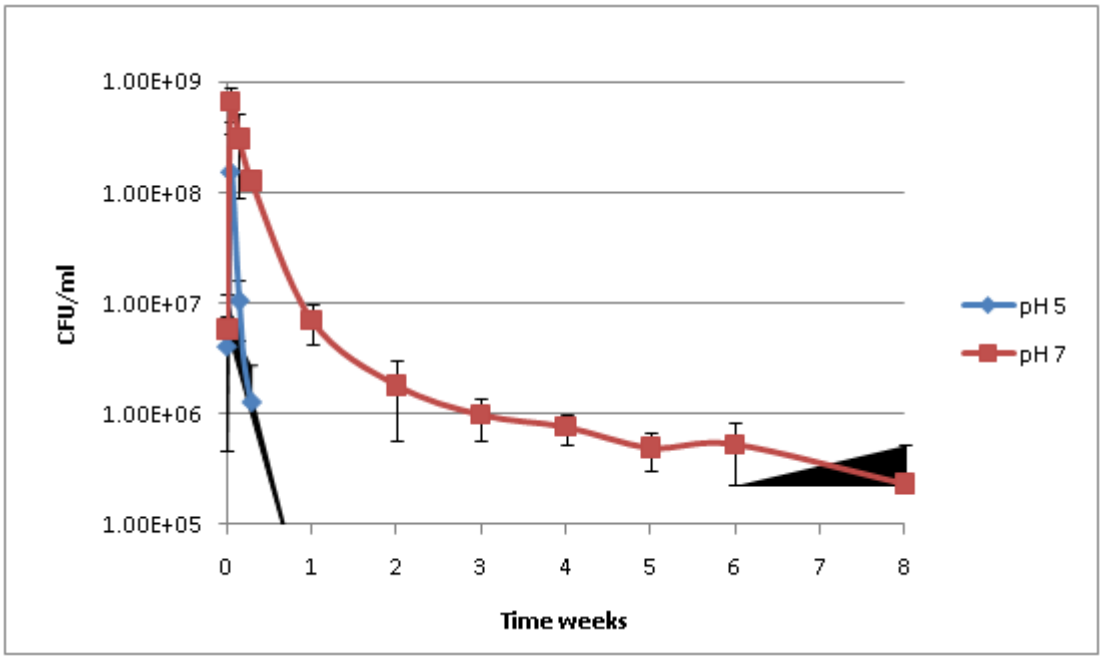


Figure 3.6. GBS A909 survival in extended stationary phase by plate counting. Results show mean and SD from triplicate experiments. (Limit of detection 1 colony in 100 µL from 10^{-4} diluted culture = 10^5 CFU/mL).

3.2.2.2. GBS Long term survival measured using 'spot' plates

GBS strains A909, 8181, 8184, 9993 and CT1 were broth cultured in pH 5 and pH 7 THB medium as standing cultures. Cell survival was measured by spot plating 5 µL samples from the cultures at different time points directly onto THA plates. GBS A909 was viable for at least 4 weeks in pH 7 THB. When strain A909 was cultured in pH 5 THB, CFU were not recovered after 3 days. The limit of detection in this assay was ≥200 CFU/mL (1 CFU per 5 µL sample taken). These results were confirmed with the 4 other GBS strains (Table 3.3).

3.2.3. Possible 'Viable but non-culturable' survival of GBS

3.2.3.1. GBS A909 percentage survival in stationary phase determined by Live-Dead staining

GBS A909 viability was determined by Live-Dead staining. The percentage survival of GBS A909 in pH 5 and pH 7 THB are shown in Table 3.4. The percentage survival of GBS A909 decreased with increased culture time in both pH 5 and pH 7 medium but the survival rate of GBS decreased faster in pH 5 than in pH 7 medium. After 6 hours culture, GBS A909 had entered the stationary phase and the stable O.D. value indicated the total biomass was approximately the same (Fig. 3.1), whereas the dramatic decline in CFU/mL showed the cells lost their culturability rapidly (Fig. 3.3). The Live-Dead staining confirmed this and showed that there were still a significant number of cells that are still alive at long-term stationary phase (>48 hr) both in pH 5 and pH 7 medium. Live-Dead staining results suggested that the live cell numbers of

Table 3.3. Long term survival of GBS strains. Results are from triplicate replicate experiments with duplicate cultures in each. Survival was determined by the spot plate method: limit of detection was 200 CFU/mL.

Strain	**Growth in pH 5 TH**							**Growth in pH 7 TH**						
	1	2	3	7	14	21	28	1	2	3	7	14	21	28
A909	+	+	+	-	-	-	-	+	+	+	+	+	+	+
	+	+	+	-	-	-	-	+	+	+	+	+	+	+
	+	+	+	-	-	-	-	+	+	+	+	+	+	+
	+	+	+	-	-	-	-	+	+	+	+	+	+	+
	+	+	+	-	-	-	-	+	+	+	+	+	+	+
	+	+	+	-	-	-	-	+	+	+	+	+	+	+
8181	+	+	+	-	-	-	-	+	+	+	+	+	+	+
	+	+	+	-	-	-	-	+	+	+	+	+	+	+
	+	+	+	-	-	-	-	+	+	+	+	+	+	+
	+	+	+	-	-	-	-	+	+	+	+	+	+	+
	+	+	+	-	-	-	-	+	+	+	+	+	+	+
	+	+	+	-	-	-	-	+	+	+	+	+	+	+
8184	+	+	+	-	-	-	-	+	+	+	+	+	+	+
	+	-	-	-	-	-	-	+	+	+	+	+	+	+
	+	+	+	-	-	-	-	+	+	+	+	+	+	+
	+	+	+	-	-	-	-	+	+	+	+	+	+	+
	+	+	+	+	+	-	-	+	+	+	+	+	+	+
	+	+	+	-	-	-	-	+	+	+	+	+	+	+
9993	+	+	+	-	-	-	-	+	+	+	+	+	+	+
	+	-	-	-	-	-	-	+	+	+	+	+	+	+
	+	+	+	-	-	-	-	+	+	+	+	+	+	+
	+	+	+	-	-	-	-	+	+	+	+	+	+	+
	+	+	+	+	+	-	-	+	+	+	+	+	+	+
	+	+	+	-	-	-	-	+	+	+	+	+	+	+
CT1	+	+	+	-	-	-	-	+	+	+	+	+	+	+
	+	-	-	-	-	-	-	+	+	+	+	+	+	+
	+	+	+	-	-	-	-	+	+	+	+	+	+	+
	+	+	+	-	-	-	-	+	+	+	+	+	+	+
	+	+	-	-	-	-	-	+	+	+	+	+	+	+
	+	+	+	-	-	-	-	+	+	+	+	+	+	+

Table 3.4. Percentage survival of GBS A909 in pH 5 and pH 7 TH medium determined by Live-Dead staining. % Survival (live cells) was determined from a total of 400 cells counted at each time point.

Time (hr)	Replicate	pH 7 (live%)	pH 5 (live%)
6	1	94.3	93.3
	2	93.0	90.3
	3	92	89
		93.1±1.5	**90.8±1.2**
24	1	83.3	59.0
	2	81.5	33.0
	3	80.3	44
		81.7±1.5	**45.3±13**
48	1	58.0	24.3
	2	61.0	24.0
	3	64.5	20.3
		61.2±3.3	**22.9±2.2**
72	1	53.3	7.8
	2	42.0	14.3
	3	45	10.8
		46.8±5.8	**11±3.3**
168	1	2.75	0.25
	2	7.25	2
	3	6.5	1.25
		5.5±2.4	**1.2±0.9**

GBS A909 in pH 5 medium at 72 hours were approximately 11% of the total cells: assuming a maximum growth to ca. 10^9 CFU/mL at 6 hours (as suggested by CFU/mL measurement, Fig. 3.3), this would correspond to approximately 10^8 live cells/mL. However, the CFU/mL measurement showed the cell number was approximately 10^6 CFU/mL at 72 hr (Fig. 3.6). This suggests that around 99% of the cells at 72 hr were alive but could not be grown on THA. At the 1 week timepoint, Live-Dead staining results suggested that cell numbers were approximately 10^7 live cells/mL (1% of the total cells) but the CFU measurement showed cells could not be detected at the 1 week timepoint. Thus a larger number of cells were shown to be alive with the Live-Dead staining method than could be grown on the agar plates. This suggested the cells may survive in a 'viable but non-cultureable' (VBNC) state (Bogosian & Bourneuf 2001; Oliver 2004).

3.2.3.2. Regrowth of extended stationary phase cells into broth

Data from triplicate experiments that examined regrowth of GBS from extended stationary phase are shown in Table 3.5 and Table 3.6. Note that here the limit of detection at t=0 (i.e. directly after passage into fresh THB) is 1 CFU/100 µL regrowth culture (= 200 CFU/20 mL = 200 CFU/mL stationary phase culture used as inoculum). Thus these limits of detection are comparable to the 'spot' plate assay (Table 3.3).

GBS A909 was cultured in 20 mL of pH 5 THB for different times in stationary phase (1 day – 4 weeks) and CFU/mL determined are shown in

Table 3.5. Regrowth of extended stationary phase cell into pH 5 THB. Light red highlighting shows improved detection of viable cell into broth compared to the 'spot' plate method. Blue highlighting shows extended lag phase for 1 and 2 weeks stationary phase cultures compared to 24 hr culture.

Time sampled	Assay	0 hr allowed for regrowth	6 hr allowed for regrowth	24 hr allowed for regrowth
24hr culture	OD	0.032±0.003	0.351±0.041	0.800±0.016
	c.f.u./mL, aerobic incubation	$22x10^4±6x10^4$	$28x10^6±27x10^6$	$24x10^7±5x10^7$
	c.f.u./mL, low O_2 incubation	$23x10^4±5x10^4$	$30x10^6±10x10^6$	$25x10^7±6x10^7$
1 week culture	OD	0.038±0.003	0.039±0.003	0.755±0.051
	c.f.u./mL, aerobic incubation	70±10	97±31	$21x10^7±9x10^7$
	c.f.u./mL, low O_2 incubation	57±25	103±21	$11x10^7±3x10^7$
2 week culture	OD	0.042±0.003	0.037±0.003	0.285±217
	c.f.u./mL, aerobic incubation	9±10	90±130 [nb no growth in 1 replicate]	$38x10^6±45x10^6$ [nb no growth in 1 replicate]
	c.f.u./mL, low O_2 incubation	7±6	100±140 [nb no growth in 1 replicate]	$20x10^6±28x10^6$ [nb no growth in 1 replicate]
4 week culture	OD	0.042±0.006	0.044±0.009	0.044±0.013
	c.f.u./mL, aerobic incubation	0	0	0
	c.f.u./mL, low O_2 incubation	0	0	0

Table 3.6. Regrowth of extended stationary phase cell into pH 7 THB. Green highlighting shows improved survival and no extended lag for pH 7 culture at later timepoints.

Time sampled	Assay	0 hr allowed for regrowth	6 hr allowed for regrowth	24 hr allowed for regrowth
24hr culture	OD	0.050±0.011	0.740±0.22	0.796±0.025
	c.f.u./mL, aerobic incubation	$44x10^5±32x10^5$	$36x10^7±16x10^7$	$27x10^7±14x10^7$
	c.f.u./mL, low O_2 incubation	$55x10^5±16x10^5$	$16x10^7±2x10^7$	$25x10^7±14x10^7$
1 week culture	OD	0.047±0.005	0.759±0.035	0.871±0.046
	c.f.u./mL, aerobic incubation	$44x10^4±4x10^4$	$23x10^7±12x10^7$	$44x10^7±15x10^7$
	c.f.u./mL, low O_2 incubation	$45x10^4±17x10^4$	$24x10^7±7x10^7$	$44x10^7±20x10^7$
2 week culture	OD	0.063±0.007	0.597±0.173	0.841±0.058
	c.f.u./mL, aerobic incubation	$16x10^4±8x10^4$	$20x10^7±15x10^7$	$31x10^7±15x10^7$
	c.f.u./mL, low O_2 incubation	$15x10^4±5x10^4$	$14x10^7±7x10^7$	$24x10^7±19x10^7$
4 week culture	OD	0.041±0.003	0.436±0.155	0.874±0.034
	c.f.u./mL, aerobic incubation	$12x10^4±9x10^4$	$57x10^6±56x10^6$	$32x10^7±13x10^7$
	c.f.u./mL, low O_2 incubation	$11x10^4±5x10^4$	$60x10^6±46x10^6$	$27x10^7±13x1\ 0^7$

Table 3.5. CFU/mL counts were determined on agar plates after incubation in aerobic and anaerobic conditions. The results showed there was an improved detection of viable cells following passage into broth compared to the 'spot' plate method. As with previous data, the results showed that the longer that stationary phase continued the lower the number of cells were detected, although recovery into broth extended the timescale for detection of low numbers of viable stationary phase cells to 2 weeks (though there was one pH 5 culture that could not be regrown at the 2 week point). As shown in Table 3.5, the lag phase of the regrowth cultures at 1 week and 2 week points were notably longer than for regrowth culture after 24 hrs.

For GBS A909 broth culture in 20 ml of pH 7 THB for different times (1 day – 4 weeks) in stationary phase, the CFU/mL determined are shown in Table 3.6 (after incubation of agar plates in aerobic and anaerobic conditions). GBS A909 cultured in pH 7 THB medium could be regrown after 4 weeks in stationary phase but, as in previous experiments, CFU/mL of GBS A909 in pH 7 TH medium decreased as time in stationary phase increased. These data and the corresponding data for pH 5 THB grown cells indicate that recovery of GBS CFU was not significantly affected by incubation in aerobic versus anaerobic (candle jar) conditions. The lag phase for regrowth of cultures at the 2 and 4 week sampling points was longer than for regrowth of culture at 24 hrs and 1 week timepoints, as shown by lower OD and CFU/mL at 6 hrs. However, this delay in regrowth was not as extensive as for pH 5 THB grown cells (Table 3.5). Samples from all regrown cultures gave Gram-positive staining results and Lancefield B typing, confirming regrowth was due to GBS and not culture

contaminants. These data again demonstrate that the culturability of GBS A909 from extended stationary phase cultures decreased in both pH 5 and pH 7 THB, but was more greatly decreased in pH 5 medium.

3.2.4. Acid adaptation

3.2.4.1. Auto acidification measurements

The growth curves of GBS A909 in THB medium with different pH (4.3, 5, 6 and 7.4) measured by O.D. at 600nm (Figure 3.7) agreed with the results measured by microtitre plate reader at 570 nm (Fig. 3.1). As shown in Table 3.7, after 6 hours GBS A909 culture, the pH of the original pH 7.4 THB dropped to 6.5, i.e. approximately 1 pH unit, and stayed at pH 6.5 during the stationary phase. After 6 hours GBS A909 culture, the pH of the original pH 6 THB dropped to 5.3 and stayed at 5.3 during the stationary phase. After 6 hours GBS A909 culture, the pH of the original pH 5 THB dropped to 4.5 and stayed at 4.5 during the stationary phase. The extent of the pH drop thus correlated with the extent of GBS growth (as measured by final OD 600). There was no significant change in medium pH value when GBS was cultured in pH 4.3 THB, consistent with a lack of GBS growth at this pH (Fig 3,1; Table 3.7; Fig. 3.7).

After 4 weeks culture, the medium pH of GBS A909 grown in pH 7.4 THB dropped to 7.1. This result was confirmed with four other GBS strains (Table 3.8). Thus after 4 weeks culture, the medium pH value of GBS strains

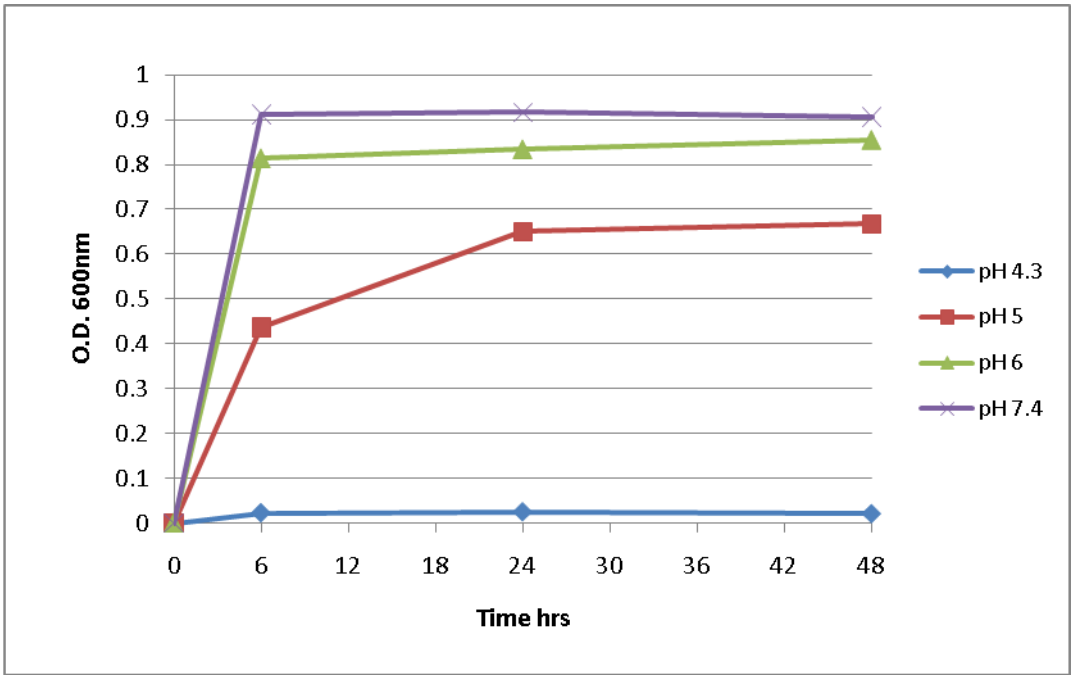


Figure 3.7. GBS A909 growth curves in pH 4.3, 5, 6 and 7.4 TH medium. GBS growth was measured at O.D. 600nm.

Table 3.7. Final medium pH after GBS A909 auto-acidification by up to 48 hours growth.

Sample /Time (hrs)	0 hrs	6 hrs	24 hrs	48 hrs
pH 4.2	4.24±0.03	4.24±0.03	4.24±0.03	4.25±0.06
pH 5	5.04±0.03	4.54±0.05	4.56±0.05	4.57±0.10
pH 6	6.04±0.02	5.36±0.09	5.25±0.18	5.26±0.18
pH 7.4	7.45±0.06	6.50±0.04	6.46±0.13	6.46±0.05

Table 3.8. Final medium pH after GBS strains growth for 4 weeks (duplicate cultures).

	pH:7.4	pH:5	pH:4
A909 (1)	7.15	4.59	4.14
A909 (2)	7.11	4.65	4.13
8181 (1)	7.07	4.59	4.14
8184 (2)	7.08	4.64	4.14
8184 (1)	7.18	4.58	4.15
8184 (2)	7.02	4.36	4.15
9993 (1)	7.15	4.47	4.14
9993 (2)	7.01	4.51	4.14
CT1 (1)	7.00	4.66	4.16
CT1 (2)	7.06	4.67	4.16

inoculated into pH 7.4 THB dropped around 0.2-0.4 pH units. In contrast, the 48 hour auto-acidification results showed the medium pH dropped around 1 pH units after 6 hours culture and kept this pH for 48 hours (Table 3.7). Similarly, after inoculation of GBS strains into pH 5 broth, pH values of the medium dropped ca. 0.3-0.4 pH units after 4 weeks culture compared to a drop of 0.5 units after 6-48 hr with strain A909. It is known that GBS are acid-forming bacteria (Michelson, 1972), which can produce lactic acid through metabolising glucose. These data suggest that when the glucose was exhausted, in extended stationary phase, the GBS are undergoing alternative metabolic pathways and some of the initial acid production is reversed.

3.2.4.2. Acid adaptation experiments: a revised strategy for exposure to acid

It is well recognised that many bacteria can undergo an adaptive 'acid tolerance response' (ATR, Cotter and Hill, 2003). An ATR is normally defined as improved survival after exposure to extreme acid stress and is often measured by exposure to a non-lethal acid pH for a short period, followed by colony counting after different exposure times under extreme acid stress (e.g. pH 3.5 or less for 0-60 min). The ATR suggests that prior growth history affects affects survival on exposure to acid stress. Previous experiments showed that GBS A909 could not be cultured from pH 5 THB medium after 3 days culture (Figure 3.6; Table 3.3). Experiments were therefore carried out to see if prior exposure of GBS to acid (e.g. from autoacidification during growth) would affect survival in stationary phase. GBS A909 were gradually exposed to pH 7 THB for 3 hrs

and pH 6 THB overnight before culture in pH 5 THB. The CFU/mL were around 10^8 at the 6 hours point, 10^7 at 1 day, 10^5 at 2 day, 10^4 at 3 day point and could not be recovered at 1 week point (Figure 3.8). There was no significant improvement in GBS A909 survival after exposure to non-lethal acid conditions followed by culture in pH 5 THB.

3.2.5. Alternative media for GBS growth

3.2.5.1 GBS A909 growth on THA plates at different pH

GBS A909 growth on pH 5 THA gave similar colony counts as GBS A909 grown on pH 7 THA regardless of whether the plates were incubated aerobically or anaerobically (Table 3.9). Importantly, the similar counts from aerobically or anaerobically incubated pH 7 THA plates after 24 hr suggest that failure to detect viable CFU in earlier experiments (Fig. 3.6; Table 3.3) to recover viable GBS from extended stationary phase was not affected by the incubation method used after sampling. However, when cultured for 24 hrs, the colony sizes of GBS A909 cultured on pH 5 THA under anaerobic conditions were noticeably smaller than those produced on pH 7 THA with aerobic/anaerobic incubation conditions (approximate colony diameters less than 0.5 mm compared to about 1 mm). There were no colonies on pH 5 THA plates after aerobic incubation for 24 hrs but colonies were visible at 48 hrs. The colony sizes of GBS A909 cultured on pH 5 THA with aerobic conditions at 48 hrs were much smaller than those on pH 5 THA incubated anaerobically.

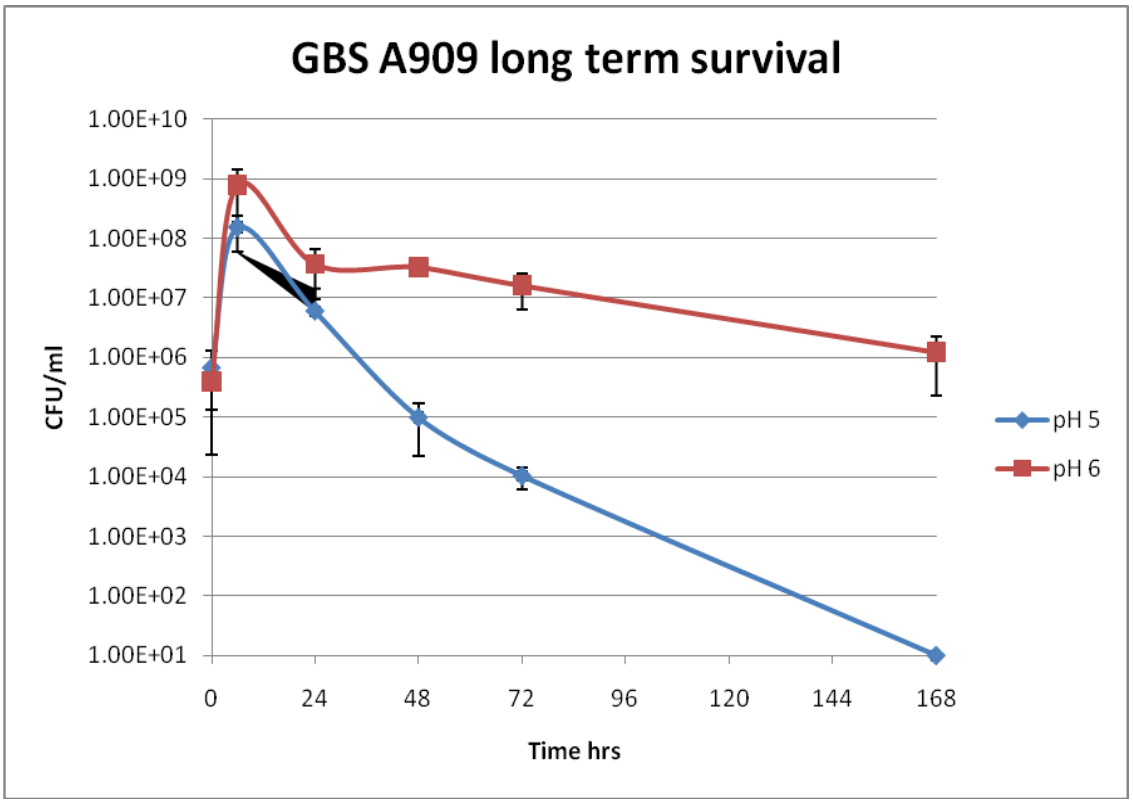


Figure 3.8. GBS A909 long term survival after acid adaptation and culture at different pH. Results shows mean and SD from triplicate experiments.

Table 3.9. GBS A909 grown on pH 5 and pH 7 TH agar plates under aerobic or anaerobic conditions. Colony counts were performed at 24 hrs except for those on pH 5 THA incubated anaerobically, which were performed at 48 hr.

Growth condition	CFU/mL (pH 5 agar plate)	CFU/mL (pH 7 agar plate)
Aerobic	$115x10^6 \pm 48x10^6$	$149x10^6 \pm 52x10^6$
Anaerobic	$148x10^6 \pm 30x10^6$	$125x10^6 \pm 63x10^6$

These data are generally consistent with results observed for ‘standing’ broth cultures (i.e. that pH 5 cultures will support growth (Fig. 3.1) but are consistent with a reduced growth rate for both anaerobically and aerobically grown GBS at pH 5, with the latter more apparent.

3.2.5.2. GBS growth in vagina simulative medium

There was no growth observed of GBS A909 in vagina simulative medium (Table 3.10) as measured by OD at 600 nm.

3.2.5.3. GBS growth on M17 agar versus M17 with Heme/MQ supplementation (M17R) plates

Growth on M17 medium agar plates gave similar CFU/mL to those observed with overnight cultures plated on THA (Table 3.11). Growth was not notably improved by medium supplementation with heme and menaquinone (M17R) compared to control medium. It has been reported that GBS can aerobically respire using externally derived haem and quinones (Yamamoto et al., 2005; Rezaïki et al., 2008). Yamamoto et al. (2005) cultured GBS strain NEM316 in M17 medium with horse blood and menaquinone added as exogenous sources of haem and quinone. They found that respiration metabolism occurred and caused an increased GBS growth (Yamamoto et al., 2005). In contrast, the data here did not show any significant growth improvement of GBS A909 grown in M17R compared with control medium. This may be because the strain used was A909, which is different to strain NEM316.

Table 3.10. GBS A909 growth in Vagina Simulative medium (duplicate experiments).

Time hrs/GBS A909	OD (sample 1)	OD (sample 2)
0	0.025	0.018
1	0.015	0.007
2	0.034	0.022
3	0.021	0.035
4	0.037	0.035
5	0.040	0.026
6	0.039	0.021

Table 3.11. GBS A909 grown on M17 plates with Heme/ MQ supplementation plates.

Medium	CFU/mL (GBS A909)
M17	$113x10^6 \pm 92x10^6$
M17R	$82x10^6 \pm 23x10^6$
M17-control	$73x10^6 \pm 48x10^6$

3.2.6. Biofilm experiments

3.2.6.1. Biofilm culture in extended stationary phase

CFU counting results showed there was biofilm formation when GBS A909 was grown in THB using tissue culture flasks to provide an abiotic surface (Table 3.12). GBS A909 biofilm culture in pH 7 TH medium showed an apparently reduced CFU/mL at each time point compared with planktonic cells. This was most likely due to the growth of GBS cells in the medium which did not form biofilm culture, which were removed when the biofilms were washed before they were assayed. GBS A909 could also form biofilms in pH 5 THB. Biofilm CFU/mL of GBS A909 in pH 5 TH medium were obtained after 7 days but could not be detected after 14 days. However, notably, this demonstrated the first measurable recovery of CFU after 1 week in stationary phase for pH 5 grown cultures (except in regrowth experiments).

Live-Dead staining results showed the percentage of live cells in biofilm cultures in pH 5 and pH 7 TH medium was improved compared with planktonic cells at all comparable time points (1, 4, and 7 days). This may suggest biofilm formation provides a protection to the cells against acid stress. Live-Dead staining showed that after 1 week in extended stationary phase in pH 5 TH medium, there were around 15.7% biofilm grown cells alive. In contrast, the colony counting method showed that at the 7 day timepoint, 127 CFU/mL were present, which is only 0.005% of the counts obtained after 1 day in stationary phase. This again suggested the percentage of live cells measured by Live-Dead staining is much higher proportionally than suggested by CFU

Table 3.12. Biofilm culture in extended stationary phase. Data in black font are the mean biofilm data (n=6 experiments, except at 14d (n=4 experiments); Data in blue font are taken from comparable planktonic growth experiments (Fig. 3.6, Table 3.4). Yellow highlighting that shows CFU/mL recovery was lower in biofilm cultures (pH 7) than in planktonic cultures. Brown highlighting shows CFU/mL recovery was improved in pH 5 TH biofilm culture compared with planctonic culture at the 1 week point. Note that the percentage of live cells was higher in biofilm cultures (both pH 5 and pH 7) than in planctonic cultures at all comparable timepoints (planctonic data are taken from Table 3.4). * Note that these data were measured at 72 hr (3 days) rather than 4 days.

Time	**pH 5 TH biofilm**	pH 5 planctonic	**pH 7 TH biofilm**	pH 7 planctonic	**pH 5 TH biofilm**	planctonic	**pH 7 TH biofilm**	planctonic	**pH 5 TH biofilm**	**pH 7 TH biofilm**
	CFU/mL	CFU/mL	**CFU/mL**	CFU/mL	**% Live**	**% Live**	**% Live**	**% Live**	**CV stain**	**CV stain**
1d	$27\pm13\times10^{5}$	$11\pm58\times10^{5}$	$565\pm197\times10^{5}$	$31\pm22\times10^{7}$	68.0	45.3	86.0	81.7	1.085±0.506 54%	2.018±0.535
4d	$95\pm64\times10^{2}$		$68\pm18\times10^{5}$		19.5	11*	62.5	46.8*	0.808±0.27 50%	1.61±0.395
7d	127±134	0	$13.5\pm3\times10^{4}$	$70\pm28\times10^{5}$	15.7	1.2	34.3	5.5	0.953±0.099 57%	1.668±0.388
14d	0±0	0	$125\pm9\times10^{3}$	$18\pm12\times10^{5}$	6.4		39.3		0.3±0.05 34%	0.892±0.314

counts (see Section 3.2.3. above). Similarly, live cells could also be detected after 14 days in pH 5 biofilm growths, whereas CFU could not be recovered by sampling and culture.

Crystal violet (CV) staining results agreed with CFU counting and indicated there was less biofilm biomass laid down at pH 5 compared with pH 7. The results also suggested biofilms are relatively stable over 1-7 days but less so after 14 days in stationary phase (a reduction to ~30%-50% of CV values compared to those obtained after 1-7 days).

These data indicated that biofilm culture may yield fewer cells but improves survival over 4-7 day timescales at pH 5. This suggests biofilm growth generates smaller but more persistent communities of bacteria which may be a better reflection of the *in vivo* situation. However, recovery of surviving cells from pH 5 grown biofilms was still limited compared to biofilms formed in pH 7 THB, consistent with the data from planktonic cultures.

3.2.6.2. Biofilm survival following 'feeding' with nutrient broth

Experiments were performed to determine if feeding biofilms with nutrient broth improved survival and recovery of GBS. As in earlier experiments, GBS A909 inoculated into pH 5 THB (planktonic cells) showed very low recovery of CFU/mL after 1 week, as there was only one culture from which cells could be recovered (Table 3.13). As in initial experiments, unfed biofilm cultured GBS A909 yielded notably lower CFU/mL after 24 hr than planktonic cells ($4.1\pm3.1\times10^4$ versus $1.9\pm0.8\times10^6$ CFU/mL i.e. 2.%). Bacteria in biofilm cultures

that were fed with 1/10th strength pH 5 THB were much more readily detected (≥10^3 CFU/mL) at the 1 week timepoint compared to previous data (as starved biofilms yielded ca. 10^2 CFU/mL after 7 days, Table 3.12). Biofilm cultured cells fed with 1/10th human serum yielded slightly lower CFU/mL than biofilm cells fed with 1/10th THB (yield 9x10^3 vs 16.7x10^3 CFU/mL i.e. 54%). These fed biofilms also gave notably higher counts (≥10^3 CFU/mL) at the 1 week timepoint compared to unfed extended stationary phase biofilms (Table 3.12). Therefore, these data suggest that feeding with low nutrient media may increase biofilm persistance, which may better reflect low delivery of nutrients *in vivo* than the simple starvation conditions of extended stationary phase cultures.

3.2.6.3. Assay for improving biofilm formation by coating culture plates with extracellular matrix molecules (hyaluronic acid and heparin)

CFU/mL of GBS A909 biofilm cultures was measured following coating of the microtitre plates with heparin, hyaluronic acid and heparin & hyaluronic acid (Table 3.14). The counts obtained were 209%, 245% and 191% of those on plates without coating. This result showed coating plates with extracellular matrix molecules could improve biofilm formation. Similar results were obtained with GBS strain NEM316, which notably could form better biofilms than A909 under all conditions. There was no obvious additive effect on biofilm formation of plate coating with heparin and hyaluronic acid combined than either one alone.

Table 3.13. Biofilm survival following 'feeding' with nutrient broth.

Cultures were established in different modes of growth in pH 5 THB. Yellow highlighting shows that the CFU/mL recovery was improved in fed biofilm cultures after 1 week point compared with unfed cultures.

Time	Sample	Replicate 1	Replicate 2	Replicate 3	Mean	S.D.
24hrs	Planktonic culture	26×10^5	20×10^5	11×10^5	19×10^5	8×10^5
24hrs	Early biofilm	41×10^2	570×10^2	610×10^2	407×10^2	317×10^2
1 week	Planktonic culture	20	0	0	6.7	11.5
1 week	THB fed biofilm	38×10^3	5×10^3	7×10^3	16.7×10^3	18.5×10^3
1 week	Human serum fed biofilm	17×10^3	8×10^3	2×10^3	9×10^3	7.5×10^3

Table 3.14. Assay for improving biofilm formation by coating culture plates with extracellular matrix molecules (hyaluronic acid and heparin)

Strain	Uncoated	Heparin	Hyaluronic acid	Heparin + Hyaluronic acid
A909	$3.3\pm0.8 \times 10^8$	$6.9\pm2.2 \times 10^8$	$8.1\pm2.9 \times 10^8$	$6.3\pm1.5 \times 10^8$
	100%	209%	245%	191%
NEM316	$4.9\pm2.6 \times 10^8$	$9.1\pm0.6 \times 10^8$	$8.5\pm2.8 \times 10^8$	$9.2\pm3.3 \times 10^8$
	148	276%	257%	278%

3.3. Discussion of GBS growth characteristics

3.3.1. GBS long term survival in stationary phase

The growth phases of GBS A909 observed in pH 7 TH medium are in agreement with the proposal of Finkel (2006) that the bacterial life cycle can be divided into five phases: lag phase, exponential phase, stationary phase, death phase and long-term stationary phase (Finkel, 2006). After 4-6 hours culture, GBS A909 entered into stationary phase with a high density of cell numbers between ca. 10^8 and 10^9 CFU/mL maintained up to 2 days. In this phase, the cells likely had a high metabolic rate and eventually exhausted the limited environmental nutrition. Due to the absence of nutrition, most of cells entered a 'death mode', in which cells lose viability, and cell numbers dropped to ca. 10^6 CFU/mL. Surviving cells can then use the components from dead cells as nutrition. After 2 weeks, cells entered into long term stationary phase, in which cells numbers were maintained at 10^5-10^6 CFU/mL for more than 8 weeks. In this phase, cells preserve a dynamic balance of cell 'birth' and 'death' (Finkel, 2006). It is reported that other bacteria, notably *S. pyogenes*, also showed long term survival and remained culturable for more than 1 year (Wood et al., 2005). Bacteria survive in long-term stationary phase by remaining metabolically active: S. *pyogenes* lost culturability in less than 72 hours when cells at long-term stationary phase were transferred from glucose depleted rich medium to phosphate buffered saline or when antibiotics were added (Wood et al., 2009). Amino acids are necessary for *S. pyogenes* long term survival as cell numbers decreased rapidly when *S. pyogenes* was cultured in nitrogen limited medium (Trainor et al., 1999). The pyruvate pathway and amino acid catabolic pathway

are the main metabolic pathways of S. *pyogenes* surviving during long-term stationary phase (Wood et al., 2009). This was supported by measuring the medium components before S. *pyogenes* entered into stationary phase and at 12 weeks. The results showed there was an increase of formate and acetate concentration and decrease of lactate levels (Wood et al., 2009). After 4 weeks of stationary phase *S. pyogenes* produced two kinds of colonies: atypical large colonies and microcolonies, which expressed both common and unique characters when analysed by proteomic methods (Wood et al., 2005). This suggested there was an accumulation of mutants surviving in long-term stationary phase and these expressed differences in more than metabolism (Wood et al., 2009). Global regulators (e.g. RelA, Rgg/RopB, CsrR/CovR, CcpA and CodY) regulate *S. pyogenes* expression of metabolic genes and virulence factors (Wood et al., 2009). There was RpoS accumulation at stationary phase, which can help cells adapt and resist stress environments. RpoS regulates genes involved in cell morphological changes, stress resistance, metabolic activity and virulence factor expression (Llorens et al., 2010).

GBS strains survived for much shorter periods in extended stationary phase in pH 5 medium compared to pH 7 medium. The data showed GBS A909 could not typically be recovered at 1 week in pH 5 medium (Figure 3.6) and these findings were supported by replicates with other GBS strains, with a highly sensitive lower limit of detection (200 CFU/mL; Table 3.3). Therefore these data show that the starting pH of the culture medium can affect long term survival of bacteria. This data was very surprising given that normal vaginal pH is lower than pH 5 and GBS colonisation may be prolonged *in vivo*. However,

how GBS arrives in the acid medium of the vagina is not known but may be important. It is generally assumed that the major reservoir for GBS is rectal colonisation but it is not known if transfer to the vagina is a 'gradual' or 'abrupt' colonisation event. GBS may mount an adaptive ATR which improves growth and long term survival. The ATR has been extensively studied in other bacteria, including streptococci (Weilin-Neilands and Svensater, 2007; Martin-Galiano et al., 2005). GBS growth experiments were therefore developed that allowed GBS to be given 'transient exposure' or 'habituation' to low pH before the extended incubations to measure cell survival at low pH. However the data showed there was no improvement of GBS A909 survival in pH 5 THB after the strategy of exposure to acid conditioning.

3.3.2. Acid Adaptation

In vitro GBS growth in unbuffered THB broths will 'autoacidify' the medium due to fermentation producing acids (for GBS, mostly lactic acid, Michelson 1972). Note that the main cause of the acid environment in pH 7 broths is streptococcal metabolism (i.e. an 'endogenous' phenomenom). Moreover, this will be a gradual acidification and should induce some acid tolerance. A different scenario is entry of streptococci into an acid environment generated by an 'exogenous' source e.g. *in vivo* movement to a new body site such as the vagina which is already acid. However, it is important to consider whether this is a gradual process down a pH gradient, perhaps similar to stepwise movement across tissue surfaces such as the perineum, which has a pH ca. 6 (Runeman et al. 2003; Runeman et al. 2005) or an abrupt exposure to acid by

direct inoculation into the new environment (e.g. rapid transfer from rectum to vagina). There is no clear evidence which of these situations (or both) reflects the mode of entry of GBS into the vagina. *In vitro* inoculation into low pH broths (as in experiments described earlier in this chapter e.g. Fig 3.6) typically models an 'abrupt' transfer. As gradual colonisation from rectum to vagina may involve transient colonisation of the perineum (typical pH 6), GBS *in vivo* may be able experience conditions allowing an ATR to develop.

It is important to note that studies of the ATR phenomena are very hard to interpret and it is clear that there may be important species and strain specific variations in the mechanisms underlying how the ATR is achieved. A good example of different ATR induction strategies is given by Papadimitriou et al. (2007), who used three main methods: (1) Autoacidification – the effect of fermentation during growth releasing acid end products to acidify non-buffered media. (2) Transient exposure to non-lethal pH – cells grown in pH 7 buffered medium were harvested and resuspended in medium at pH 5.5 or 6 for 1 hour. (3) Acid "habituation" – growth of inoculum overnight at pH 6 (i.e. to stationary phase) followed by subculture into medium at pH 6. The cells were then challenged by exposure to pH 3.5 for 15 or 30 minutes. The data of Papadimitriou et al. (2007) showed that all three strategies induced an ATR, although autoacidification was less effective at the 15 min time point. Another important point is that many studies focus on ATR of log phase cells. However, Papadimitriou et al. (2007) showed that entry to stationary phase induced an ATR, although this was ineffective after prolonged incubation (48hr) in stationary phase. There are also previous reports of a strain variable stationary

phase ATR in *Strep. mutans* (Svensäter et al. 2001) and *Lactococcus lactis*. The strategy here for exposure to acid showed there was no improvement of GBS A909 long term survival in pH 5 THB (Figure 3.8). This result was consistent with the data of Papadimitriou et al. (2007).

3.3.3. Possible 'viable but non-culturabale' survival of GBS

Bacteria in the VBNC state are unable to grow on standard media and form colonies but are still alive (Bogosian & Bourneuf 2001; Oliver 2004; Oliver, 2009). Live-Dead staining with acridine orange/ethidium bromide can be used to determine whether the cells are in a living or dead state based on cell membrane integrity. Viable cells have intact cytoplasmic membranes and a functional electron transport system. The total number of bacteria was determined by acridine orange staining. Acridine orange is a cell permeable fluorescent dye, which can interact with DNA and RNA present and gives a green fluorescence. Dead cells that have lost membrane integrity are stained as red flourescence by ethidium bromide. The staining of ethidium bromide dominates over acridine orange. The viable cells were visualized as green fluorescence under Simens Olympus BX40 microscope in fluorescence mode, while dead cells were visualized as red fluorescence.

The Live-Dead staining results showed that there were still a significant number of cells that are still alive at long-term stationary phase (>48 hrs) both in pH 5 and pH 7 medium, which are much higher than the live cells recovered by THA plate sampling methods. Thus a large number of cells were shown to

be alive with the Live-Dead Staining method than could be grown on agar plates, which suggested the cells may survive in a ‘viable but non-culturable’ (VBNC) state.

VBNC cells also can be confirmed by using reverse transcriptase (RT)-PCR to detect gene expression. Nonculturable cells continued to express bacterial mRNA, which has a half life of only 3-5 mins. Expression of bacterial mRNA was deemed as an indicator of cells being alive (Oliver, 2009). Other methods, including redox indicators and flow cytometry, have also been used to detect VBNC cells (Bogosian & Bourneuf, 2001).

Environmental stresses such as starvation, thermal stress, oxidative stress and pH changes have been reported to induce bacteria to enter into a VBNC state (Oliver 2009). Bacteria in the VBNC state are considered to be in a dormant phase, accompanied by very low levels of metabolic activity, macromolecular synthesis and respiration, but ATP levels and membrane potential are still high. VBNC cells can be resuscitated in favourable environments (Oliver 2009).

Bacterial entrance into a VBNC state is accompanied by metabolic changes including in nutrient transport, respiration rates and macromolecular synthesis (Oliver 2004). Proteomic analysis of *E. faecalis* revealed there are different metabolic levels including glycolysis, oxidative phosphorylation and phospholipid biosynthesis between growing and VBNC cells (Heim et al., 2002). Muela et al. (2009) found the *E. coli* outer membrane subproteome changed under stress conditions (Muela et al 2009). *E. coli* underwent modification of the peptidoglycan when undergoing the transition from a growth

state to the VBNC state. These changes of bacterial metabolism and cellular components may be explained as a cellular adaptation to the stress environment. RT-PCR experiments showed nonculturable *E. coli* continually expressed the genes of *mobA, rfbE, stxl, gfp* and for 16S rRNA synthesis (Muela et al., 2009). These suggested the cells were viable. Continued expression of the stress σ factor RpoS was found by *E. coli* in the VBNC state. The expression of the virulence factors CagA, VacA and UreA by *Helicobacter pylori* in the VBNC state suggested the bacteria remains infectious in this state (Muela et al., 2009).

The possibility identified here that GBS cells entered into a 'VBNC' state should be further confirmed by other methods, such as RT-PCR and proteomics.

3.3.4. Biofilm formation contributes to GBS survival

The results obtained here showed that there was biofilm formation when GBS A909 was grown in TH broth medium using tissue culture flasks and microtitre trays. Bacterial biofilms are composed of surface-associated bacteria surrounded by an extracellular polysaccharide matrix (Kaur et al., 2009; Hall-Stoodley and Stoodley 2009). This three dimensional complex is a coordinated community and allows bacteria to adapt to and survive in host environments (Hall-Stoodley and Stoodley 2009).

Live-Dead staining results showed the percentage of live cells in biofilm cultures was improved compared with planktonic cells. This may be explained

as the biofilms may provide a protection to the cells against stress environments. Hall-Stoodley and Stoodley (2009) reviewed that bacteria in biofilms can detect environment changes and respond to it in order to survive in diverse and stressful conditions. Biofilms protect bacteria against antibiotics by decreasing the antibiotic's penetration rate and mediating bacterial gene expression. Transmission electron micrographs reveal biofilms protect bacteria against phagocytes (Hall-Stoodley and Stoodley 2009). S. *mutans* biofilm cells expressed significant greater acid tolerance ability compared with planktonic cells both at 3 hours and 3 day time points and bacterial acid tolerance was increased with increased biofilm density (Welin-Neilands and Svensäter 2007).

Biofilm formation coating culture plates with extracellular matrix molecules (hyaluronic acid/ heparin) results showed GBS strain NEM316 could notably form better biofilm than A909 under all conditions. Konto-Ghiorghi et al. (2005) characterized the *gbs1479-1474* locus of NEM316, which encoding the pilus proteins, and found that PilA and PilB are necesary for biofilm formation. There was an impaired biofilm formation in pilus deletion stains compared with wild type strains (Konto-Ghiorghi et al., 2005).

Previous results (Section 3.2.2) showed GBS A909 grown in pH 5 TH medium without feeding could not be recovered after 1 week point. Biofilm cultures fed with 1/10th THB pH 5 or human serum were readily detected at the 1 week time point (Table 3.13). Vaginal fluid is the main source of host derived nutrients for the vaginal bacteria and is produced by reproductive age women at a rate of 1-3 g per day. Vaginal fluid is an acid complex mixture, which is composed of cervical mucus, endometrial fluid, desquamated vaginal epithelial

cells and leukocytes. Other nutrients come from menstrual fluid, which is composed of 30-50% whole blood, carbohydrates, amino acids, proteins, urea, lipids and fatty acids. Both pH and components of vaginal fluid are variable during menstruation cycle. Moreover, transepithelial delivery of nutrients may be relevant depending on the precise location of GBS *in vivo* which appears to be as yet unknown. An *in vitro* model of GBS A909 feeding with low concentration medium (1/10th strength) is therefore arguably more like *in vivo* conditions, in which bacteria can continue to acquire fresh nutrition from the environment.

The most important finding of the present chapter is that GBS long term survival is affected by pH. GBS could survive in pH 7 THB for more than 8 weeks and maintained high cell numbers. GBS could not be recovered from pH 5 THB after 1 week using THA plates, except on a few occasions (Table 3.5 and 3.13), and never after longer than 1 week. Acid adaptation experiments showed there was no significant improvement of GBS growth in pH 5 THB. These data suggest that it will be important to study further how GBS is able to colonise the low pH environment of the vagina for apparently long periods of time. Live-dead staining results suggested that a large number of cells might enter into 'VBNC' state. Biofilm results showed there was biofilm formation of GBS and the biofilms were improved when 'feeding' with low nutrient media and coating surfaces with extracellular matrix molecules.

Chapter 4

Results and Discussion of proteomic investigation of GBS grown under conditions associated with neonatal exposure

4.1. Background

The rapid progression of early onset GBS disease (typically within 24 hr of birth) suggests that the most common route of vertical transmission is ascending infection, with the bacteria gaining access to the amniotic fluid followed by aspiration into neonatal lung. During the development of GBS disease, bacteria must adapt from conditions associated with vaginal carriage to those of neonatal infection and invasion. Proteomic investigations of GBS protein expression under conditions representing those associated with benign maternal colonisation and foetal exposure may help our understanding of the molecular basis of GBS virulence. To do this, an *in vitro* model of GBS growth under conditions reflecting material colonisation and neonatal exposure has been established. Proteins expressed under each growth condition were separated by 2D SDS-PAGE. Gel images were analyzed using PDQuest 8.0 and differentially expressed proteins were determined. Individual proteins were subjected to in-gel trypsin digestion and identified using LC/ESI-MS with peptide fragment fingerprinting. Proteins were then identified using Mascot searches and investigated using NCBI Blast searches and Uniprot searches.

4.2. Results

4.2.1. An *in vitro* model of GBS growth under conditions associated with neonatal exposure

An *in vitro* model of GBS growth under conditions associated with maternal colonization and neonatal exposure was established. GBS A909 grown in pH 5 THB with standing at early stationary phase reflected bacterial maternal colonization as the vagina is very acidic (pH normally less than 4.5), with low oxygen and, likely, limited nutrition. Inoculation of GBS A909 from pH 5 THB at early stationary to pH 7 THB medium with shaking and harvesting at mid-late exponential phase reflected GBS passage from the mother to the neonate as the neonatal lung and blood are near neutral pH and high oxygen conditions, with plenty of nutrients (as indicated by the known rapid growth of GBS *in vivo*).

GBS growth curves were determined by measuring the OD at 600 nm. The experiments were performed in triplicate. Figure 4.1 shows GBS A909 growth in pH 5 THB medium reached the onset of stationary phase at ca. 6 hours, with an OD_{600} of 0.6. GBS A909 growth in pH 7 THB medium reached mid-late exponential phase at 3 hours with an OD_{600} of 0.6 and entered stationary phase at 4 hours with OD_{600} of 0.9. These data are consistent with that previously presented in Figure 3.1. For proteomic analysis, GBS cells were harvested following growth to points P1 and P2 (Fig. 4.1). Thus cells at P1 represent GBS A909 grown in pH 5 THB with standing (i.e. low O_2) and were harvested at early stationary phase. Cells at P2 represent GBS A909 grown in pH 7 THB with shaking and were harvested at mid-late exponential phase. Growth of GBS A909 yielded the same biomass at points P1 and P2. This *in*

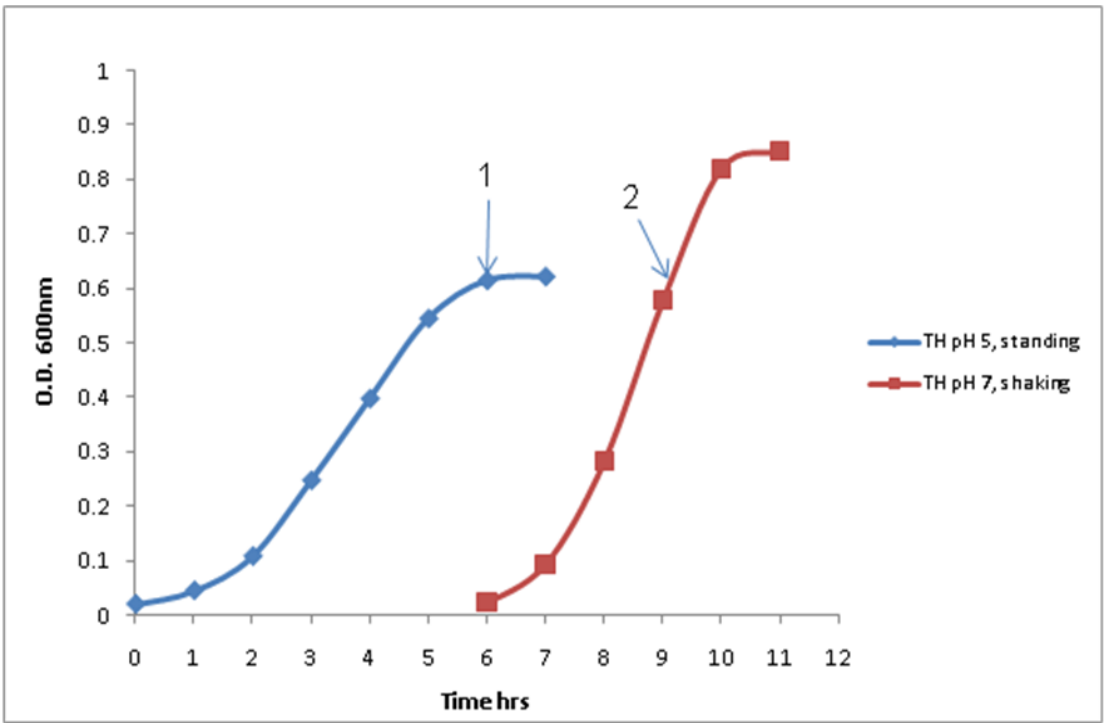


Figure 4.1. Representative GBS A909 growth curves under different growth conditions. Arrows indicate the two growth conditions considered relevant for proteomics.

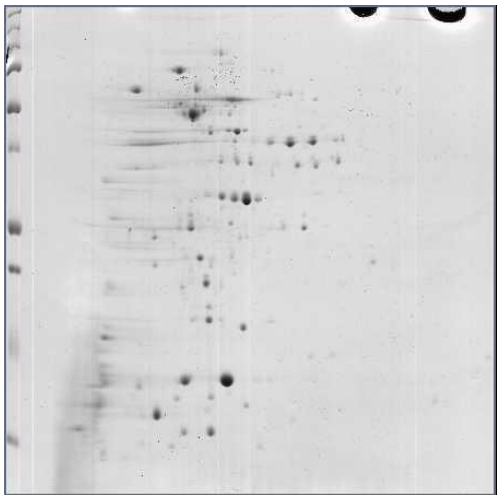
Condition 1: 50 ml THB pH 5 broth with microaerophilic conditions (standing culture). Condition 2: 50 ml THB pH 7 broth with aerated conditions (shaking, 150 rpm). Data are representative of triplicate experiments.

vitro model of the growth conditions such that cells harvested at P2 are adapted directly from cells grown under the conditions that prevail at point P1 also mimics the bacterial progression from colonisation to ascending infection via the amniotic fluid.

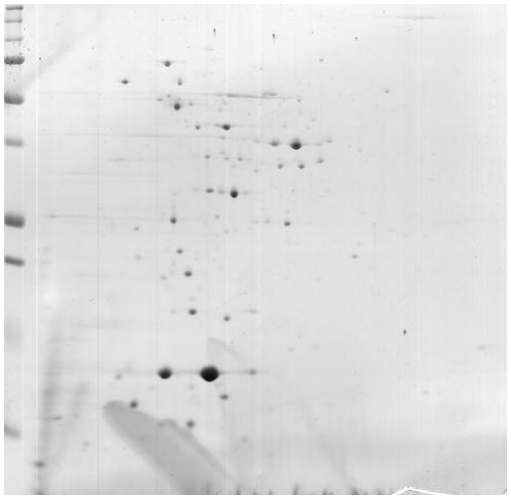
4.2.2. 2D SDS-PAGE and gel analysis

The optimised conditions of 2D SDS-PAGE for samples from GBS A909 were found to be (1) 50 ml of GBS A909 culture from each condition (P1 and P2, Fig. 4.1). (2) 100 µl of lysis solution (urea 8M, CHAPS 4% (w/v)) with 2% (w/v) IPG buffer (pH 4-7) was used to extract proteins. (3) a 2D clean-up kit (GE Healthcare) was used to precipitate protein and reduce interfering substances. (4) 350 µl rehydration solution was used to resuspend protein samples and load them on to Immobiline DryStrips. (5) Immobiline DryStrips pH 4-7, 18cm were used for IEF. (6) The first dimension was run for 8.5 hrs. (7) 14% SDS-PAGE was performed for the second dimension. (8) Colloidal Coomassie blue stain was used to visualize the gels.

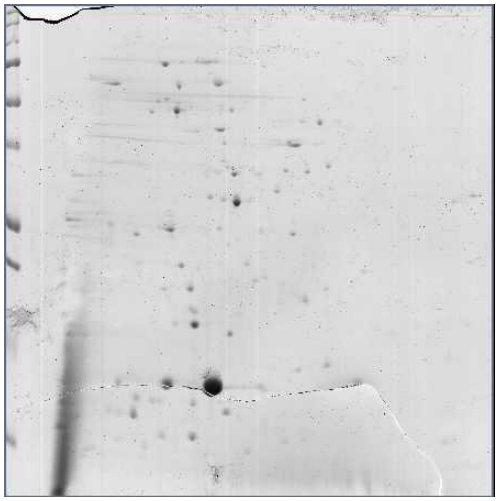
Six good quality 2DE gels from independent experiments for each growth condition were acquired (Figures 4.2 and 4.3). The gels were digitally photographed using a Bio-Rad GB-710 calibrated imaging densitometer and analyzed using PDQuest V8.0 software. After detecting and editing spots, the MatchSet analysis showed approximately 85 and 95 protein spots that were reproducibly visible on the gels from P1 and P2 harvested cells, respectively, and 89% of the protein spots were matched between the gels, with a correlation



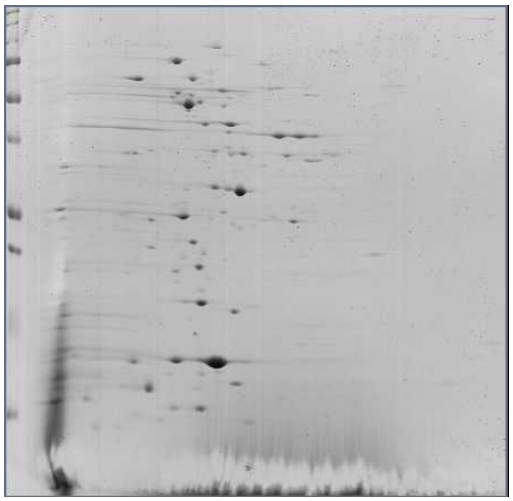
Condition P1 (1)



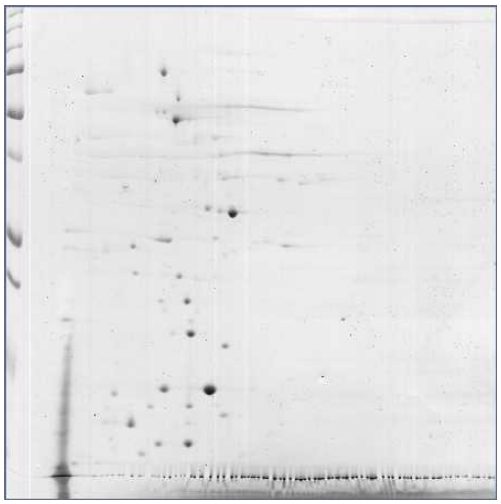
Condition P1 (2)



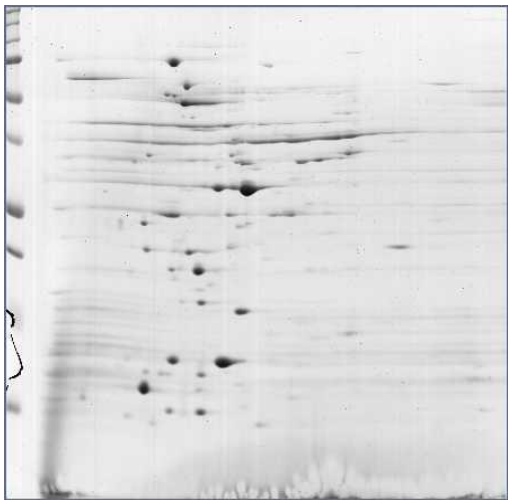
Condition P1 (3)



Condition P1 (4)

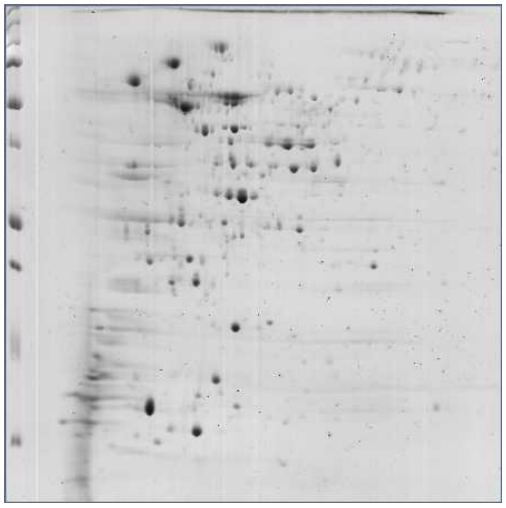


Condition P1 (5)

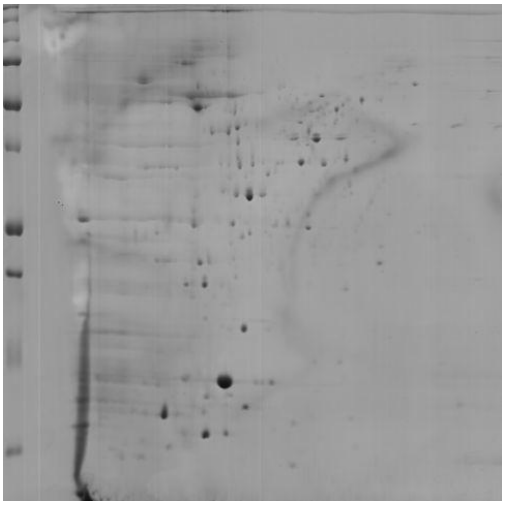


Condition P1 (6)

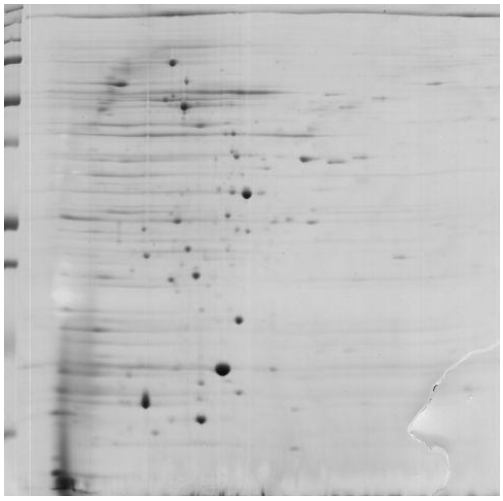
Figure 4.2. The six good quality 2D gels showing protein spots from cells harvested at condition P1. 100 µl lysis solution (urea 8M, CHAPS 4% (w/v)) was used to extract cells. Immobiline DryStrips pH 4-7, 18 cm were used for the first dimension (IEF), 14% (w/v) SDS-PAGE for the second dimension. Colloidal Coomassie blue stain was used to visualize the gel. Protein standards are shown at the left hand side of each gel (250kDa, 150kDa, 100kDa, 75kDa. 50kDa, 37kDa, 25kDa, 20kDa, 15kDa, 10kDa).



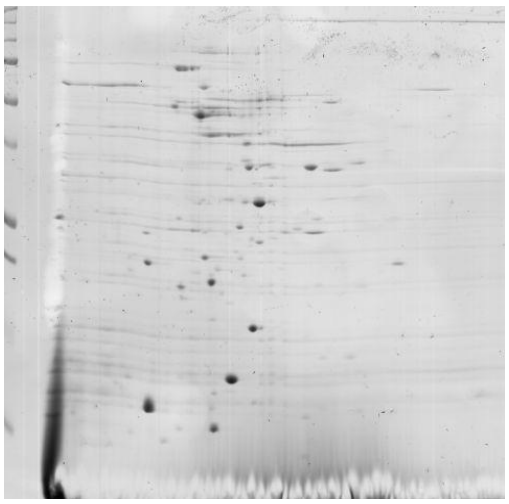
Condition P2 (1)



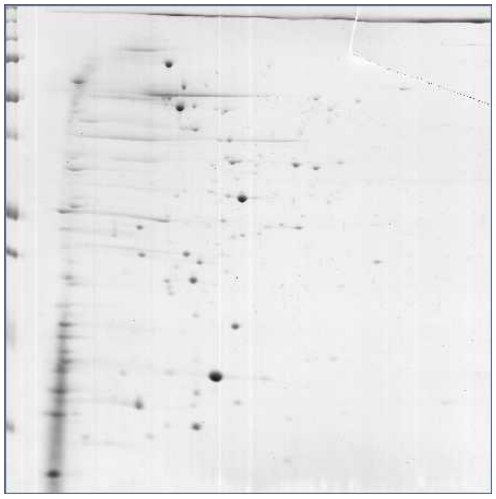
Condition P2 (2)



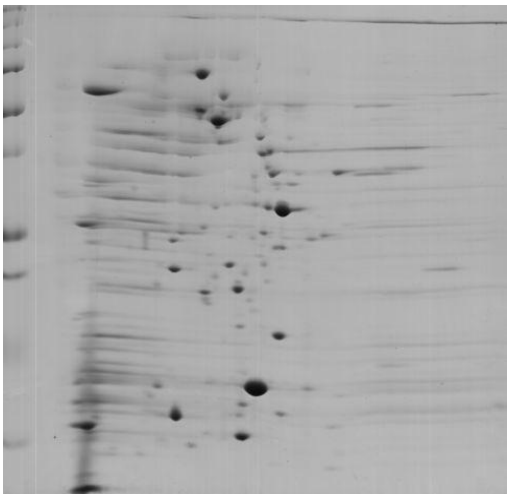
Condition P2 (3)



Condition P2 (4)



Condition P2 (5)



Condition P2 (6)

Figure 4.3. The six good quality 2D gels showing protein spots from cells harvested at condition P2. 100 µl lysis solution (urea 8M, CHAPS 4% (w/v)) was used to extract cells. Immobiline DryStrips pH 4-7, 18 cm was used for the first dimension (IEF), 14% (w/v) SDS-PAGE for the second dimension. Colloidal Coomassie blue stain was used to visualize the gel. Protein standards are shown at the left hand side of each gel (250kDa, 150kDa, 100kDa, 75kDa. 50kDa, 37kDa, 25kDa, 20kDa, 15kDa, 10kDa).

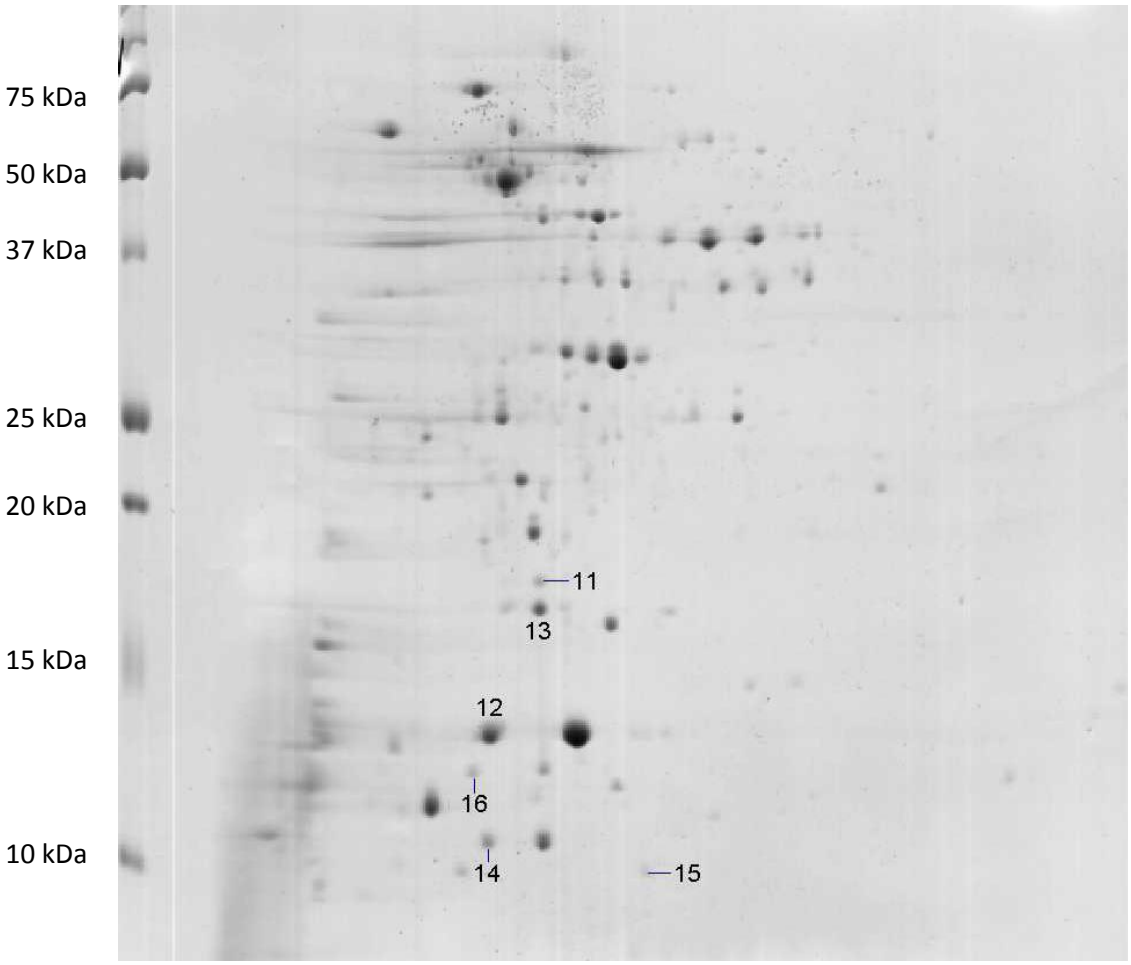


Figure 4.4. Merged master 2D gel of proteins from GBS A909 cultured in 50 ml pH 5 TH medium. Protein spots up-regulated at this growth condition (P1) are numbered (6 of the 16 differentially expressed proteins identified). Protein molecular weight standards are indicated at the left hand side of the gel.

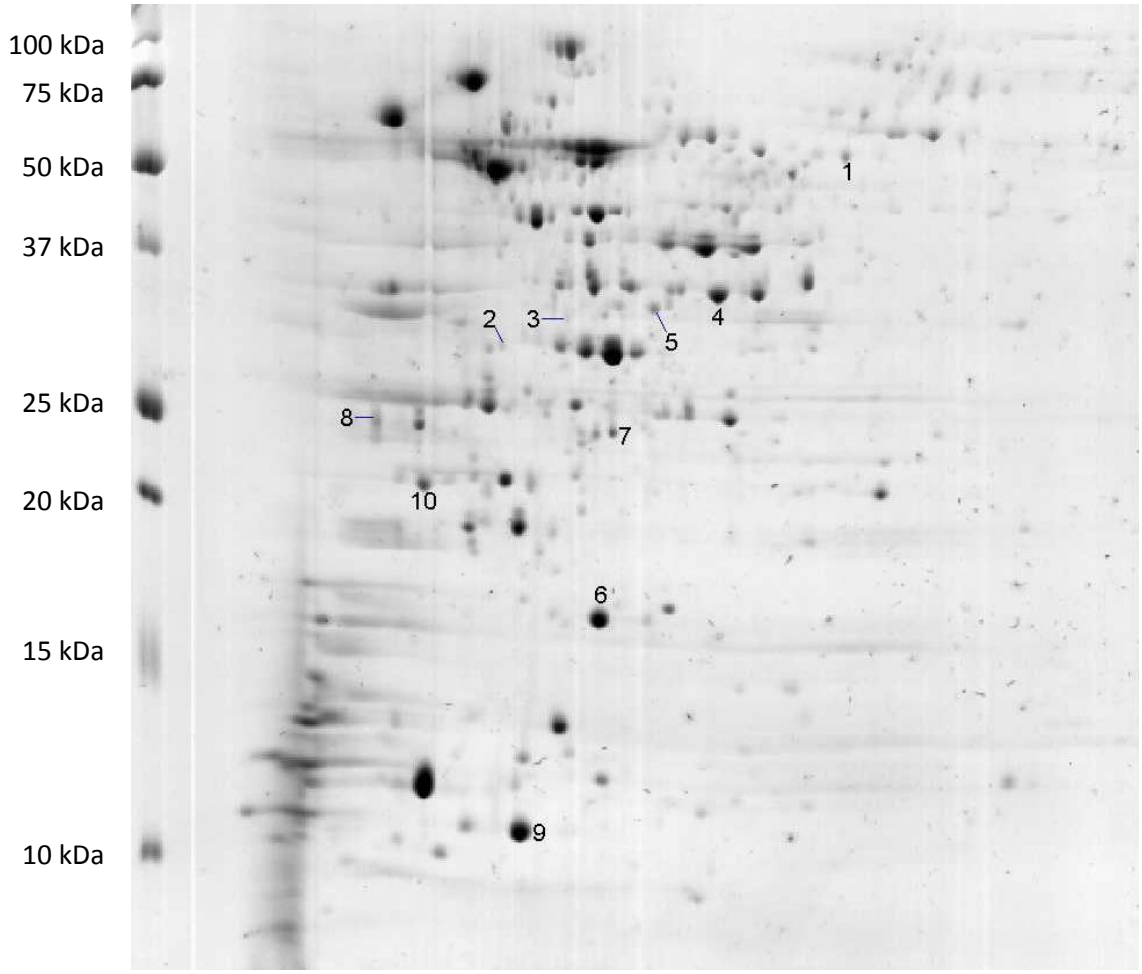


Figure 4.5. Merged master 2D gel of proteins from GBS A909 cultured in 50ml pH 7 TH medium. Protein spots up-regulated at this growth condition (P2) are numbered (10 of the 16 differentially expressed proteins identified). Protein molecular weight standards are indicated at the left hand side of the gel.

coefficient for 2 representative gels of 0.73. Merged master gels from this image analysis are shown in Figures 4.4 and 4.5. The quantitation of all of the protein spots was reviewed using the analysis tool of the Spot Quantity Table Report based on the 'spots' density. All protein spots from one condition expressed more strongly than the other condition were listed (Table 4.1 and Table 4.2) and the expression ratio (Table 4.3) was calculated. The Independent-sample t-test was used to determine statistically significant differences in the spot quantities from the two different conditions. Since $p<0.05$, there is a statistically significant difference in the mean scores of spot quantities from two different conditions.

4.2.3. Protein in-gel trypsin digestion, mass spectrometry analysis and protein identification

Following in-gel trypsin digestion, 7 µl of digested peptide sample with buffer was loaded and analysed by LC/ESI-MS (Section 2.2.6.). The mass spectrum data produced were up-loaded into Mascot search (MS/MS Ion Search) software. Protein identification, accession number, protein mass, score, matched peptides and percent amino acid coverage of the entire protein were produced for each spot. These data are summarised in Table 4.4 (proteins expressed without significant difference between the two growth conditions), Table 4.5 (proteins upregulated under condition P2) and Table 4.6 (proteins upregulated under condition P1).

Table 4.1. Spot quantification of 2D gels analyzed by PDQuest (all differentially expressed protein spots, quantified from cells harvested at condition P1). (SSP is sample spot protein.)

SSP	pH 5(1)	pH 5(2)	pH 5(3)	pH 5(4)	pH 5(5)	pH 5(6)	Mean	S.D.
2321	6.2	0	0	0	0	0	1.0	2.5
3307	31.8	19.9	22.9	46.8	24	30.7	29.4	9.7
4101	31.2	19.9	22.9	46.8	24	30.7	29.4	9.7
4103	85.8	25.2	73.7	71.3	3.4	185.8	79.4	57.3
4205	393.1	529.2	212.3	297.5	65.5	896.4	399.0	290.1
4423	7.7	0	0	0	0	0	1.3	3.1
5104	163	107.5	14.1	195.4	45.8	228.6	125.7	84.9
5203	37.4	27.5	13.9	25.7	35.8	15.2	25.9	9.9
5204	175.8	180.8	300	308.5	68	288.8	210.3	89.8
5413	0	0	0	0	0	0	0	0
6206	152.3	89.9	81.5	118	42.4	132.4	102.8	39.6
6310	8.6	7.7	4.3	11.6	0.6	4.7	6.3	3.9
7007	11.9	29.7	10.7	39.8	29.3	53.7	29.2	16.5
7515	74.8	42.1	25.0	65.7	27.9	77.9	52.2	23.6
7530	1.1	0	0	0	0	0	0.2	0.5
8722	0.1	0	0	0	0	0	0	0

Table 4.2. Spots quantification of 2D gel analyzed by PDQuest (all differentially expressed protein spots, quantified from cells harvested at condition P2).

SSP	pH 7(1)	pH 7(2)	pH 7(3)	pH 7(4)	pH 7(5)	pH 7(6)	Mean	S.D.
2321	15.5	2.7	4.2	4.1	1.7	22	8.4	8.4
3307	62.7	63.8	129.3	88.9	25.9	162.8	88.9	49.8
4101	0	0	0	0	0	0	0	0
4103	15.3	25.2	16.9	14	29.1	11.1	18.6	7
4205	29.3	7.6	0	74.7	90.2	31.6	38.9	36.2
4423	4.4	6	17	8.6	0	13.4	8.2	6.2
5104	211.2	379	206.1	519.5	426.4	394	356	124.2
5203	0	3.2	0	0	4.7	3.8	2.0	2.2
5204	15.9	5.1	22.6	10.4	13	29.2	16.0	8.7
5413	7	6	5.4	3.3	9.1	13	7.3	3.4
6206	159.4	269.9	229.7	298.5	226.7	288.4	245.4	51.5
6310	15.2	24	65	31.5	29.3	47.7	35.5	18
7007	0	0	0	0	0	0	0	0
7515	88.6	146.5	179.1	233.2	163.3	85.0	149.3	56.5
7530	0	21.3	22.5	14.6	24.2	34.1	19.5	11.4
8722	11.4	15.3	0	11.4	68	0	17.7	25.5

Table 4.3. Spot quantification and expression ratios of proteins expressed differentially between conditions P1 and P2. The final column shows the spot number for each protein as indicated on Figs. 4.4. and 4.5.

SSP	Protein	Spot density		Ratio P1:P2	Spot number	T test P value
		P1	P2			
2321	Uracil phosphoribosytransferase	1	8.4	0.12	8	0.065
3307	Alkyl hydroperoxide reductase, subunit C	29.1	88.9	0.33	10	0.016
4101	Co-chaperonin GroES	21.9	0	↑	16	0.001
4103	30S ribosomal protein S6	79.4	18.6	4.27	14	0.058
4205	Ribosomal protein L21	399	38.9	10.26	12	0.013
4423	ABC transporter, ATP-binding protein	1.3	8.2	0.16	2	0.034
5104	30S ribosomal protein S6	125.7	356	0.35	9	0.004
5203	Adenine phosphoribosyltransferase	25.9	1.95	13.28	11	0.002
5204	Hypothetical protein SAG1654	210.3	16	13.14	13	0.003
5413	Thioredoxin reductase	0	7.3	↓	3	0.000
6206	50S ribosomal protein L10 Hypothetical protein SAG0739	102.8	245.4	0.42	6	0.003
6310	Glutamine ABC transporter, ATP-binding protein Hypothetical protein SAG1211	6.3	35.5	0.18	7	0.003
7007	PTS system, IIB component, putative	29.2	0	↑	15	0.001
7515	Cysteine synthase A	52.2	149.3	0.35	4	0.003
7530	Dihydro orotate dehydrogenase 1A F_0F_1 ATP synthase subunit gamma	0.2	19.5	0.01	5	0.002
8722	C protein beta antigen	0	17.7	↓	1	0.120

A total of 76 protein spots could be confidently identified. Of these, 57 spots showed no statistically significant difference in expression between the 2 growth conditions (Table 4.4). These included 2 separate spots identified as glyceraldehyde-3-phosphate dehydrogenase. In addition to the 57 stably expressed proteins, 21 protein spots were reproducibly differentially expressed, 19 of which could be identified by LC-MS (Table 4.3). Of these, 13 proteins were more abundantly expressed in cells harvested at point P2 (Figure 4.5; Table 4.5), where as 6 proteins were more abundantly expressed in cells harvested at point P1 (Figure 4.4; Table 4.6).

4.3. Discussion

4.3.1. An *in vitro* model of GBS grown under conditions associated with neonatal exposure

The growth experiments demonstrated that growth of GBS at pH 5 is both slower and yields less biomass (lower final OD_{600} nm) compared to growth at pH 7, although the maximum growth rate at exponential phase was similar in each case (Figure 4.1.). These data are consistent with that in Chapter 3 (e.g. Fig 3.1), which showed slower GBS growth at pH 5 and lower final yields. In contrast, at each pH, GBS growth with aeration (shaking) was the same as that observed without aeration, as observed previously for GBS strain NEM316 grown in M17 medium (Yamamoto et al., 2005).

For proteomic analysis, GBS cells were harvested following growth to points P1 and P2 (Fig. 4.1). Cells at P1 represent GBS A909 grown in pH 5

Table 4.4. Proteins identified without change in expression level under growth conditions P1 and P2. Proteins in bold were also identified in a proteomics study of GBS surface proteins (Hughes et al., 2002).

SSP	Protein identification	Accession number	Score	Protein code	Matched peptides	% protein coverage
2111	Hypothetical protein SAG2091	gi|22538226	107	SAK_2030	2	21%
2704	Trigger factor	gi|76788439	255	SAK_0155	5	22%
3103	50S ribosomal protein L7/L12	gi|22537448	141	SAK_1334	2	19%
3119	30S ribosomal protein S16	gi|22537505	80	SAK_1391	2	33%
3305	Co-chaperone GrpE	gi|76788374	127	SAK_0146	2	12%
4204	Transcription enlongation factor GreA	gi|22537752	266	SAK_1627	4	35%
4214	Thiol peroxidase	gi|22537289	103	SAK_1217	2	15%
4305	ATP-dependent Clp protease proteolytic subunit	gi|22537725	101	SAK_1600	2	23%
4409	Triosephosphate isomerase	gi|22536927	411	SAK_0888	5	31%
4410	Peptidyl-prolyl cis-trans isomerase	gi|22536506	268	SAK_0393	5	18%
4420	Phosphoglycerate mutase	gi|22536928	206	SAK_0889	4	26%
4421	50S ribosomal protein L3	gi|22536243	148	SAK_0091	3	30%
4605	**Phosphopyruvate hydratase**	gi|22536801	369	SAK_0713	4	17%
4612	Dipeptidase PepV	gi|76787443	769	SAK_1305	10	29%

4614	Cell dividion protein FtsZ	gi|22536658	813	SAK_0581	14	46%
4804	**Molecular chaperone DnaK**	gi|22536282	734	SAK_0147	15	34%
5202	Gls24 protein, putative	gi|22537295	798	SAK_1223	10	72%
5218	Methionine sulfoxide reductase A	gi|22537653	122	SAK_1534	3	24%
5304	Hypothetical protein gbs1202	gi|22511251	300	SAK_1221	5	45%
5307	**Manganese-dependent superoxide dismutase**	gi|2765187	106	SAK_0931	2	13%
5313	Deoxyribose-phosphate aldolase	gi|22538205	69	SAK_2009	1	5%
5609	Elongation factor Ts	gi|22537970	683	SAK_1851	9	39%
5617	Aldehyde dehydrogenase family protein	gi|22537282	229	SAK_1211	4	17%
5621	F_0F_1 ATP synthase subunit beta	gi|22537026	1289	SAK_0986	20	65%
5701	**Chaperonin GroEL**	gi|22538209	1435	SAK_2013	20	54%
6112	Hypothetical protein SAG1760	gi|22537899	173	SAK_1782	4	67%
6113	Hypothetical protein SAG2138	gi|22538272	68	SAK_2096	2	42%
6205	Hypoxanthine-guanine phosphoribosyltransferase	gi|22536200	73	SAK_0014	2	13%
6305	dTDP-4-keto-6-deoxyglucose-3,5-epimerase	gi|22537357	280	SAK_1286	5	36%
6309	Hypothetical protein SAG1751	gi|22537890	509	SAK_1774	9	52%
6402	Transcriptional repressor CodY	gi|76786992	178	SAK_1687	4	22%
6403	Acetoin reductase	gi|22536701	571	SAK_0674	6	31%
6406	Adenylate kinase	gi|76787497	677	SAK_0122	12	63%

6412	Fructose-bisphosphate aldolase	gi|22536312	525	SAK_0178	7	31%
6521	Acetoin dehydrogenase, thymine PPi dependent	gi|22537042	496	SAK_1002	9	41%
6522	L-lactate dehydrogenase	gi|22537120	311	SAK_1054	5	21%
6524	Phosphotransacetylase	gi|22537250	376	SAK_1177	9	26%
6604	**Glucose-6-phosphate isomerase**	gi|22536585	706	SAK_0475	14	36%
6607	DNA-directed RNA polymerase subunit alpha	gi|76787566	369	SAK_0166	9	31%
6609	**Phosphoglycerate kinase**	gi|19171967	1075	SAK_1788	14	48%
7225	Hypothetical protein SAG1677	gi|22537817	301	SAK_1689	4	37%
7311	Dihydroxyacetone kinase family protein	gi|22537791	277	SAK_1663	5	44%
7406	Uridylate kinase	gi|22537656	328	SAK_1537	5	35%
7413	Purine nucleoside phosphatase	gi|19171971	308	SAK_1267	4	32%
7513	**Glyceraldehyde-3-phosphate dehydrogenase**	gi|22537907	507	SAK_1790	8	34%
7613	**Glyceraldehyde-3-phosphate dehydrogenase**	gi|22537907	312	SAK_1790	4	18%
7526	UDP-glucose 4-epimerase	gi|22538061	71	SAK_1882	1	3%
7731	Pyruvate kinase	gi|22537102	704	SAK_1073	10	30%
7810	ATP-dependent Clp protease, ATP-binding subunit	gi|22537450	683	SAK_1336	13	30%
8205	50S ribosomal protein L6	gi|22536258	229	SAK_0106	4	43%
8304	Ribosome recycling factor	gi|22537655	271	SAK_1536	4	34%
8401	Hypothetical protein SAG0963	gi|22537124	268	SAK_1058	5	22%

8416	1-Phosphofructokinase	gi|22537494	184	SAK_1378	3	13%
8502	6-Phosphofructokinase	gi|22537101	466	SAK_1036	6	25%
8609	Phosphocarrier protein HPr	gi|22536985	68	SAK_0945	1	11%
6603	Inositol-5-monophosphate dehydrogenase	gi|22536517	639	SAK_2117	9	34%
9107	Hypothetical protein SAG0302	gi|22536486	190	SAK_0373	4	48%

Table 4.5. Proteins upregulated in cells harvested at growth point P2. Cysteine synthase A (bold) was also identified in a proteomics study of GBS surface proteins (Hughes et al., 2002).

Spot #[a]	Protein identification	Accession number[b]	Score[c]	Protein code	Matched peptides[d]	% Protein coverage[e]	Expression ratio
1	c protein beta antigen	gi|46521	113	SAK_0186	2	2%	↓
1	c protein beta antigen	gi|37572662	111	SAK_0186	2	2%	↓
2	FeS assembly ATPase SufC	gi|22536326	221	SAK_0199	4	23%	0.16
3	Thioredoxin reductase	gi|22536478	130	SAK_0366	6	26%	↓
4	**Cysteine synthase A**	gi|22536517	616	SAK_0404	13	53%	0.35
5	Dihydroorotate dehydrogenase 1A	gi|22536686	446	SAK_0657	9	36%	0.01
6	Hypothetical protein SAG0739	gi|22536903	74	SAK_0865	1	11%	0.42
5	F_0F_1 ATP synthase subunit gamma	gi|22537025	57	SAK_0985	1	6%	0.01
7	Hypothetical protein SAG1211	gi|22537369	91	SAK_1297	2	16%	0.18
6	50S ribosomal protein L10	gi|22537449	512	SAK_1335	27	60%	0.42
7	Glutamine ABC transporter, ATP-binding protein	gi|22537610	266	SAK_1498	8	25%	0.18
8	Uracil phosphoribosytransferase	gi|22537726	105	SAK_1601	2	17%	0.12
9	30S ribosomal protein S6	gi|22537853	98	SAK_1722	1	16%	0.35
10	Alkyl hydroperoxide reductase, subunit c	gi|22537972	186	SAK_1853	5	31%	0.33

[a] Spot number refers to the proteins labelled in Fig 4.3.

[b] Accession ID of each protein is the GenInfo number in the NCBI protein database.

[c] Mascot score.

[d] Number of nonredundant peptides identified for each protein.

[e] Percent amino acid coverage of entire protein.

[f] Ratio of the protein quantity in condition P1 versus condition P2 (values less than 1 indicate greater expression at P2).

Table 4.6. Proteins upregulated in cells harvested at growth point P1.

Spot #[a]	Protein identification	Accession number[b]	Score[c]	Protein code	Matched peptides[d]	% Protein coverage[e]	Expression ratio
11	Adenine phosphoribosyltransferase	gi|22537363	58	SAK_1292	1	8%	13.28
12	Ribosomal protein L21	Q3D9Y2_STRAG	91	SAK_1403	1	16%	10.26
13	Hypothetical protein SAG1654	gi|22537794	192	SAK_1666	4	34%	13.14
14	30S ribosomal protein S6	gi|22537853	68	SAK_1722	2	16%	4.27
15	PTS system, IIB component, putative	gi|22538072	280	SAK_1894	16	59%	↑
16	Co-chaperonin GroES	gi|22538210	109	SAK_2014	3	41%	↑

[a] Spot number refers to the proteins labelled in Fig. 4.4.

[b] Accession ID of each protein is the GenInfo number in the NCBI protein database.

[c] Mascot score.

[d] Number of nonredundant peptides identified for each protein.

[e] Percent amino acid coverage of entire protein.

[f] Ratio of the protein quantity in condition P1 versus condition P2.

THB with standing (low O_2, microaerophilic) and were harvested at early stationary phase. The autoacidification data (Chapter 3, Table 3.7.) indicate that at early stationary phase the pH will have dropped to approximately 4.5. Cells at P2 represent GBS A909 grown in pH 7 THB with shaking and were harvested at mid-late exponential phase. An important feature of the experimental design was the sequential nature of the growth conditions such that cells harvested at P2 are adapted directly from cells grown under the conditions that prevail at point P1. This was designed to mimic the bacterial progression from colonisation to ascending infection via the amniotic fluid. Conditions at P1 reflect GBS colonisation in the maternal vagina, which is acid and predominately anaerobic. Conditions at P2 reflect GBS infection of the neonate, which typically occurs at neutral and aerobic environments (e.g. the neonatal lung and blood).

There are some spots which were identified as containing more than one individual protein. Where this mixed spot is considered to be significantly differentially expressed between two conditions, this will affect the interpretation of the results as only one protein or both proteins in the spot may be expressed differentially. This needs further studies on this mixed spot by protein quantification. Protein quantification in MS-based proteomics is most commonly achieved by using the stable isotope method. Isotope-coded affinity tag (ICAT) and stable isotope labelling with amino acid in cell culture (SILAC) have been recently used to label proteins in protein quantification experiment (Lane, 2005). Moreover, it is also conceivable that some spots identified as containing more than one protein may in fact contain unrecognised differentially expressed

proteins, if one protein is induced and the other reduced, thereby cancelling any change in total spot levels.

Confidence in the protein identification was achieved by applying the following criteria: 1) the protein hits (positive peptides) were from Group B *Streptococcus* ; 2) only peptides for which the MS/MS spectra exceeded Mascot's significance level (mascot score >40) were included; 3) the query matched peptides ≥2. The mass spectra detected by MS was compared with the MS/MS fragment masses calculated from peptide sequences in the database and a match score is calculated (Lane, 2005). The Mascot peptide score reflects the statistical significance of the match between the experimental and theoretical spectra. Each peptide ID is an independent measurement and the protein score is a total of the peptide scores. A high confidence protein identification can be assumed with a high mascot score (Chepanoske, et al., 2004). Average Peptide Score (APS) has recently been proposed for determining accurate protein identification. This method is approached by first filtering out low-scoring peptide identifications and then eliminating protein identifications with APS scores lower than the level of a correct score. Thus a high average APS reflect a more confident protein identification (Shadforth, et al., 2005).

4.3.2. Expressed proteins that showed no significant differences between the two growth conditions

57 protein spots showed no statistically significant difference in expression between the two growth conditions (Table 4.5). These included 2 separate

spots identified as the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) which has been identified as a surface associated virulence factor in GBS and other streptococci, being notable for its ability to bind human plasminogen and fibrinogen (Hughes et al., 2002; Madureira et al., 2007; Magalhães et al., 2007). Although lacking a signal peptide directing protein secretion, GAPDH has previously been identified as a GBS surface protein (Hughes et al., 2002). Both the data here and that of Hughes et al. (2002) suggest that GAPDH is produced as at least two isoforms. Surface associated GAPDH may contribute to GBS virulence by binding host plasminogen and activating it to the plasmin protease. GAPDH is also involved in an indirect plasmin binding pathway via initial binding of fibrinogen at the cell surface and assisting plasminogen activator complex formation. GBS GAPDH binding to plasminogen by either the direct or indirect pathway may contribute to bacterial invasion and infection in the host (Magalhães et al., 2007). Madureira et al. found that GAPDH could induce B cell activity in both *in vitro* and *in vivo* models and that recombinant GADPH could increase CD69 expression on B lymphocytes. This effect was abolished in the presence of antibodies to recombinant GAPDH or inactive recombinant GAPDH. GAPDH can increase bacterial growth in mice, as mice were more susceptible to a GBS GADPH over-expression strain than the wild type strain (Madureira et al., 2007). These data suggested GBS GADPH may have an immunomodulatory function (Madureira et al., 2007). Terao et al. (2006) revealed *S. pyogenes* soluble GAPDH binds C5a directly and enhances ScpA cleavage of C5a. GAPDH also inhibited C5a induced neutrophil chemotaxis and H_2O_2 production in a dose-dependent manner. Since C5a plays an important role in the complement pathway of the immune system, GAPDH

binding and further cleavage of C5a may help *S. pyogenes* escape host immune cleance (Terao et al., 2006). GAPDH is an essential enzyme of bacteria involved in carbohydrate metabolism. *S. pneumoniae* GAPDH also showed affinity for plasmin and plasminogen (Bergmann et al., 2003).

As well as GAPDH, this study identified 8 other proteins (Table 4.4, Table 4.5) from the 14 GBS proteins previously identified by Hughes et al. (2002). All of these proteins are cytoplasmic proteins that, despite lacking signal peptides, are also apparently present as cell surface proteins (Hughes et al., 2002). Notably, in the present study, these proteins were recovered from whole cells but their localisation (cytoplasmic or surface associated) was not determined.

4.3.3. Proteins more abundantly expressed in cells harvested at point P2

The putative GBS virulence factor C protein β antigen (Bac, SAK_0186) was expressed more abundantly in cells from point P2. This analysis detected a ca. 55-kDa form of Bac (Fig. 4.5, spot 1). Mature Bac is predicted to be a protein of ca. 123 kDa, which is wall anchored as the protein contains a canonical C-terminal LPXTG motif for anchoring of proteins to the peptidoglycan by sortase (Lindahl et al., 2005). However, release of a ca. 55 kDa form of Bac by GBS strains, including A909, has been reported by Brady and Boyle (1989). Thus, it is likely that it is this form of the protein has been detected in the present study. Moreover, the tryptic peptides detected here were localised to the N-terminus of the protein, i.e. that region likely to be released by proteolysis of the C-terminally anchored mature form.

The Bac protein C-terminal part includes a proline-rich region, a highly periodic XPZ sequence of amino acid residues, which is an important structure for protein-protein interactions. However, the function of the XPZ region in GBS is not clear. This sequence varied in length among different GBS strains (Areschoug et al., 2002). NCBI Blast analysis of the Bac sequence showed there are other functional domains located in Bac (Fig. 4.6): a YSIRK motif is located within the N-terminal signal peptide, which is strongly correlated with the C-terminal LPXTG Gram positive anchoring motif; the immunoglobulin-like region is located in the central part of Bac; and a fibronectin-binding motif is located close to the C-terminus, which can bind to host fibronectin in the extracellular matrix (Fig. 4.6).

The approximate cleavage site of the low molecular weight Bac from whole Bac is shown in Fig. 4.6. The presence of low molecular weight Bac may be caused by cleavage of Bac during protein extraction or proteolysis by enzymes produced by GBS itself.

The upregulation of Bac under condition P2, where cells were growing at a faster growth rate (mid-exponential phase) is clearly consistent with the data of Ross et al. (1999) who observed 5.5 fold more Bac production in strain A909 at faster growth rates than at slower growth rates. Expression of *bac* (*SAK_0186*) is under control of a two component regulatory system (*sak_0188-sak_0189*) associated with *bac* in a ca. 9 kb genomic island (Tettelin et al.,

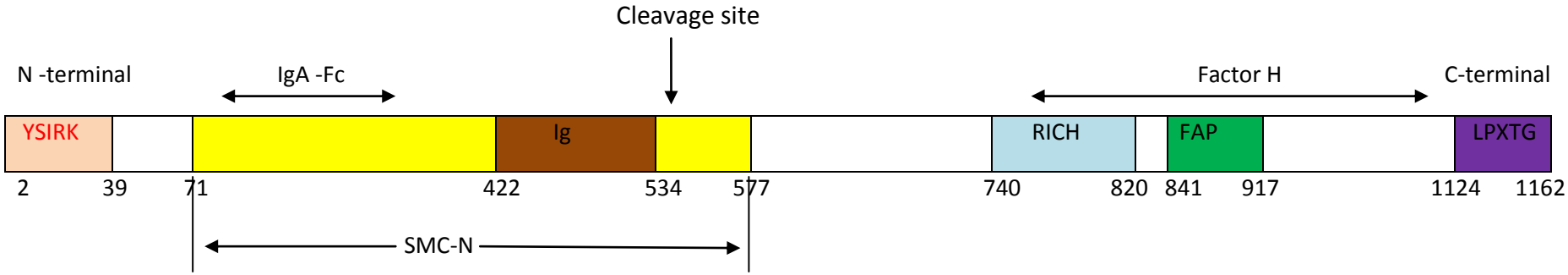


Figure 4.6. Schematic diagram of GBS A909 C protein beta antigen structural domains.

YSIRK: Gram positive signal peptide, YSIRK family, (2-39).

SMC-N: SMC N-terminal domain, (71-577). IgA-Fc: Immunoglobulin binding domain

Ig: Immunoglobulin-like domain, (442-534).

RICH: RICH ('rich in charged residues') domain, (740-820).

FAP: Fibronectin-attachment domain, (841-917).

LPXTG: LPXTG cell wall anchor domain, (1124-1162).

2005; Dmitriev et al., 2006; Nagano et al., 2006; Rozhdestvenskaya et al., 2010). Two component regulatory systems (TCS) are composed of a sensor histidine kinase and a DNA-binding response regulator that control gene expression in response to environmental signals such as stress. Higher levels of Bac expression have been detected in invasive GBS strains compared to vaginal carriage strains (Nagano et al., 2002) but the data presented here and that of others (Ross et al., 1999; Rozhdestvenskaya et al., 2010) clearly indicate that the levels of this protein can be regulated in response to specific growth conditions in individual strains. Consequently, growth enviroment rather than simply strain origin (carriage versus invasive) may be the key to Bac gene expression.

Bac is able to simultaneously bind the Fc region of IgA antibodies and the complement regulator factor H, which suggests a role in immune evasion (Areshoug et al., 2002; Lindahl et al., 2005). Bac specifically binds to the Fc region of IgA, the binding site for which is located at the Bac N-terminal region (the region detected here; Figure 4.6). Human IgA is an important immunoglobulin on mucosal surfaces and protects the host against bacterial invasion. Bac binding to the Fc portion of IgA may also block IgG or complement deposition on the GBS surface. Alternatively, this binding may inhibit IgA binding to the human IgA receptor, CD89, to allow GBS to evade immune elimination (Pleass et al., 2000; Nagano et al., 2002). Bac also binds human complement regulator factor H (FH), a 150 kDa single chain plasma glycoprotein. The Bac binding site for FH is localized in the C-terminal part of the protein, which is separate and non-overlapping with the IgA binding site

(Fig. 4.6). FH plays an important role in regulation of complement activation via the alternative pathway. FH bound to Bac retains its complement regulating ability and may lead to the destruction of the surface bound complement component C3b, which in turn may prevent phagocytosis (Areshoug et al., 2002b). Bac binding to IgA and FH may therefore play an important role in GBS disease by contributing to immune evasion. As condition P2 is hypothesised to mimic conditions associated with invasive foetal GBS exposure (e.g. entry into the amniotic fluid or blood), it is clearly of interest that this putative virulence factor linked to immune evasion is upregulated despite the absence, under the *in vitro* conditions used, of the actual components of the immune system that might be predicted to upregulate Bac expression. However, it is unclear why a mutant in which the *sak_0188-sak_0189* TCS was inactivated showed decreased *bac* expression but also showed moderate increase in virulence in a mouse intraperitoneal infection model (Rozhdestvenskaya et al., 2010).

Although Bac elicits protective antibodies in mice (Yang et al., 2007), its utility as a vaccine candidate is limited by its restricted distribution within the GBS population, being associated primarily with serotype Ib, some sereotype II, Ia and V strains (Nagano et al., 2002, 2006; Lindahl et al., 2005; Kong et al., 2006; Manning et al., 2006), and the recently described serotype IX (Slotved et al., 2007). This restricted distribution was confirmed in a recent typing study of 238 GBS isolates from both clinical and bovine sources (Sørenson et al., 2010). The *bac* gene was only associated with the largest cluster identified (cluster A, 117 isolates in total) and was detected in only 36 isolates (31% of ‘cluster A’ isolates; 15% of the total isolates studied).

Several of the other proteins upregulated at P2 can be linked to responses to oxidative stress, consistent with the shift from microaerophilic to aerobic culture of the cells harvested at this point. SAK0199 is a predicted SufC ATPase belonging to a putative SUF cluster for the biogenesis of [Fe-S] proteins, as in other *Firmicutes* (Ayala-Castro et al., 2008; Riboldi et al., 2009). Fe-S clusters provide redox-active cofactors for a wide variety of biological processes and so this machinery may therefore be needed to assist in the adaptive response to growth in oxidative conditions. Exposure of *E. coli* to oxidative stress environments leads to a significant upregulation of *suf* transcription (Ayala Castro et al., 2008; Riboldi et al., 2009). Interestingly, the SAK0404 cysteine synthase (CysK) was also upregulated along with SAK0199. CysK has also been implicated as part of the stress response of several bacteria, including *Lactococcus lactis*, and may also as a source of sulphide for (Fe-S) cluster biogenesis (Fernandez et al., 2002).

SAK0366 is a predicted thioredoxin reductase (TrxB). The thioredoxin-thioredoxin reductase interaction is likely to be of significance in sensing oxidative stress in streptococci as thioredoxin reductase has been shown to be a component of the oxidative stress response of lactococci and lactobacilli (Vido et al., 2005; Serrano et al., 2007). TrxB is a member of the flavoprotein oxidoreductase family, which provide protection against oxygen species. NAD(P)H TrxR-dependent Trx reduction provides electrons to peroxidases for conversion of peroxide to alcohols. Serrano et al. (2007) found overproduction of TrxB in *L. plantarum* triggered the induction of 16 gene transcripts which were associated with oxidative stress responses (Serrano et al., 2007). TrxB

was also found to be upregulated under respiration conditions in *L. lactis*. A TrxB inactive *L. lactis* strain grown in aerobic conditions had a longer lag phase compared with wild type strain (Vido et al., 2005). This suggests *trxB1* inactivation may lead to hydrogen peroxide (H_2O_2) accumulation. The growth defect was restored by adding antioxidants including catalase, glutathione, cysteine and pyruvate. Thus the Trx system provides protection against oxidative stress (Vido et al., 2005).

SAK1853 is a putative alkyl hydroperoxide reductase, which is encoded in an operon with SAK1854, the associated AhpF subunit. AhpCF is thought to be involved in the response to peroxide stress of other Gram-positive bacteria (Brenot et al., 2005; Vido et al., 2005; La Carbona et al., 2007). In an *S. pyogenes* AhpC deletion strain, peroxide degradation was 60% slower than in the wild type strain (Brenot et al., 2005). The GBS *ahpCF* operon has been shown to be part of the CsrRS (CovRS) regulon, which has an important impact on virulence factor expression (Jiang et al., 2008; Santi et al., 2009). Recently, GBS AhpC has been shown to be a haem-binding protein that may influence both respiration metabolism, which depends on exogenous haem and menaquinone (Yamamoto et al., 2005) and may affect a haem dependent catalase activity (Lechardeur et al., 2010). AhpC can bind haem without change in its catalytic activity. Respiration was decreased in a GBS *ahpC* mutant (Lechardeur et al., 2010). Thus is it is likely that the observed induction of AhpC in this proteomics study reflects the increased oxidative stress of growth in aerated culture. In contrast to these observations, it was notable that the SodA superoxide dismutase (SAK0913) was identified among the proteins not found

to be differentially expressed in the present study, consistent with previous observations that expression of SodA is not affected by aerobic growth or growth at lowered pH (Gaillot et al., 1997).

Several of the proteins upregulated in cells at P2 can be linked to the increased growth rate of mid-exponential phase cells. These include dihydroorotate dehydrogenase 1A (SAK0657) and uracil phosphoribosytransferase (SAK1601), which are needed for pyrimidine nucleotide biosynthesis, the F_0F_1 ATP synthase gamma subunit (AtpG, SAK0985), and ribosomal proteins L10 and S6 (SAK1335, SAK1722). The upregulation of the SAK1498 ATPase component of the putative glutamine ABC transporter (SAK1497-SAK1498) also emphasises the likely need for increased amino acid uptake to support growth.

In addition to these proteins, two proteins of unknown function were upregulated in cells grown under condition P2. SAK0865 is a predicted secreted protein and its gene is located in the predicted 4-gene operon *sak0862-sak0865*, also encoding HPr kinase, the lipoprotein biosynthetic enzyme Lgt (Bray et al., 2009) and a conserved hypothetical protein belonging to PFAM family PF06103. SAK_0862 is HPr kinase/phosphorylase, which catalyzes phosphorylation of the phosphocarrier protein HPr of the GBS phosphotransferase system. Bioinformatic analysis and biochemical evidence have demonstrated that SAK_0863 is the GBS prolipoprotein diacylglyceryl transferase (Lgt protein), which lipidates lipoprotein precursors and is involved in Lpp anchoring into plasma membrane (Bray et al, 2009). SAK_0864 is a conserved hypothetical protein of unknown function, which belongs to protein

family PF06103. This genetic locus is well conserved in streptococcal genomes and although none of the other 3 proteins encoded were detected as differentially expressed in this study, it is intriguing that PF06103 proteins may be associated with general stress responses and a GBS Lgt deletion strain also showed sensitivity to oxidative stress (Bray et al, 2009). The protein shown here to be induced at P2, SAK0865, has also been identified as a component of the GBS phosphoproteome that is responsive to the eukaryotic type serine/threonine kinase Stk1 (Silvestroni et al., 2009). Stk1 has been well established as one of the key GBS regulators of both virulence factor expression (Rajagopal et al., 2005; Rajagopal, 2009) and purine metabolism (see below; Rajagopal et al., 2005). However, it is also noted that 3 proteins belonging to the Stk1-responsive phosphoproteome (SAK0373, SAK0581, and SAK2030; Silvestroni et al., 2009) were identified among those proteins stably expressed in the present study (Table 4.4). Two of these proteins (GpsB, SAK0373; FtsZ, SAK0581) are linked to the cell division process whereas the function of SAK2030 remains unknown, although this gene is apparently not essential (Silvestroni et al., 2009).

The gene for the other protein of unknown function identified, SAK1297, is apparently located in an operon with SAK1298, which encodes an HflX family GTPase. This operon structure is well conserved in streptococcal genomes and it is noted that the N-terminus of SAK1297 is distantly related to putative galactose-1-phosphate uridylytransferase proteins, most likely as it represents a nucleotide-binding domain which could suggest a functional interaction with the GTPase.

4.3.4. Proteins more abundantly expressed in cells harvested at point P1

Only six proteins were identified as being more abundant in cells harvested at point P1 (Table 4.6), which is hypothesised to reflect conditions associated with GBS colonisation (i.e. low pH and O_2, nutrient stress). Consistent with this, the increased expression of the SAK2014 GroES chaperonin was observed in these cells. Mascot search results showed GroES is composed of 94 amino acids and the theoretical molecular weight was 10 kDa (Figure 4.7). GroES has previously been linked to the stress responses of diverse lactic acid bacteria, including acid tolerance or adaptation of *S. mutans* (Lemos et al., 2001), *L. lactis* (Frees et al., 2003), and *Lactobacillus bulgaricus* (Fernandez et al., 2008), and so its induction in early stationary phase (following auto-acidification of the medium to pH 5) is expected. As in *S. mutans, S. pneumoniae* and *S. pyogenes* (Lemos et al., 2001; Chastanet et al., 2001; Woodbury and Haldenwang, 2003), the GBS groES-groEL locus is preceded by a perfect CIRCE ('controlling inverted repeat of chaperone expression') element suggesting comparable regulation by the stress response regulator HrcA (SAK0145 in GBS strain A909). Sequence analysis has shown that the sequence of CIRCE is TTAGCACTC-N9-GAGTGCTAA (Woodbury and Haldenwang, 2003), which is located 37 bp upstream from the *groES* start codon (Figure 4.8). HrcA binding to the CIRCE element negatively regulates *groES* and *groEL* expression in Gram positive bacteria. mRNA levels of *groES* and *groEL* were up-regulated in a *S. mutans hrcA* inactivation strain compared with the wild type strain (Lemos et al., 2001). GroES and GroEL are stress proteins, which respond to environmental changes. These proteins also play

important roles in assisting the folding of new or denatured proteins and protein assembly, transport and degradation (Lemos et al., 2001). Moreover, as in *S. pneumoniae* (Chastanet et al., 2001), a binding site for the CtsR regulator (SAK1849 in GBS strain A909) is located 16 bp upstream from the highly conserved CIRCE regulator of *groESL*, suggesting this operon may be regulated by both CtsR and HrcA (Fig. 4.8). However, whilst upregulation of GroES was observed at point P1, GroEL was identified among the stably expressed proteins, suggesting some differences in the regulation of the expression of these proteins, which are typically cotranscribed (Lemos et al., 2001; Woodbury and Haldenwang, 2003).

Also upregulated in cells harvested at point P1 were SAK1292 and SAK1894. SAK1292 is the putative GBS adenine phosphoribosyltransferase, which is linked to both the salvage and *de novo* pathway of purine biosynthesis (Rajagopal et al., 2005). The regulation of GBS purine biosynthesis by the Stk1 serine/threonine kinase (Rajagopal et al., 2005) suggests that it is important for GBS to be able to respond to nutrient stress *in vivo*.

SAK1894 is a predicted PTS phosphotransferase protein, which Sutcliffe et al. (2008) have previously speculated is a part of a galactitol uptake system that supplies precursors needed for the biosynthesis of the GBS Group B carbohydrate. The PTS pathway is composed of four proteins, EI, HPr, EIIA and EIIB, which can catalyze the transportation and phosphorylation of carbohydrates and regulate their activity (Rajagopal et al., 2005).

Two other proteins (SAK1403 and SAK1722) were identified as the ribosomal proteins L21 and S6. Of these, a distinct protein spot upregulated at

MLKPLGDR**VI ISFVETEEK**S VGGFVLAGAS HDATKTAKVL AVGDGIR**TLT**
GELVAPSVAE GDTVLVENGA GLEVKDGNEK VTVVRESDIV AVVK

Figure 4.7. GroES (SAK_2014) amino acid sequence. Sequences highlighted in red: matched peptides identified by LC-MS; Protein sequence coverage is 41%.

```
2009161 tcttttgcca taatttaata cccaggccta aaatttaaca aagtctactt tctttacaaa
2009221 gcaacttttc tctaataggc ctatatcctt tcttgcttaa taaaacctat ttaacgacag
2009281 caacaatgtc actttcacga acaactgtca ctttttcgtt accgtcttta acttccaaac
2009341 cagcaccatt ttcgactaaa acagtatcac cctctgcaac gctaggtgca actaattccc
2009401 ctgttaaggt acgaataccg tcacccacag caagaacttt ggctgtcttt gtcgcatcat
2009461 ggcttgctcc tgcaagaaca aaaccaccga cagatttctc ttcagtttca acaaacgaaa
2009521 tgatcacacg atcacctaat ggttttaaca taataaacga acctccatac gagatatatt
2009581 ttgatttttt agcactccta tacagagagt gctaactaca aaaactattc tatcacttgg
2009641 tcagaattag tcaagagaaa aggcacgttt caaacaagaa aaatttccct agttatcctg
2009701 tccattcact agagaaattt atatttaac ctaagacaag ggcatcatct atcaactgtt
2009761 gacctgagtt tttatggaaa agttccatta attctgcaac tgttaagttt ctctttgctt
```

Figure 4.8. DNA sequence of groES and groEL operon of GBS showing the intergenic CIRCE element. Blue highlight: CIRCE element; Grey highlight: *groES* (*sak_2014*) coding sequence (encoded on the complementary strand); Green highlight: start of the *groEL*/*SAK_2013* coding sequence (encoded on the complementary strand); Purple highlight: heptad binding sites for the CtsR regulator; Yellow highlights: partial downstream coding sequences for SAK_2015, a putative ABC transport system ATP-binding protein.

P2 was also identified as the ribosomal protein S6 (Table 4.6; Fig. 4.4), indicating that different isoforms of this protein are produced under the conditions examined here.

Finally, a conserved hypothetical protein, SAK1666, a member of the cupin super family was identified. The function of this protein is difficult to predict as cupins are a very diverse protein superfamily (Dunwell et al., 2004). The cupin domain is composed of conserved motifs connected with β-strands, which form a small β-barrel (Dunwell et al., 2003). SAK1666 has a distant sequence similarity to the cupin sugar-phosphate isomerase subfamily. Cupin type phosphoglucose isomerases (cPGIs), play an important role in sugar metabolism, catalyzing the reversible isomerisation of glucose-6-phosphate to fructose-6-phosphate (Hansen et al., 2004). Notably, the closest protein homologues of SAK1666 identified by BLAST searches are archaeal proteins from member of the genus *Methanosarcina*, which exhibit very high (75%) amino acid sequence identity and it is intriguing that lateral gene transfer of cupin phosphoglucoisomerases between Archaea and *Proteobacteria* has been postulated previously (Hansen et al., 2005). The possibility that SAK1666 is a cupin sugar-phosphate isomerase and the similar induction of the predicted PTS phosphotransferase protein SAK1894 at condition P1 suggest that changes to carbohydrate metabolism may be relevant during colonisation.

4.3.5. Comparison with transcriptome studies and other proteomic studies

The proteomic data presented here can be compared with several recently published GBS transcriptome analyses (Mereghetti et al., 2008a, 2008b; Sitkiewicz and Musser, 2009; Santi et al., 2009; Sitkiewicz et al., 2009). Firstly, it is clear that the majority of the proteins detected here as stably expressed are differentially regulated under other conditions: 53 out of these 57 proteins (93%) respond to at least one of the stimuli studied in transcriptome experiments (Table 4.7), confirming the likely importance of flexibility in gene regulation to the adaptability of GBS (Rajagopal, 2009; Santi et al., 2009).

With regard to upregulation of virulence determinants, it can be assumed that proteins upregulated at P2 would likely be also upregulated in transcriptomic studies modelling host invasion i.e. growth in blood (Mereghetti et al., 2008a) or amniotic fluid (Sitkiewicz et al., 2009), growth at elevated temperature (Mereghetti et al., 2008b) and exposure to near-neutral pH (Santi et al., 2009). As Bac is absent from the GBS strains NEM316 (serotype III) and 2603.V (serotype V) used in the transcriptome studies, it is not possible to relate the data for this putative virulence factor to that from the previous transcriptomic profiling studies of GBS. Seven out of the 12 other proteins identified in the present study as more abundant at P2 were upregulated in at least one of the transcriptome studies (Table 4.7). Six of 12 proteins abundant at P2 were up-regulated either at mid-log or stationary phase at 40^{o}C compared with 30^{o}C. These results are consistent with the bacteria having the capacity to survive in a wide range of environments. Conversely, it would also be expect

Table 4.7. Compare proteins differentially expressed in P1 and P2 conditions with transcriptome studies. C protein beta antigen (Bac) is not encoded in the strains studied by transcriptomics (purple highlighting). Genes that were significantly down regulated (blue highlighting) or upregulated (green highlighting) in transcriptome studies are indicated.

Proteins identified in GBS strain A909	Mereghtti et al., 2008a				Mereghtti et al., 2008b		Sitkitwicz & Musser 2009	Sitkitwicz et al., 2009		
	Exposure to blood				**Elevated temperature**		**Growth phase**	**Growth in Amniotic fluid**		
Up regulated at P2	37°C, 30min	40°C, 30min	37°C, 90min	40°C, 90min	40vs30°C, Mid-log	40vs30°C, Stat.	Stat. vs mid-log	AF at Mid-log	AF at Late-log	AF at Stat.
C protein beta antigen	absent	Absent	absent	absent	absent	absent	absent	absent	absent	absent
FeS assembly ATPase SufC	same	Same	same	down	same	same	same	same	down	same
Thioredoxin reductase	same	Same	same	same	same	same	same	same	down	same
Cysteine synthase A	same	Down	same	down	same	up	down	down	down	same
Dihydroorotate dehydrogenase IA	same	Same	up	up	same	down	down	up	up	up
Hypothetical protein GAG 0739	same	Same	up	up	same	same	up			
F_0F_1 ATP synthase, subunit gamma	down	Down	same	same	same	up	same	same	same	down
Hypothetical protein SAG 1211	down	Down	same	same	same	same	same	same	down	down
50S ribosomal protein L10	same	Same	same	same	same	up	down	same	up	same
Glutamine ABC transporter, ATP binding protein	down	Down	down	down	same	up	down	same	up	down
Uracil phosphoribosytransferase	same	Same	same	same	same	same	down	same	same	down

30S riboxomal protein S6	same	Same	same	same	same	same	same			
Alkyl hydroperoxide reductase, subunit C	same	Same	same	same	up	same	up			
Up regulated at P1										
Ademine phosphoribosyltransferase	down	Down	same	same	same	same	down	same	down	same
Ribosomal protein L21	same	Same	same	same	same	same	down	same	down	same
Hypothetical protein SAG1654	up	Up	same	same	same	same	up	same	same	down
30S ribosomal protein S6	same	Same	same	same	same	same	same			
PTS system, IIB component, putative	same	Same	same	same	same	same	up	down	down	same
Co-chaperonin GroES	same	Same	same	same	same	same	down			

that those proteins that have here been associated with colonisation conditions (i.e. upregulated at P1) would not to be induced in conditions associated with virulence in transcriptome studies. Consistent with this (Table 4.7), none of the 6 proteins identified as induced in condition P1 (Table 4.6) were induced by elevated temperature (Mereghetti et al., 2008b) and only one out of 6 was upregulated in blood (Mereghetti et al., 2008a). Likewise, 2 of these 6 proteins were found to be upregulated in response to 30 min. exposure to pH 5.5, although the GroES co-chaperonin was found to be down regulated under these conditions (Santi et al., 2009). None of the 4 genes for which data is available were induced in amniotic fluid (Sitkiewcz et al., 2009). Proteins abundant at P1 are thus less induced in conditions reflecting disease (exposure to blood, high temperature and growth in amniotic fluid). This is consistent with the proteomic results here as condition P1 is considered to reflect maternal colonization.

Cumulatively, the transcriptome data available for the genes encoding proteins that were here identified as being differentially expressed can be considered to be in reasonable agreement with the present hypothesis that cells at point P1 express proteins that might be associated with maternal colonisation whereas cells at point P2 express proteins that might be associated with GBS growth *in utero* and/or during neonatal invasive disease.

Hughes et al. (2001) first identified GBS surface proteins using 2D and MALDI mass spectrometry methods. A total of 14 proteins were identified in their study, among which phosphopyruvate hydratase, molecular chaperone DnaK, managanese-dependent superoxide dismutase, chaperonin GroEL,

glucose-6-phosphate isomerase, phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase were also identified in the present study. Johri et al. (2006) studied the cell wall-associated and membrane proteins expressed by a GBS type V strain using transcriptomic and proteomic methods. They compared GBS at a fast growth rate of 1.8 h (with oxygen and without oxygen) and at low growth rate time of 11 h. The proteins were separated by 1D SDS-PAGE and a fused-silica capillary column and further identified by tandem mass spectrometry. 130 proteins were found to expressed by GBS and 78 of these proteins were detected as exclusively expressed at the condition associated with high cell invasion (fast growth, with oxygen). 111 of the 158 total proteins identifed were found to be exclusively expressed by GBS at the fast growth rate compared with the low growth rate. The results showed GBS proteins are expressed differentially at different growth rates and oxygen levels. However, the study did not show which method was used to compare protein expression and what criteria were set for the significance of the changes.

Chapter 5

A proteomic investigation of *Streptococcus agalactiae* grown in the presence of human serum

5.1. Background

In early onset GBS disease, the organism is transferred from mother to neonate and so disease progression is characterised by bacterial adaptation from conditions associated with commensal maternal vaginal colonisation to those associated with neonatal disease, including exposure to amniotic fluid and blood. GBS exposure to human serum is highly relevant to its pathogenesis as septicaemia is an important disease manifestation and mechanism for GBS dissemination (Henneke and Berner, 2006; Mereghetti et al., 2008; Rajagopal, 2009). To explore this *in vitro*, this study used a proteomic approach to identify proteins differentially expressed following growth on Todd Hewitt agar in the presence or absence of 10% human serum. Biomass from the plate surfaces was harvested and the proteins expressed under these conditions were analysed. Specific proteins were also investigated by using western blotting. These data may contribute to understanding GBS virulence factor expression under conditions reflecting those *in vivo* and thus their contribution to bacterial survival and invasion.

5.2. Results

5.2.1. GBS growth on pH 6 THA square plates in the presence or absence of 10% human serum

Previous experiments (Chapter 3) have shown that GBS grows well in human serum. However, centrifugation of serum grown cultures also spun down large amounts of serum derived materials which might interfere with proteomic analyses. To expose GBS to human serum, and in order to facilitate recovery of bacterial cells away from medium components, GBS strain A909 was grown as lawns on 132 cm^2 plates containing 100 mL Todd Hewitt agar (THA) or THA supplemented with 10% human v/v serum (THA-HS). After inoculation for 48 hrs, biomass from the plate surfaces was removed by scraping and washed three times by resuspension into 10 ml PBS and centrifugation. Biomass equivalent to 25% of the bacteria from the surface of 1 plate was used as the sample for each gel (Section 2.2.1.2.1).

Prior to harvesting the biomass, the cell numbers were determined by CFU/ml counting (Table 5.1). A 0.38 cm^2 circular plug from each plate was removed and colony forming units/cm^2 determined to confirm similar growth yields were obtained on each medium. The CFU counts of GBS A909 grown on pH 6 THA for 48 hrs was $227\pm37\times10^6/cm^2$ while on THA-HS was $194\pm87\times10^6/cm^2$. SPSS independent-sample t-test results showed the Sig. P=0.579 >0.5. Thus, there was no significant difference in GBS A909 biomass formation on the THA medium between presence and absence of human serum.

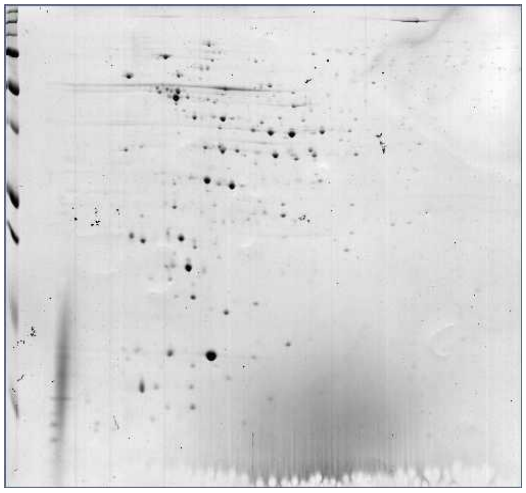
Table 5.1. CFU/cm^2 of GBS A909 grown on pH 6 THA square plate in the presence or absence of 10% human serum.

Results are shown for 3 separate plate growths for each condition, used to generate biomass for proteomics.

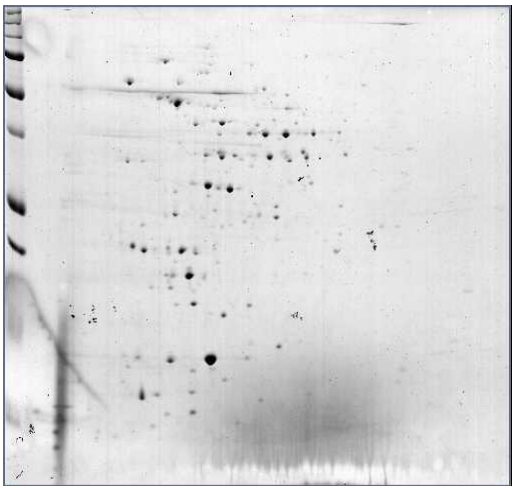
pH 6 plate	CFU/ cm^2 (1)	CFU/ cm^2 (2)	CFU/ cm^2 (3)	Mean CFU/ cm^2	S.D.
THA	216×10^6	268×10^6	197×10^6	227×10^6	37×10^6
THA-HS	142×10^6	145×10^6	295×10^6	194×10^6	87×10^6

5.2.2. 2D SDS-PAGE gel and gel analysis

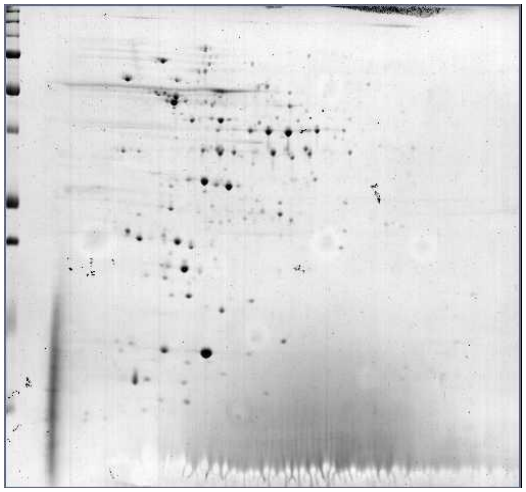
The optimised conditions of 2D SDS-PAGE were the same as described in Chapter 4.2.2. except that the protein samples were precipitated by using acetone. Six replicate 2D gels (Figure 5.1 and Figure 5.2) for each growth condition were image analysed and differentially expressed spots identified using the PDQuest^{TM} V 8.0 software. After detecting and editing spots, there were more than 200 proteins present on each of the master gel sets (Figure 5.3 and 5.4). The MatchSet analysis showed approximately 130 and 150 protein spots were reproducibly visible on the gels from condition 1 (THA) and condition 2 (THA-HS) cells, respectively, and approximately 80% of the spots were matched between the gels, with an average correlation coefficient for gels from the two different conditions of 0.75. All protein spot quantities were reviewed based on their density in the Spot Quantity Table Report. All spots from one condition that expressed stronger than the other condition are listed (Table 5.2 and Table 5.3) and the expression ratios were calculated (Table 5.4). The Independent-sample t-test was used to determine statistically significant differences in the spot quantities from the two different conditions. Since $p<0.05$, there is a statistically significant difference in the mean scores of spot quantities from two different conditions.



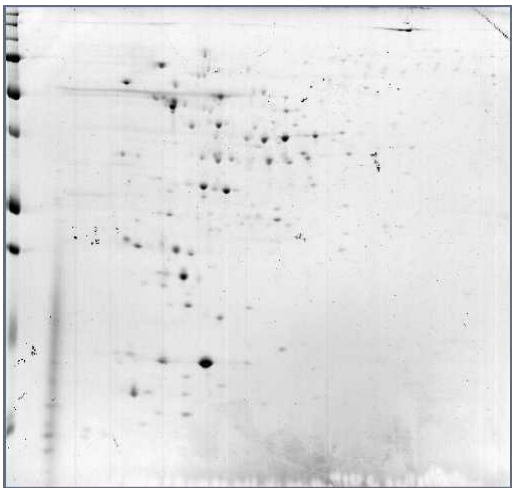
(1) 25 ml THA.



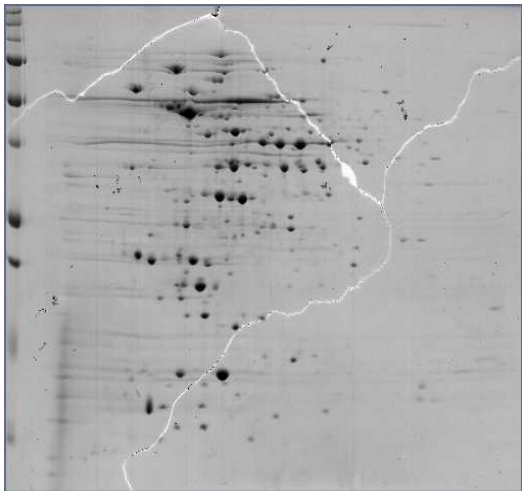
(2) 25 ml THA.



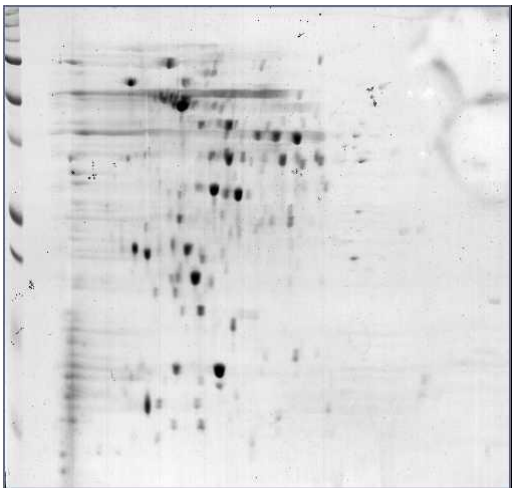
(3) 25 ml THA.



(4) 25 ml THA.

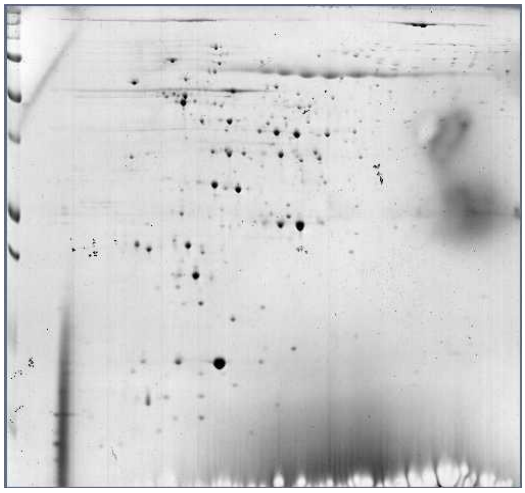


(5) 25 ml THA.

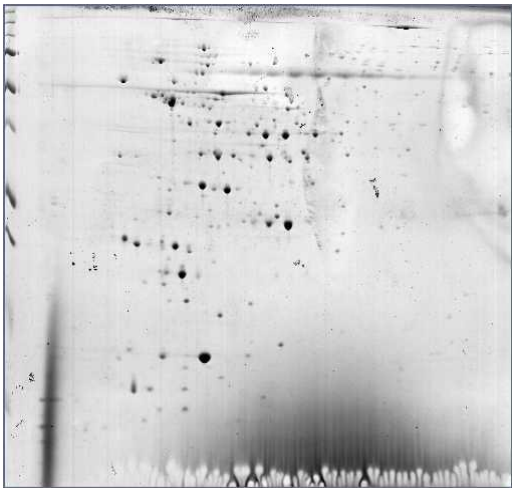


(6) 25 ml THA.

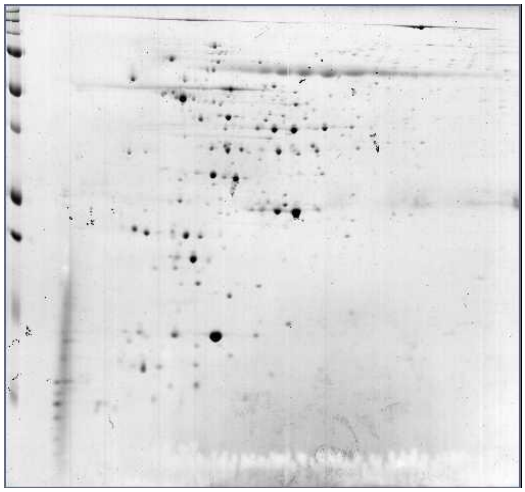
Figure 5.1. The six good quality 2D gels showing protein spots from cells harvested after growth on THA (condition 1). 100 µl lysis solution (urea 8M, CHAPS 4%) was used to extract cells. Immobiline DryStrips pH:4-7, 18cm were used for the first dimension (IEF), 14% SDS-PAGE for the second dimension. Colloidal Coomassie blue stain was used to visualize the gels. Protein standards are shown at the left hand side of each gel (150kDa, 100kDa, 75kDa. 50kDa, 37kDa, 25kDa, 20kDa, 15kDa, 10kDa).



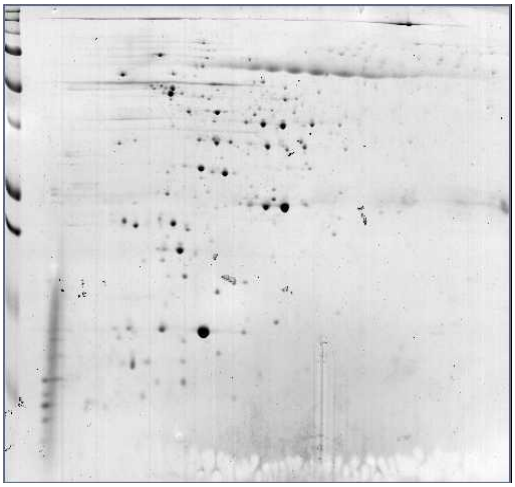
(1) 25 ml THA- HS.



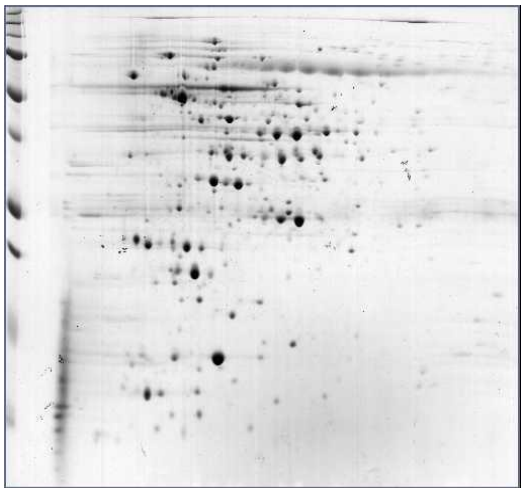
(2) 25 ml THA- HS.



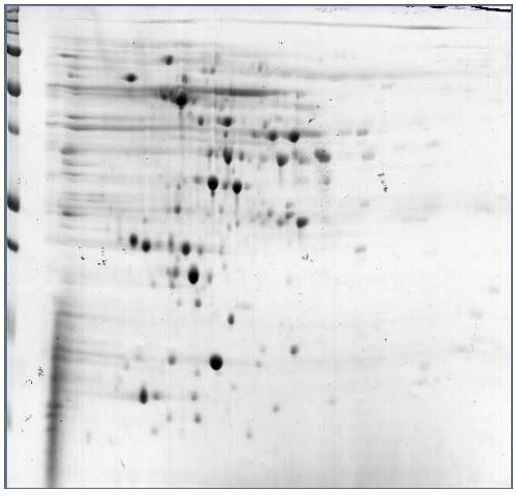
(3) 25 ml THA- HS.



(4) 25 ml THA- HS.



(5) 25 ml THA- HS.



(6) 25 ml THA- HS.

Figure 5.2. The six good quality 2D gels showing protein spots from cells harvested after growth on THA-HS (condition 2). 100 µl lysis solution (urea 8M, CHAPS 4%) was used to extract cells. Immobiline DryStrips pH:4-7, 18cm were used for the first dimension (IEF), 14% SDS-PAGE for the second dimension. Colloidal Coomassie blue stain was used to visualize the gels. Protein standards are shown at the left hand side of each gel (150kDa, 100kDa, 75kDa. 50kDa, 37kDa, 25kDa, 20kDa, 15kDa, 10kDa).

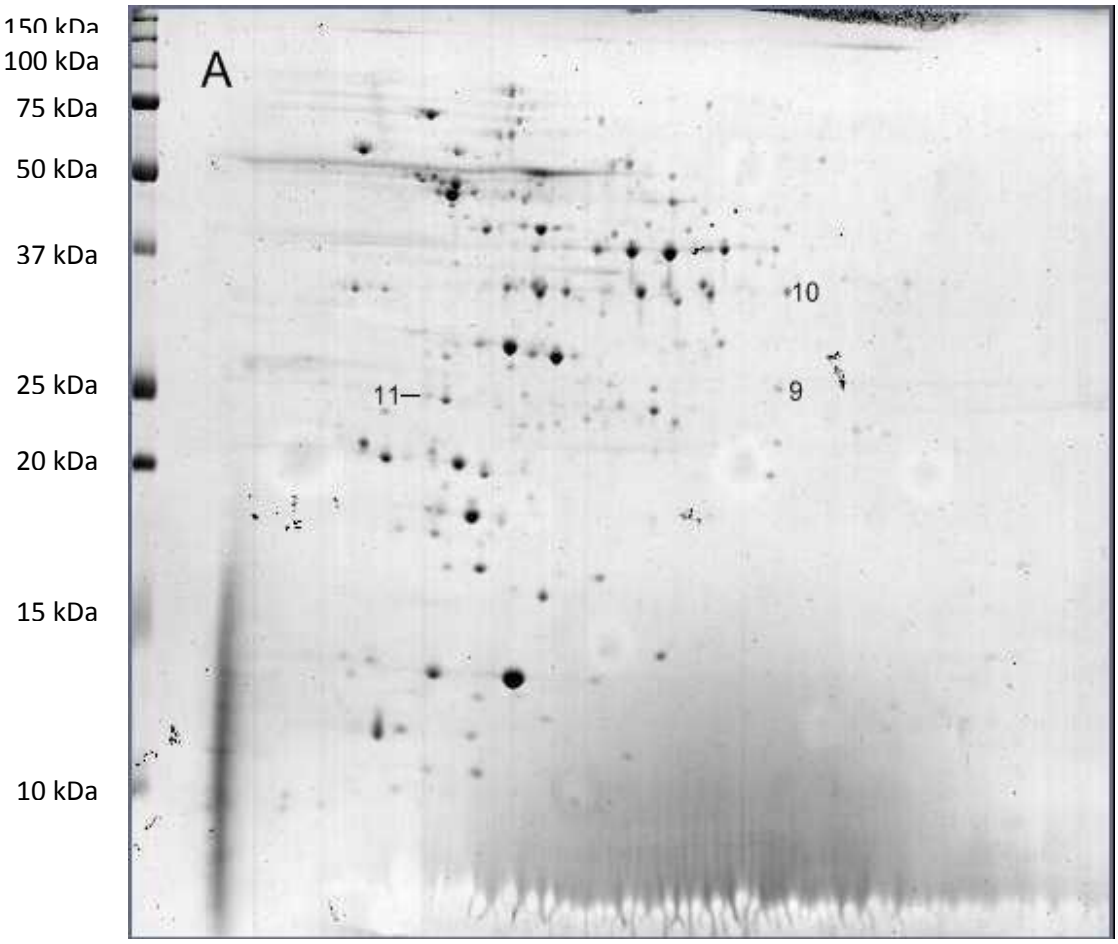


Figure 5.3. Master 2D gel of proteins from GBS A909 cultured on pH 6 THA plates. Gel image is the merged master from the six gels shown in Fig. 5.1. Protein standards (left hand side): 150kDa, 100kDa, 75kDa. 50kDa, 37kDa, 25kDa, 20kDa, 15kDa, 10kDa.

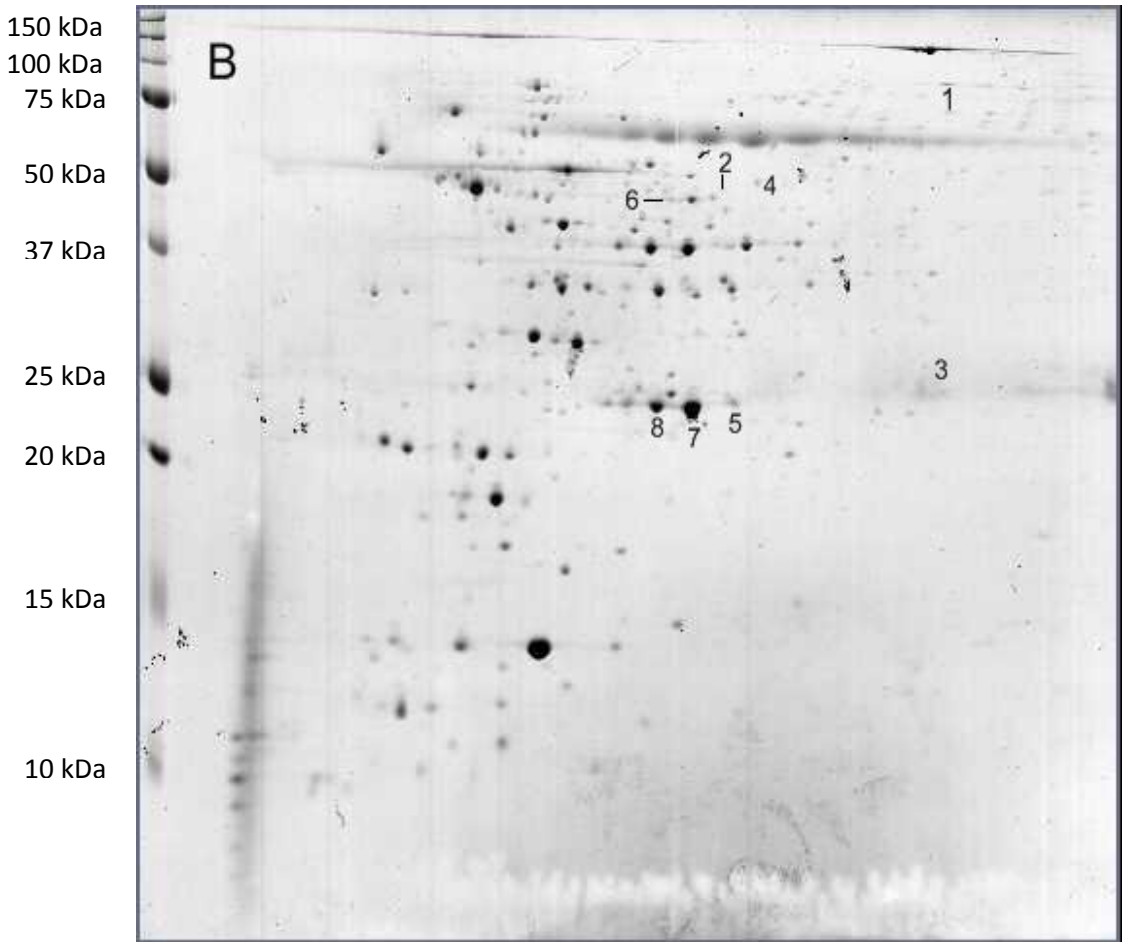


Figure 5.4. Master 2D gel of proteins from GBS A909 cultured on pH 6 THA-HS plates. Gel image is the merged master from the six gels shown in Fig. 5.2. Protein standards (left hand side): 150kDa, 100kDa, 75kDa. 50kDa, 37kDa, 25kDa, 20kDa, 15kDa, 10kDa.

Table 5.2. Spot quantities of the differentially expressed protein spots on the master 2D gel shown in Figure 5.3. Spots were analyzed by PDQuest.

SSP	THA-HS(1)	THA-HS(2)	THA-HS(3)	THA-HS(4)	THA-HS(5)	THA-HS(6)	Mean	S.D.
2221	185.1	141.1	40.9	76.1	57.6	119.8	103.4	55.0
4209	5946.7	2940.3	3206.6	2487.9	5357.6	5283.4	4203.8	1488.0
5212	9506.3	7603.1	2429.2	6255.8	10388.8	10681.8	7810.8	3136.4
5213	1337.3	55.0	871.3	557.1	1221.9	1026.7	844.9	474.7
5527	260.6	260.6	218.2	297.9	364.3	99.2	250.1	88.7
5528	109.7	0	73.2	96.4	40.1	72.0	65.2	39.9
6316	350.7	112.2	239.2	288.6	87.7	276.5	225.8	104.2
6419	1175.4	529.3	370.0	509.1	621.6	379.0	597.4	298.8
6615	294.2	11.3	376.2	535.4	305.1	338.9	310.2	170.6
7310	166.6	0.7	126.7	76.4	93.6	163.0	104.5	62.4
8820	223.6	80.1	362.6	255.0	419.8	314.5	275.9	119.4

Table 5.3. Spots quantities of the differentially expressed protein spots on the 2D gel shown in Figure 5.4. Spots were analyzed by PDQuest.

SSP	THA(1)	THA(2)	THA(3)	THA(4)	THA(5)	THA(6)	Mean	S.D.
2221	258.3	289.3	412.1	74.5	687.6	586.8	384.8	206.0
4209	0	0	186.1	74.7	0	0	43.5	69.4
5212	200.9	63.9	692.8	423.3	16.1	0	232.8	251.2
5213	22.4	21.8	36.5	0	74.4	24.7	30.0	22.6
5527	31.0	47.8	117.9	9.2	35.4	0	40.2	38.3
5528	0	0	0	0	0	0	0	0
6316	344.7	353.3	318.7	321.6	385.6	322.5	341.1	23.7
6419	765.8	737.4	917.8	730.8	780.8	877.2	801.6	70.8
6615	208.2	115.7	137.9	213.2	51.0	0	121.0	77.4
7310	0	0	0	0	0	0	0	0
8820	0	0	0	0	0	0	0	0

Table 5.4. Spot quantification and expression ratios for the differentially expressed protein spots between the two growth conditions.

SSP	Protein	Mean spot density		Ratio THA-HS:THA	Spot number Figs 5.3 and 5.4	T test P value
		THA-HS	THA			
2221	Alkyl hydroperoxide reductase, subunit C	103.4	384.8	0.3	11	0.014
4209	30S ribosomal protein S3	4203.8	43.5	96.6	7	0.001
5212	30S ribosomal protein S3	7810.8	232.8	33.6	8	0.002
5213	Peptide deformylase	844.9	30	28.2	5	0.002
5527	Arginine deiminase	250.1	40.2	6.2	6	0.001
5528	Acetate kinase	65.2	0	↑	2	0.002
6316	AtsA/ElaC family protein	225.8	341.1	0.7	9	0.025
6419	3-hydroxybutyryl-CoA dehydrogenase	597.4	801.6	0.7	10	0.136
6419	conserved hypothetical	597.4	801.6	0.7	10	0.136
6615	Glyceraldehyde-3-phosphate dehydrogenase NADP-dependent	310.2	121	2.6	4	0.035
7310	ABC transporter, substrate-binding protein	104.5	0	↑	3	0.002
8820	C protein beta antigen	275.9	0	↑	1	0.000

5.2.3. Protein in-gel trypsin digestion and mass spectroscopy analysis

Proteins spots were excised from the gels and identified by in-gel trypsin digestion followed by liquid chromatography-electrospray ionisation mass spectrometry. The data for protein identification, accession number, protein mass, score, matched peptides and percent amino acid coverage of the entire protein are shown in Table 5.5 (proteins expressed without significant difference between two conditions) and Table 5.6 (proteins upregulated after growth on THA-HS and after growth on THA). Protein functions were explored using NCBI BlastP searches (with conserved domain analysis) and their UniProtKB entries.

A total of 103 proteins in 84 distinct protein spots could be confidently identified by LC/ESI-MS. Of these, 73 protein spots showed no statistically significant difference in expression between the two growth conditions (Table 5.5). In addition to these stably expressed proteins, 11 protein spots were reproducibly differentially expressed (Table 5.6). Of these, 8 proteins were more abundantly expressed in cells harvested after growth on THA-HS (Figure 5.4, Table 5.6), which included 2 separate spots that were identified as 30S ribosomal protein S3, whereas 4 proteins were more abundantly expressed in cells harvested after growth on THA (Figure 5.3, Table 5.6).

Table 5.5. Proteins without change in expression under condition 1 and condition 2 identified using LC/ESI-MS. Condition 1: GBS grown on pH 6 THA. Condition 2: GBS grown on pH 6 THA-HS.

SSP	Protein identification	Accession number	Score	Protein code	Matched peptides	% protein coverage
1013	Hypothetical protein SAG0490	gi|22536669	58	SAK_0591	1	30%
1104	50S ribosomal protein L7/L12	gi|25011416	212	SAK_1334	3	42%
1107	Hypothetical protein SAG0739	gi|22537903	103	SAK_0865	1	11%
1108	Hypothetical protein SAG2091	gi|22538226	104	SAK_2030	2	45%
1114	Thiol peroxidase	gi|22537289	323	SAK_1217	6	43%
1115	Gls 24 protein, putative	gi|22537295	226	SAK_1223	4	36%
	Hypothetical protein SAG0377	gi|22536560	101	SAK_0451	1	14%
1220	Hypothetical protein gbs1202	gi|25011251	537	SAK_1221	3	22%
1410	Cell division protein DivIVA, putative	gi|22536663	389	SAK_0586	6	33%
2101	Hypothetical protein SAK_0613	gi|76786947	335	SAK_0613	3	46%
2113	Co-chaperonin GroES	gi|22538210	97	SAK_2014	2	41%
2116	30S ribosomal protein S6	gi|22537853	164	SAK_1722	2	33%
2120	Hypothetical protein gbs1204	gi|25011253	511	SAK_1223	7	47%
2125	Glycerophosphoryl phosphodiesterase, putative	gi|77405398	61	SAK_1307	1	1%

2213	Manganese-dependent superoxide dismutase	gi|2765187	229	SAK_1223	3	22%
2219	50S ribosomal protein L4	gi|22536244	321	SAK_0092	6	44%
2307	Acetoin reductase	gi|22536701	704	SAK_0674	8	50%
2309	Glucose-1-phosphate thymidylytransferase	gi|22537385	490	SAK_1287	6	28%
	Chaperonin GroEL	gi|22538209	269	SAK_2013	4	12%
	Phosphopyruvate hydratase	gi|22536801	261	SAK_0713	3	11%
2313	Fructose-bisphosphate aldolase	gi|22536312	160	SAK_0178	2	12%
2516	Phosphopentomutase	gi|76787408	201	SAK_1269	5	17%
2629	Cell dividion protein FtsZ	gi|22536658	725	SAK_0581	12	45%
	Dipeptidase PepV	gi|76787443	379	SAK_1305	4	14%
	Aldehyde dehydeogenase family protein	gi|22537282	312	SAK_1211	7	29%
	Elongation factor Tu	gi|22536926	179	SAK_0887	2	7%
3215	Adenylate kinase	gi|76787497	844	SAK_0112	10	55%
	50S ribosomal protein L1	gi|22537662	192	SAK_1543	2	13%
4106	30S ribosomal protein S8	gi|22536257	143	SAK_0105	2	24%
4108	Hypothetical protein SAG1677	gi|22537817	603	SAK_1689	6	50%
4109	Hypothetical protein SAK_0181	gi|76787208	382	SAK_0181	4	69%
4110	PTS system, IIB component, putative	gi|22538072	311	SAK_1894	5	80%

4111	Hypothetical protein SAG0793	gi|22536903	552	SAK_0865	12	47%
4211	Purine nucleoside phosphorylase	gi|22537336	465	SAK_1537	7	45%
4212	Uridylate kinase	gi|22537656	268	SAK_1537	3	21%
4213	Phosphomethylpyrimide kinase	gi|22536288	226	SAK_0151	3	23%
4214	Uridine phosphorylase	Q3D7D3STRAG	649		9	57%
	Phosphoglycerate mutase family protein	Q3C2R1STRAG	289	SAK_0889	5	27%
	Phosphate transport system regulatory protein PhoU	Q3CYA6STRAG	212	SAK_1082	2	15%
	Ribosomal protein L1	Q3DKXDSTRAG	151	SAK_1543	2	14%
4310	F_0F_1 ATP synthase subunit gama	gi|22537025	583	SAK_0985	8	32%
4312	Fructose-bisphophate aldolase	gi|22536312	715	SAK_0178	10	39%
4316	Glucokinase	gi|22536650	709	SAK_0573	9	35%
4420	Glyceradehyde-3-phosphate dehydrogenase	gi|22537907	549	SAK_1790	10	41%
	Ornithine carbamoytransferase	gi|22538299	206	SAK_2123	5	26%
4421	Nephritis-associated plasmin receptor	gi|71040526	462	SAK_1790	8	31%
4425	Cysteine synthase A	gi|22536517	722	SAK_0404	12	60%
4427	UDP-glucose 4-epimerase	gi|22538061	197	SAK_1882	4	25%
4428	L-lactate dehydrogenase	gi|22537120	348	SAK_1054	4	20%
4431	Tagatose 1,6-diphophate aldolase	gi|22538066	654	SAK_1887	11	41%

	Acetoin dehydrogenase, thymine PPi dependent, E1 component, beta subunit	gi|22537042	581	SAK_1002	11	37%
4518	Malate oxidoreductase	gi|22538057	777	SAK_1878	9	35%
5102	Riboflavin synthase, beta subunit	gi|22536913	143	SAK_0875	3	33%
5106	Dihydroxyacetone kinase family protein	gi|22537791	410	SAK_1663	6	31%
5107	Single-strand DNA-binding protein	gi|22537852	178	SAK_1721	2	11%
	50S ribosomal protein L6	gi|22536258	97	SAK_0106	1	6%
5214	Phosphoglyceromutase	gi|22536928	597	SAK_0889	8	57%
5319	Aldo/keto reductase family oxidoreductase	gi|22537619	288	SAK_1506	5	23%
5320	1-Phosphofrutokinase	gi|22537494	337	SAK_1378	4	19%
5432	Alcohol dehydrogenase	gi|22536239	300	SAK_0087	5	27%
5429	PTS system, mannose-specific IIAB components	gi|22536544	537	SAK_0436	9	43%
5524	3-oxoacyl-(acyl carrier protein) synthase II	gi|22536532	345	SAK_0423	17	37%
6205	Ribosome recycling factor	gi|22537655	368	SAK_1536	5	34%
6416	Alcohol dehydrogenase, zinc-dontaining	gi|22537777	437	SAK_1651	6	22%
7102	Galactose-6-phosphate isomerase subunit LacA	gi|22538069	477	SAK_1890	6	54%
7103	Hypothetical protein SAG0302	gi|22536486	215	SAK_0373	4	48%
7104	GntR family transcriptional regulator	gi|22537099	133	SAK_1034	2	19%
7611	Inositol-5-monophosphate dehydrogenase	gi|22538293	490	SAK_2117	7	20%

8108	Hypothetical protein gbs0457	gi|25010530	276	SAK_0503	4	32%
8109	Adc operon repressor AdcR	gi|22536339	78	SAK_0217	1	16%
8110	PTS system mannose/fructose/sorbose family IIB subunit	gi|76788194	441	SAK_1910	8	65%
9104	Galactose-6-phosphate isomerase subunit LacB	gi|22538068	269	SAK_1889	3	30%
9208	Universal stress protein	gi|22537872	351	SAK_1741	4	32%

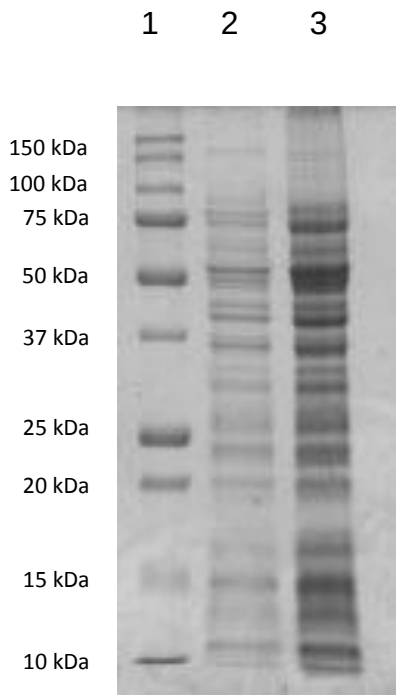
Table 5.6. Proteins expressed differentially under condition 1 or condition 2 identified by LC/ESI-MS. Condition 1: GBS grown on pH 6 THA. Condition 2: GBS grown on pH 6 THA-HS.

Spot	Protein identification **Proteins up-regulated in condition 2**	Accession number	Score	Protein code	Matched peptides	% protein coverage	Expression ratio
1	C protein beta antigen	gi|76150609	249	SAK_0186	4	6%	↑
2	Acetate kinase	gi|22536352	179	SAK_0234	4	16%	↑
3	ABC transporter, substrate-binding protein	gi|22536474	142	SAK_0362	2	10%	↑
4	Glyceraldehyde-3-phosphate dehydrogenase NADP-dependent	gi|22536987	952	SAK_0749	16	40%	2.56
5	Peptide deformylase	gi|22538033	128	SAK_1863	2	18%	28.16
6	Arginine deiminase	gi|76787000	321	SAK_2121	5	13%	6.22
7	30S ribosomal protein S3	gi|76798223	395	SAK_0097	6	39%	96.63
8	30S ribosomal protein S3	gi|76798223	131	SAK_0097	3	19%	33.55
	Proteins up-regulated in condition 1						
9	AtsA/ElaC family protein	gi|22536582	168	SAK_0473	6	36%	0.66
10	3-hydroxybutyryl-CoA dehydrogenase	gi|76788407	396	SAK_1685	9	27%	0.74
10	Conserved hypothetical	gi|22537295	192	SAK_1223	2	21%	0.74
11	Alkyl hydroperoxide reductase, subunit C	gi|22537972	397	SAK_1853	6	32%	0.27

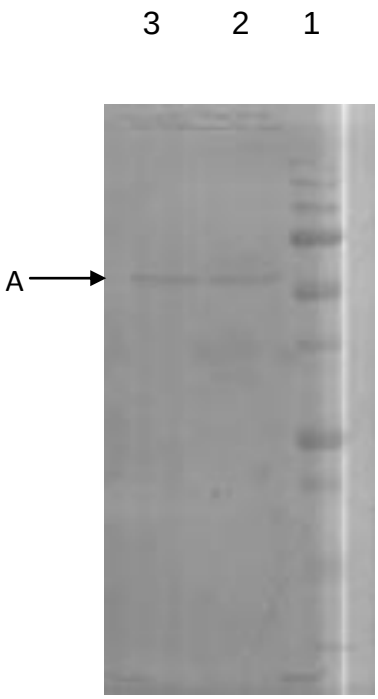
5.2.4. Western blotting

Western blotting was performed to confirm the differential expression of Bac (spot 1 Figure 5.4, Table 5.6). Protein samples from GBS A909 pH 6 THA and THA-HS were extracted using the same procedure as for preparation of proteomics samples. Proteins were separated by 12% 1D SDS-PAGE and transferred onto nitrocellulose membranes. Anti-Bac, anti-Sip and anti-HPr were used as the primary antibodies. Anti-rabbit IgG alkaline phosphatase conjugated secondary antibody (Sigma) was used to develop blots for Bac and HPr and alkaline phosphatase conjugated anti-mouse IgG was used as secondary antibody for the Sip blot.

There were bands with MW of approximately 53 kDa on the anti-Sip western blot membrane (Figure 5.5B) from both GBS A909 THA culture and THA-HS cultures (the calculated molecular weight of mature Sip is 53 kDa [Brodeur et al., 2000]). Likewise, the anti-HPr western blot membrane (Figure 5.5C) showed there were bands with MW of approximately 15 kDa from both THA culture and THA-HA cultures (the calculated molecular weight of HPr is 9 kDa,). This indicated that both proteins were expressed in GBS A909 during growth on both THA and THA-HS. There was no pronounced difference in the expression of these proteins between these two conditions. Neither protein was detected in the stably expressed protein group by proteomics (Table 5.5), suggesting Western blot detection is more sensitive than Coomassie Brilliant Blue. Expression of HPr is consistent with the expression of several other PTS transport system components in the stably expressed protein group (SSP 4110, 5429, 8110; Table 5.5). In contrast to HPr and Sip, a band of MW around 120

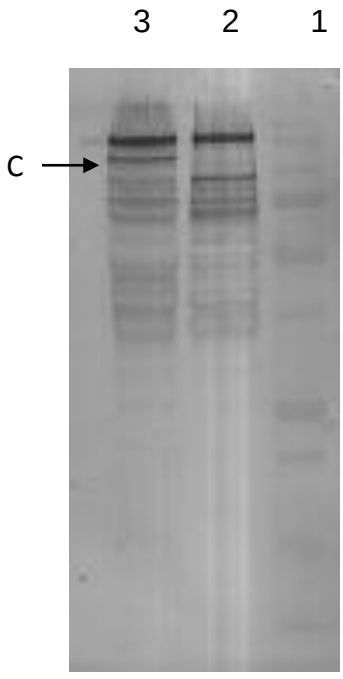


(A) 12% 1D SDS-PAGE.



(B) Western blotting of anti-Sip.

(C) Western blotting of anti-HPr.



(D) Western blotting of anti-Bac.

Figure 5.5. Western blotting analysis of proteins expressed following growth on THA and THA-HS. In each panel Lane 1 contains prestained protein MW standards (labelled in Figure A, left hand side); Lane 2: GBS A909 cultured in pH 6 THA plate; Lane 3: GBS A909 cultured in pH 6 THA-HS. Cross reacting bands of expected MW for the target proteins are labelled with arrows A (Sip), B (HPr) and C (Bac)

kDa was detected on the anti-Bac Western blot membrane (Figure 5.5D) from the THA-HS grown culture but not from the THA grown culture. This suggested C protein beta antigen was expressed differentially between GBS A909 THA-HS and THA cultures i.e. THA-HS can induce *bac* expression, confirming the data from the proteomics experiments. However there were also some additional cross-reacting bands on the anti-Bac western blot membrane, suggesting some lack of specificity of the antibody.

5.3. Discussion

5.3.1. An *in vitro* model of GBS growth with human serum and 2D gel analysis

The work in this chapter describes how an *in vitro* model of exposure of GBS to human serum was established. In order to facilitate recovery of bacterial cells away from medium components, notably components of human serum that pelleted with bacterial cells on centrifugation, GBS strain A909 was grown as lawns on square plates containing THA or THA-HS. These experiments should allow proteins induced by human serum to be detected. The growth experiment showed there was no significant difference in GBS A909 biomass formation on the THA medium following growth in the presence and absence of human serum. As plates were incubated for identical lengths of time, this strongly suggests bacterial cells from each plate condition are in the same phase of growth.

The optimised conditions for the 2D gel handling were the same as in the previous proteomic experiment except that the protein samples were

precipitated by using acetone. There were more than 130 protein spots visible on each gel. As some protein spots contained more than one identifiable protein, a total of 103 proteins in 84 protein spots could be confidently identified by LC/ESI-MS. Of these, 73 spots showed no statistically significant difference in expression between the two growth conditions. In addition to the 73 stably expressed proteins, 11 protein spots were reproducibly differentially expressed.

The Mascot search showed the theoretical molecular weight of each identified protein, which is listed in Table 5.7. The observed molecular weight of protein was determined by comparing migration positions with protein MW standards on the left side of the 2D gels (Figure 5.3 and Figure 5.4) and are listed in Table 5.7. For example, Mascot search showed the MW of C protein β antigen is around 135 and the protein spot of Bac on 2D gel (Figure 5.4), spot number 1 is located between 100kDa and 150kDa. More precisely, the MW of mature Bac without its signal peptide and its C-terminal cell wall sorting (LPXTG) signal can be calculated as 124 kDa. The observed MW for 10 of 12 proteins correlates well with the theoretical MW. The theorical MW of protein SAK_1685, 3-hydroxybutyryl-CoA dehydrogenase is 42kDa, but the observed MW is around 25-37kDa. This may be caused by loss of some peptide during protein extraction or aberrant mobilty on SDS-PAGE. Protein SAK_1223, a conserved hypothetical, may be present as dimers as the observed MW (25-37kDa) is approximately two times the theoretical MW (19kDa).

5.3.2. Proteins more abundantly expressed in cells harvested after growth on THA-HS

Seven proteins were expressed more abundantly after growth on THA-HS. These proteins included the Bac protein, a putative virulence factor linked to immune evasion (Lindahl et al., 2005; Chapter 4). However, growth under *in vitro* conditions mimicking those associated with invasive disease previously identified the soluble 55-kDa form of Bac as being upregulated (Chapter 4). In the present experiments, the mature ca. 120 kDa form of Bac was detected (Figure 5.4, spot 1). The upregulation of a ca. 120 kDa protein was confirmed by Western blotting using a polyclonal anti-Bac antibody (Fig. 5.5) although extensive cross-reactivity of this antibody with other high molecular weight proteins was noted. Cumulatively, these *in vitro* data are consistent with the likely *in vivo* upregulation of Bac expression in response to the shift from maternal colonisation to neonatal colonisation and disease. Bac is able to simultaneously bind the Fc region of IgA antibodies and the complement regulator factor H with separate binding sites (Figure 4.6). Bac binding to IgA may allow GBS to evade immune elimination and binding to factor H may inhibit complement attack and phagocytosis, thus contributing to the ability of GBS to evade the immune system (see Chapter 4). Gene expression of *bac* (*sak_0186*) has recently been shown to be under the control of an adjacent two component system, *sak_0188-sak_0189* (Rozhdestvenskaya et al., 2010). Clearly, in those strains that possess the *bac* gene (notably GBS serotype Ib and some serotypes Ia and II strains (Lindhahl et al., 2005; Sørenson et al., 2010)), Bac antigen expression is likely to be closely coordinated during infection and further studies are needed to elucidate this process. Deletion of 51 amino acid residues, including part of Bac IgA-binding region might cause strain loss the IgA-binding ability in strain Ib separated from infact crebrospinal

Table 5.7 Comparison between observed proteins molecular weight for differentially expressed proteins with their theoretical molecular weight.

Spot [a]	Protein identity	Calculated MW	Observed MW
1	SAK_0186, C protein beta antigen (Bac)	135 full	100-150
2	SAK_0234, Acetate kinase	43	top end of 37-50 range
3	SAK_0362, ABC transporter substrate-binding protein	30	~25
4	SAK_0947, Glyceraldehyde-3-phosphate dehydrogenase	51	~50
5	SAK_1863, Peptide deformylase	22.8	20-25
6	SAK_2121, Arginine deiminase	46	~50
7	SAK_0097, 30S ribosomal protein S3	23	20-25
8	SAK_0097, 30S ribosomal protein S3	23	20-25
9	SAK_0473, metal dependent hydrolase of the beta-lactamase superfamily	29	~25
10	SAK_1685, 3-hydroxybutyryl-CoA dehydrogenase	42	25-37
10	SAK_1223, conserved hypothetical	19	Top end of 25-37 range
11	SAK_1853, alkyl hydroperoxide reductase, subunit C	20	~25

fluid (CSF) (Nagano et al., 2006), further complicating the contribution of Bac to GBS virulence.

In addition to Bac, the proteomic data indicated that arginine deiminase (ArcA, SAK_2121) was also upregulated by growth on THA-HS. These data are consistent with a previous preliminary report of GBS ArcA induction by human serum as determined by RT-PCR analysis (Aymanns et al., 2006). Transcriptome analyses have also shown expression of GBS ArcA was induced 5- to 15-fold by exposure to human blood (Mereghetti et al., 2008); at later stages of growth in amniotic fluid (Sitkiewicz et al., 2009); and at stationary phase in Todd Hewitt broth (Sitkiewicz and Musser, 2009). However, Sitkiewicz et al. (2010) also found recently that in *S. pyogenes*, gene expression of arginine deiminase was down-regulated about 900 fold in amniotic fluid. They proposed the lack of arginine deiminase may link to the decreased host immune recognition and help bacteria survival in an amniotic fluid environment (Sitkiewicz et al., 2010). However, this explanation is not consistent with the observation that these two streptococci regulate ArcA differently in amniotic fluid. It is notable that GBS, as an organism whose pathogenicity more commonly results in exposure to amniotic fluid up-regulates rather than down-regulates ArcA.

ArcA is an enzyme of the ADI pathway, by which bacteria convert L-arginine to ornithine and with the production of ammonia (Ryan et al., 2009, Figure 5.6). The enzymes of the ADI system include arginine deiminase, catabolic ornithine carbamoyltransferase and carbamate kinase, which are encoded by the *arcA*, *arcB* and *arcC* genes. ArcA catalyzes L-arginine conversion to citrulline and ammonia. The citrulline is then converted to

ornithine and carbamoyl phosphate, catalysed by *arcB*. Carbamoyl phosphate is converted to carbon dioxide, ammonia and ATP by the action of *arcC.* Ornithine is secreted out of the cell by ArcD in exchange for arginine in an energy independent manner (Ryan et al., 2009, Degnan et al., 1998) (Figure 5.6). ATP is used to extrude protons across the membrane and maintain pH homeostasis and also provides energy for growth. ATP generation is likely to be an important source of energy during carbohydrate limitation (hence its induction in stationary phase) and ArcA may also contribute to acid tolerance by generating ammonia (Liu et al., 2008; Ryan et al., 2009). The production of ammonia can neutralize acid and protect bacteria from lethal acidic environments. RT-PCR analysis of *L. monocytogenes* revealed ADI genes were upregulated in both acid and anaerobic environments with the addition of arginine (Ryan et al., 2009). ArcA may also contribute to immune evasion by inhibiting the proliferation of blood mononuclear cells (Degnan et al., 2000). Degnan et al. (2000) found in *S. pyogenes*, that cell extracts originally identified as streptococcal acid glycoprotein were associated with arginine deiminase activity and were able to inhibit human peripheral blood mononuclear cell proliferation in a dose dependent manner. The inhibition could be restored by adding L-arginine. Bioinformatic analysis showed 31% to 39% protein sequence identity of streptococcal acid glycoprotein to arginine deiminase of *Mycoplasma hominis, Mycoplasma arginini, Pseudomonas putida* and *Pseudomonas aeruginosa* (Degnan et al., 2000). ArcA expression in *E. coli* also inhibited the growth of mouse tumour cells (Misawa et al., 1994). Morphologic analysis showed that ArcA induced human T lymphoblastoid cell death via the apoptosis pathway (Komada et al., 1997). A possible explanation is that the

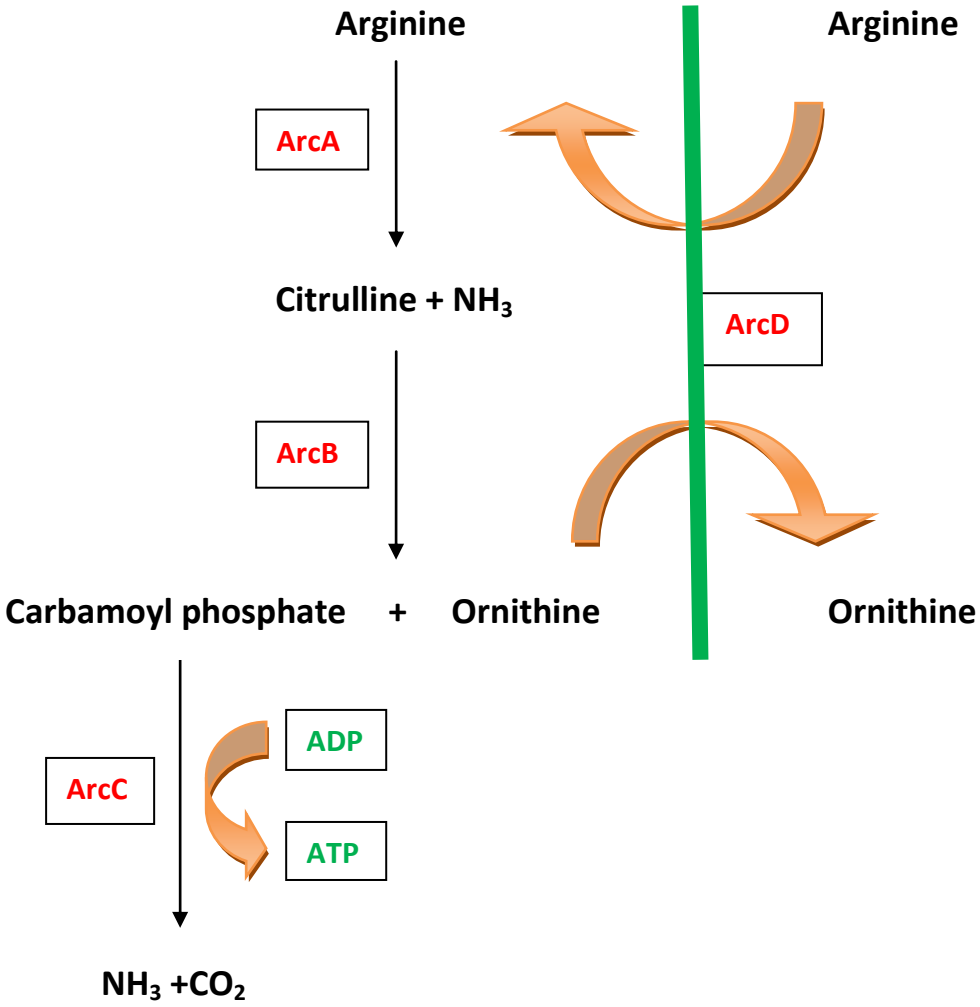


Figure 5.6. Arginine deiminase metabolic pathways. The main pathway is cytoplasmic but exchange of ornithine and arginine across the cell membrane (green) is also shown.

presence of ArcA will deplete L-arginine, which is essential for the optimal cell growth (Degnan et al., 2000).

Cumulatively, these data point to an important role of ArcA in adaptation of GBS to the neonatal environment. In GBS strain 090R, ArcA expression appears to be under the control of the RgfAC two component system (Spellerberg et al., 2002; Aymanns et al., 2006). However in GBS strains A909 and H36B, a single nucleotide substitution in the RgfA sequence (SAK_1918 in A909) prematurely truncates this putative response regulator such that it may be non-functional. Moreover, whereas ArcA is part of the GBS core genome, the RgfAC two component system is not. Consequently, ArcA expression in different GBS strains is likely to be regulated by several interacting mechanisms involving both two components systems and other transcriptional regulators (such as Flp and ArgR), as in other streptococci (Liu et al., 2008; Liu and Burne, 2009). Notably, Flp (SAK_2119) and ArgR (SAK_2118) transcriptional regulators are encoded adjacent to GBS *arcA*.

Two other notable proteins were induced by THA-HS: SAK_0362 is a putative substrate binding protein for an ABC transport system for amino acids. ABC transporters are involved in import and export of substrates through the cell membrane, and are typically composed of two ATP-binding proteins (ATPase) and two membrane-spanning proteins (permeases). Substrate import across the cell membrane is directed by binding to substrate-binding proteins which then transfer the substrate into the cell through the two membrane-integrated permeases (Eitinger et al., 2010). ABC transporters are not only involved in nutrient acquisition but also involved in adhesion, protein secretion, stress adaption, cell to cell communication and cell integrity maintenance

(Basavanna et al., 2009; Eitinger et al., 2010; Samen et al., 2003). Normally the substrate binding proteins of ABC import systems in GBS are bacterial lipoproteins (Sutcliffe and Harrington, 2004). However, SAK_0362 is unusual in being a predicted secreted (non-lipoprotein) substrate binding protein. Johri et al. (2007) previously observed induction of the SAK_0362 protein in GBS strain 2603/V grown under *in vitro* conditions associated with increased cervical epithelial cell invasion. Similarly, the proteomic data showed induction of glyceraldehyde-3-phosphate dehydrogenase (SAK_0947; GapN, GADPH) by growth on THA-HS and Johri et al. (2007) reported increased expression of this gene (by microarray analysis) by GBS strain 2603/V grown under *in vitro* conditions associated with increased cervical epithelial cell invasion. In contrast, Mereghetti et al. (2008) observed a ca. 2.5-fold decrease in *gapN* expression following 90 minutes exposure to human blood (Table 5.7). GBS GapN is bacterial surface protein which can interact with plasminogen and fibrinogen *in vitro*. Although lacking a signal peptide, surface associated GapN may contribute to GBS virulence by directly or indirectly binding host plasminogen and activating it to the plasmin protease (see section 4.3.2). Streptococcal GAPDH plays multiple roles in bacterial survival by interaction with plasminogen, cleaving C5a to evade complement system clearance and is involved in energy metabolism (see section 4.3.2).

Acetate kinase (SAK_0234) was also expressed abundantly following growth in THA-HS. Acetate kinase is involved in the glucose metabolic pathway, which converts glucose to pyruvate, and then converts pyruvate to acetyl phosphate. Acetate kinase catalyses acetyl phosphate conversion to acetate, with the production of ATP. Taniai et al. (2008) proposed that in

aerobic conditions, *S. pneumoniae* pyruvate oxidase (SpxB) catalyzed pyruvate conversion to acetyl phosphate with the production of H_2O_2. Since H_2O_2 may play an important role in helping bacteria avoid immune clearance, so glucose metabolism to acetate not only provides energy for bacterial growth but also helps an organisms survival in host. However, GBS lacks a clear homologue of *S. pneumoniae* SpxB and so the role of this pathway and the acetate kinase SAK_0234 in GBS is still unclear.

Peptide deformylase (SAK_1863) and two protein spots identified as the 30S ribosomal protein S3 (SAK_0097) were upregulated following growth in THA-HS suggesting there was a need for new protein synthesis. Peptide deformylase is an essential enzyme in protein synthesis, which has the function of removing of the formyl group from the N-terminal methionine of newly synthesized proteins. Peptide deformylase has been recognized as a potential drug target for antibiotics (Lee et al., 2010).

5.3.3. Proteins that were more abundantly expressed in cells harvested after growth on THA

In addition to those protein spots that were more abundant following growth on THA-HS, three spots (containing 4 proteins) were less abundant in this growth condition (Table 5.6). These included SAK_1853, the putative alkyl hydroperoxide reductase which was previously observed to be induced under conditions modelling neonatal exposure (Chapter 4). Notably, SAK_1853 was not induced by brief (up to 90 min.) exposure to blood (Mereghetti et al., 2008; Table 5.7). One spot up regulated at condition 1 was identified as two proteins, 3-hydroxybutyryl-CoA dehydrogenase (SAK_1685) and a conserved

hypothetical protein (SAK_1223). SAK_1685 is involved in fatty acid and phospholipid metabolism and transport. SAK_1223 is annotated as a putative alkaline shock protein 23 (PFAM PF03780; domain of unknown function DUF322), which may be involved in stress responses (Sitkiwicz & Musser, 2009, Mereghetti et al., 2008a). Also upregulated on THA was SAK_0473, a metal dependent hydrolase of the beta-lactamase superfamily protein. Beta-lactamases can protect bacteria by inactivating penicillins (Brook & Gober, 2008) but it remains unclear whether this is the function of SAK_0473 or whether this protein has a more distantly related function in relation to cell wall peptidoglycan metabolism.

5.3.4. Comparison of proteomics data with transcriptome studies

The proteins expressed differently after growth in the presence and absence of human serum were compared with recently published GBS transcriptome analyses (Mereghetti et al., 2008a, 2008b; Sitkiewicz and Musser, 2009; Sitkiewicz et al., 2009) as listed in Table 5.8. Proteins abundant in THA-HS were upregulated at mid log phase compared with stationary phase (except for arginine deiminase; Sitkiewicz and Musser, 2009). The data showed proteins expressed in the presence of human serum have the characteristics expected for a fast growth phase. The capability of GBS adherence and invasion to epithelial cells is higher at fast growth rate than at low growth rate (Johri et al., 2005). Transcriptome analyses have also shown expression of GBS *arcA* was induced 5- to 15-fold by short (up to 90 min.) exposure to human blood (Mereghetti et al., 2008b); at later stages of growth in amniotic fluid (Sitkiewicz

et al., 2009), and at stationary phase in Todd Hewitt broth (Sitkiewicz and Musser, 2009). In contrast to the proteomics data, Mereghetti et al. (2008b) observed a ca. 2.5-fold decrease in *gapN* expression following 90 min exposure to human blood. SAK_1223, a conserved hypothetical protein, which may be involved in stress response, was abundantly expressed following growth on THA and was observed to be up-regulated when GBS cells were exposed to blood at 90 mins, elevated temperature at mid log phase and stationary phase (Mereghetti et al., 2008a, 2008b; Sitkiewicz and Musser, 2009). Three proteins abundant following growth on THA-HS were up-regulated at 40^{o}C compared with 30^{o}C at stationary phase. The transcriptome data showed GBS is able to modulate its gene expression in response to changing temperature, which may be relevant to increased temperature (fever) during infection (Mereghetti et al., 2008b).

5.3.5. Concluding commments

These data add to the growing body of knowledge on GBS gene and protein expression. They confirm the previous observations (Chapter 4) that Bac likely has a prominent role *in vivo* in those GBS strains that encode this protein. Likewise, they extend previous studies implicating arginine deiminase specifically and arginine catabolism generally in GBS adaptation to host environments (Mereghetti et al., 2008; Sitkiewicz and Musser, 2009; Sitkiewicz et al., 2009). Although these experiments were designed to mimic GBS exposure to fluid phase human serum components, surface growth as lawns on agar plates might also be relevant as a model for topologically defined *in vivo* biofilm/microcolony growth with transepithelial nutrient delivery, which has

recently been shown to be relevant for growth of *Helicobacter pylori* (Tan et al., 2009).

Table 5.8. Comparison of proteins differentially expressed following growth on THA and THA-HS with transcriptome studies. Green highlighting: protein up-regulated in the transcriptome study. Blue highlighting: Protein down-regulated in the transcriptome study. Purple highlight: Protein absent from the strain assayed in the transcriptome studies.

Proteins identified in GBS strain A909	Mereghtti et al., 2008a				Mereghtti et al., 2008b		Sitkitwicz & Musser 2009	Sirkitwicz et al., 2009		
	Exposure to blood				**Elevated temperature**		**Growth phase**	**Growth in Amniotic fluid**		
Up regulated on THA-HS	37°C, 30min	40°C, 30min	37°C, 90min	40°C, 90min	40vs30°C, Mid-log	40vs30°C, Stat.	Stat. vs mid-log	AF at Mid-log	AF at Late-log	AF at Stat.
C protein beta antigen	absent	absent	absent	absent	absent	absent	absent	absent	absent	Absent
Acetate kinase	same	same	same	same	same	same	down	same	down	Same
ABC transporter, substate-binding protein	same	same	same	same	same	up	down	down	down	Same
Glyceraldehyde-3-phosphate dehydrogenase GapN	same	same	down	down	same	up	down	same	same	Up
Peptide deformylase	same	same	same	Same	same	same	down			
Arginine deiminase	up	up	up	up	same	up	up	same	up	Up
30S ribosomal protein S3										
30S ribosomal protein S3										

Up regulated on THA										
Metal dependent hydrolase of the beta-lactamase superfamily	same	same	same	same	up	same	up	same	same	Down
3-hydroxybutyryl-CoA dehydrogenase	same	same	up	up	up	up	up	same	down	Down
Conserved hypothetical	same	same	up	up	up	same	up			
Alkyl hydroperoxide reductase, subunit C	same	same	same	same	up	same	up			

Chapter 6

Conclusions and future work

GBS is a major neonatal pathogen that is able to survive in several very different host environments, including both the women's vagina, amniotic fluid and the neonate's lung, blood and cerebral fluid. A considerable amount of work has been done on the GBS pathogen in relation to specific virulence factors (Doran and Nizet, 2004), but only recently has the research has been focused on the ability of bacteria to adapt to different environments and few proteomic studies of GBS have been carried out. Proteomic investigations of GBS may help us understand what kind of mechanisms help the bacteria to be able to survival in these very different environments and to cause a serious disease in a short time of onset.

Long term survival of GBS under conditions of nutrient limitation and acid exposure, including planctonic and biofilm modes of growth were investigated. GBS A909 could survive in pH 7 THB for more than 8 weeks and maintained high cell numbers. In contrast, GBS A909 could not be recovered from pH 5 THB after the 1 week time point using THA plate. Acid adaptation experiments showed there was no significant improvement of GBS A909 growth in pH 5 THB after acid exposure. However, Live-Dead staining results suggested that the live cell numbers were comparatively higher than the live cells counted by CFU/mL. This suggested that cells might have entered into a 'viable but non-culturable' state. Biofilm results showed there was biofilm formation of GBS and the biofilms were improved by feeding with low nutrient

media and coating plate with extracellular matrix molecules.

An *in vitro* model of GBS growth under conditions reflecting those associated with maternal vaginal carriage (low pH, low oxygen, nutrient stress) or exposure to body fluids during invasive disease (neutral pH, aeration, nutrient sufficient) has been established and followed by proteomic investigation of proteins differentially expressed under these two conditions. A total of 76 proteins were identified and 16 of these were expressed differentially. The putative virulence factor C protein β antigen (in its ca. 55 kDa low MW form), which is linked to evading immune elimination, and proteins involved in responses to oxidative stress including Fe-S assembly protein SufC, cysteine synthase A and thioredoxin reductase were up-regulated under the conditions reflecting neonatal exposure. The GroES chaperonin, which is linked to stress responses and acid tolerance, and adenine phosphoribosyltransferase, which is linked to nutrient stress, were found to be abundant under conditions reflecting maternal carriage.

Proteomic investigation of GBS growth on Todd Hewitt agar in the presence or absence of 10% human serum was used to model conditions associated with GBS neonatal septicaemia. A total of 84 proteins were identified, 11 of which were expressed differentially. The putative virulence factor C protein β antigen (in its high MW ca. 123 kDa form); arginine deiminase, which is induced by human serum and may contribute to immune evasion; an ABC transporter substrate-binding protein; and glyceraldehyde-3-phosphate dehydrogenase were each up-regulated in the presence of human serum.

These data help us understand the molecular basis of GBS adaption to different environments and disease development. The data are important as the only previous report on differential protein expression by GBS appears to be the study of Johri et al. (2006). Although there were not as many proteins differentially expressed under the different conditions in the present study (Chapters 4 and 5) as was hoped for, two important classes of proteins may be under-represented in this study: membrane proteins and secreted proteins. Future work may use alternative methods to extract membrane proteins and to recover secreted proteins and analyse their differential expression. Some new techniques, such as multi-dimensional protein identification technology (MudPIT) may be used for proteomics to increase the identification of proteins. MudPIT has been used in a wide range of proteomics including identification of protein complexes, membrane proteins and post-translational modifications of protein expression (Claire and Yates, 2007).

The use of mutant strains may help further in establishing the significance of key proteins in the cause of GBS disease. The mutant strains are constructed by deletion of pathogen genes by strategies such as insertional inactivation or allelic replacement. Comparing the characteristics between mutant strains and wild strains will help determine the functions of the gene of interest. Investigations of key putative virulence factors identified in this study, notably Bac and arginine deiminase, are now needed.

As part of this study a proteomic model reflecting the long term survival, comparing GBS growth at 6 hours and 1 week time points was designed. However due to the limited time available, the proteins expressed between

these conditions were not able to be identified by LC-ESI/MS. Once these proteins are identified, the data can be added to the knowledge of GBS protein expression in conditions reflecting colonisation.

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