ABSTRACT

Group B Streptococcus (GBS) is a leading cause of neonatal meningitis and septicaemia. During the progression of invasive disease, GBS must be able to detect and adapt to a diverse range of environments. One of the challenging environments the organisms will encounter is the antimicrobial phagosome of cells of the innate immune system.

Combining microscopy with pharmaceutical approaches, I have been able to show that GBS is residing within a vacuole that acquires phagolysosomal markers and, that acidification of the phagosome is required for GBS to survive. In addition this work has demonstrated that GBS induces only a weak reactive oxygen burst in macrophages and consequently reactive oxygen species are of limited importance. Interestingly, however, the GBS acid response regulator CovS/R is crucial for the organism’s ability to survive within murine macrophages. This is most likely due to the regulation of genes required for adaption to the intracellular environment.

Lastly, to facilitate investigations into the interaction of GBS with the phagosome it would be desirable to be able to visualise live organisms within cells. In the final part of this thesis, I describe an evaluation of different approaches to generate a suitable, fluorescently labelled, strain of GBS.
Although it is my name on the front of this thesis, really it has been a team effort. There are so many people who have helped me along the way, both in the last four years and previously. People who have taught me, inspired me, given practical support, shown that science is fun and sometimes a shoulder to cry on. There are far too many to do justice to here.

I would like to acknowledge the help that I have obtained from GBS researchers across the country and the world in sending strains and advice; Mark Anthony, Craig Rubens, Christian Gillie, Barbra Spellerberg, Shaynoor Dramsi and Mike Wessels. I would particularly like to thank Angela Nobbs and the Jenkinson lab who provided strains and were always prepared to share protocols and advice. I would like to thank my examiners Professor Howard Jenkinson and Professor Charles Penn for their thorough examination of the work and for allowing me to improve it. I would also like to thank the various people that I persuaded to read bits of the thesis for me, Anne-Maire, Dave, Lewis, Simon and Rebekah, your comments were very helpful and gratefully appreciated.

I would like to thank Dr. Robin May for being a brilliant supervisor, without your constant optimism and support I would probably be hiding under a table somewhere. Your ability to bring out the best in people and offer guidance and advice whilst still allowing us to maintain independence are skills that I hope I can learn from you.
The May lab have not only been invaluable for their skills, expertise and cake but also for being a fantastic group to work with. I want to mention Simon, Elizabeth, Kerstin and Leanne who were with me throughout. Not only did they practically show me how to do everything in the lab they offered many thought provoking scientific conversations and new directions in which to take my project, not to mention being great friends. Thank-you Leanne, team strep! The plentiful streppy conversations, your eagle eyed proof reading and knowledge of the literature have been greatly appreciated. Everyone past, present and passing through the May lab and the members of the fourth floor have made this project possible and the last four years a real pleasure, thank-you.

My friends and family, especially my parents and Sue and Ray have given me constant support and maintained interested, even when the explanations may have been long and boring or short and hostile (when things were not going well) and I am truly thankful to them for their support.

It is not really possible to thank Steve enough, this is his PhD too. Thank-you for sharing the highs and lows; for putting up with me when the macrophages would not grow, for helping me keep things in perspective and for reminding me how to have fun when the going got tough.
Group B *Streptococcus* is the leading cause of neonatal septicaemia and meningitis in the UK. During the natural course of invasive disease the organism must be able to overcome the efforts of the host immune system. Chapter One reviews potential methods that the organism can use to overcome barriers to infection, both physical and immunological, with a particular focus on the organism’s ability to persist within phagocytic cells.

An intracellular survival assay was developed to quantitatively analyse GBS survival within phagocytic cells, specifically macrophages. Chapter Three describes work performed using this assay to investigate intracellular survival from the organism’s perspective. The contribution of known virulence factors to intracellular survival was measured by looking at strains deleted in these genes.

Chapter Four focuses on the host response to GBS, by first asking whether this is actively modified by the organism and then looking specifically at the role of reactive oxygen species and phagosome acidification on control of intracellular organisms.

Chapter Three and Four should be considered together to gain a better understanding of the host pathogen interactions of GBS and consequently chapter five draws together and discusses the data from the two previous chapters. This chapter combines the results obtained from this study with published evidence to suggest possible explanations for my findings and also proposes future lines of enquiry.
Chapter Six is a “stand alone” chapter describing an on-going project throughout this study; the development and evaluation of a strain of GBS expressing GFP. The availability of such a tool would be greatly beneficial to the study of the fate of GBS within host cells.
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ABBREVIATIONS

AMP  Antimicrobial peptide
ANOVA  Analysis of variance
ATR  Acid tolerance response
BBB  Blood brain barrier
CAMP  Cationic antimicrobial peptides
CFU  Colony forming unit
CNS  Central nervous system
CR  Complement receptor
DMEM  Dulbecco’s modified Eagle’s Medium
DMSO  Dimethyl sulfoxide
DNA  Deoxyribonucleic acid
dNTP  Deoxynucleoside triphosphates
EOD  Early onset disease
ESAT-6  Early secreted antigenic target
FBS  Foetal Bovine Serum
FeNTA  Ferric nitritotriacetate
FITC  Fluorescein isothiocyanate
GBS  Group B Streptococcus
GFP  Green Fluorescent Protein
GM-CSF  Granulocyte Macrophage Colony Stimulating Factor
H2DCFDA  2’,7’-Dichlorodihydrofluorescein diacetate
hBMEC  human Brain Microvascular Endothelial Cells
hMDMs  Human monocyte derived macrophages
HRP  Horse radish peroxidase
IAP  Intrapartum antibiotic prophylaxis
IFNγ  Interferon gamma
IL  Interleukin
iNOS  Inducible nitric oxide synthase
LB  Luria Broth
LOD  Late onset disease
LPS  Lipopolysaccharide
LTA  Lipoteichoic acid
MOI  Multiplicity of infection
NRAMP  Natural Resistance Associated Macrophage Protein
OD  Optical density
PBS  Phosphate buffered saline
PCR  Polymerase Chain Reaction
PFA  Paraformaldehyde
PI  Post infection
PKC  Protein Kinase C
PMA  Phorbol-12-myristate 13-acetate
RNA  Ribonucleic acid
RNS  Reactive nitrogen species
ROI  Reactive oxygen intermediates
ROS  Reactive oxygen species
RPMI Roswell Park Memorial Institute media
SDS  Sodium dodecyl sulfate
TCS  Two component system
THY  Todd Hewitt yeast extract broth
TLR  Toll like receptor
TNFα Tumour necrosis factor α
TRITC Tetramethylrhodamine-5(and 6) isothiocyanate
UV  Ultraviolet radiation
WT  Wild type
β-h/c β-haemolysin/cytolysin
CHAPTER 1: INTRODUCTION
1.1 GROUP B STREPTOCOCCUS

Lancefield group B streptococcus (GBS) or *Streptococcus agalactiae* is a Gram positive encapsulated bacterium exhibiting β haemolysis on blood agar. The organism is found as a commensal in the gastrointestinal and the genitourinary tract of up to 30% of healthy adults. However, GBS is a significant cause of neonatal meningitis and septicaemia; in 2010 the rate of bacteraemia (England, Wales and Northern Ireland) in neonates up to 90 days was 0.64 per 1000 live births (HPA, 2011). Infection is seen increasingly in adults, especially those with underlying diseases, particularly diabetes mellitus, and in 2005 two thirds of all invasive GBS infections in the US were in older adults (Edwards and Baker, 2005). GBS is also a significant agricultural and veterinary problem, since it can colonise the mammary glands of ruminants and is the major course of subclinical mastitis (Sorensen et al., 2010). Rarely, it has been isolated from dogs, horses, guinea pigs and fish (Elliott et al., 1990)
1.1.1 The Genus *Streptococcus*

Bacteria, Firmicutes, Bacilli, Lactobacillales, Streptococcaceae, *Streptococcus*, *Streptococcus agalactiae*.

Functionally, Group B *Streptococcus* are classified as homofermentative lactic acid bacteria, since they produce lactic acid as the sole or major fermentation product. As catalase-negative, non-sporulating, Gram positive cocci with a low GC content they, along with most of the other lactic acid bacteria, fall in the phylum Firmicutes, order Lactobacillales (Figure 1). Lactobacillales is a diverse order containing industrially important genera including *Lactococcus*, *Enterococcus*, *Streptococcus*, *Oenococcus*, *Pediococcus*, *Leuconostoc* and *Lactobacillus* (Makarova et al., 2006).

The family *Streptococcaceae* (Figure 2) includes the species *Enterococcus*, generally associated with faeces, *Lactococcus*, associated with plants and dairy products, and *Streptococcus* (Makarova and Koonin, 2006).
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Phylogenetic trees of the order Lactobacillales constructed on the basis of concatenated alignments of ribosomal proteins. Species are coloured according to family: Lactobacillaceae, blue; Leuconostocaceae, magenta; Streptococcaceae, red. The Genus Streptococcus represented by the species *Streptococcus thermophilus* and the genus Lactococcus with the species *Lactococcus lactis* subspecies *cremoris* can be seen on different branches of the family Streptococcaceae. Modified from (Makarova, Slesarev et al. 2006)
Figure 2: The position of *Streptococcus agalactiae* (GBS) within the Lactobacillales

A phylogenetic tree of the order Lactobacillales constructed on the basis of concatenated alignments of four subunits of the DNA-dependent RNA polymerase. The family Streptococcaceae is highlighted in red, the two organisms that have been used in this study *S.agalactiae* and *L.lactis* are boxed. Modified from Makarova *et al* (Makarova and Koonin 2007)
Streptococcal species of clinical importance are split into six groups based on pathogenic and clinical features. *Streptococcus agalactiae* falls into the pyogenic group. The pyogenic streptococci are identified on blood agar plates by the classical zone of β haemolysis surrounding colonies. Further classification of the β-haemolytic streptococci is by serological typing of the polysaccharide capsule, a method developed by Rebecca Lancefield in the 1940s. *Streptococcus agalactiae* is serologically classified as Lancefield Group B, hence it being commonly called Group B *Streptococcus* or GBS (Madigan *et al.*, 2006, Greenwood *et al.*, 2002).

Despite being described as homofermentative, the organism is predicted to be able to ferment a variety of carbon sources to produce a number of different by-products, including lactate, acetate, ethanol, formate and acetoin. Bovine strains are able to ferment lactose, present at high levels in the mammary glands; this is less often seen in strains isolated from humans, with only 13% of 128 clinical isolates having this ability, including the strain used in this work NEM316 (Sorensen *et al.*, 2010). The organism is aero-tolerant and can undergo respiration if the environment can supply quinone or haem which will activate an electron transport chain (Yamamoto *et al.*, 2006, Rezaïki *et al.*, 2008). GBS is auxotrophic for several essential amino acids. Based on genome sequencing it appears that the organism has a genetically similar bioenergetic metabolism to *Lactococcus* (Glaser *et al.*, 2002).
1.1.2 Group B streptococci show considerable intraspecies diversity

Group B streptococci are sub classified into ten serotypes according to the immunological reactivity of the polysaccharide capsule. Of these, serotypes Ia, Ib, II, III and V are responsible for the majority of human invasive disease. Serotype III is the serotype most often isolated from neonatal disease and is associated with 80% of cases of neonatal meningitis. Serotype V is the most common serotype isolated from GBS infection in non pregnant adults (Glaser et al., 2002, Johri et al., 2006). Multi-locus sequence typing has identified six main clonal complexes; CC1, CC10, CC23, CC19, CC17 and CC67 (Tazi et al., Sorensen et al., 2010, Jones et al., 2003c, Brochet et al., 2006, Tettelin et al., 2005). Sequence type 17 (ST17) is the predominant sequence type isolated from neonatal meningitis, this lineage appears to represent a highly successful invasive clone and is described as hypervirulent (Manning et al., 2009, Brochet et al., 2006, Jones et al., 2003c, Poyart et al., 2008). The emergence of ST17 is thought to have occurred via stepwise genome recombination from other clonal types, this group appear to be host adapted and show less diversity than other sequence types (Sorensen et al., 2010). The genomes of Streptococcus agalactiae NEM316 (serotype III), A909 (serotype Ia) and 2603v/r (serotype V) have been fully sequenced.

The GBS genome is approximately 2100Kb in size with 2000 predicted protein coding genes. From the sequenced strains it is estimated that approximately 80% of genes form a core genome, with a large and still increasing pan genome (Tettelin et al., 2005). The genome contains a large number (14) of putative chromosomal
pathogenicity islands, which are not seen in other streptococcal genomes sequenced to date (Glaser et al., 2002). However, despite carrying putative virulence determinants none of the identified pathogenicity islands have been specifically linked to disease phenotype (Herbert et al., 2005).
2. GROUP B STREPTOCOCCAL DISEASE

Group B Streptococcus emerged as a cause of neonatal meningitis, sepsis and pneumonia in the 1970’s (Anthony and Okada, 1977). GBS had been previously been identified as having the potential to cause human infection, but it is possible that during this period the clonal expansion of a successful host adapted lineage increased the prevalence of the disease. This has been seen recently with ST-17 clones (Sorensen et al., 2010). In England, Wales and Northern Ireland the rate of GBS bacteraemia including neonatal disease is 2.8 per 100 000 population (HPA, 2011).

1.2.1 Colonisation

Group B Streptococcus is part of the normal human microbiota and approximately 30% of the population are colonised at any one time. Colonisation is important as it appears to precede invasive disease. GBS has been isolated from the rectum, perianal area, vagina, cervix and urethra (Melin, 2011). Many of the prevalence studies looking at colonisation have been carried out on pregnant women and from these it is known that colonisation rates are higher in sexually active individuals, especially those with multiple sexual partners, and increases with maternal age (Sendi et al., 2008, Rocchetti et al., 2010). It is known that the vaginal flora can be an important factor in pregnancy outcome and GBS colonisation is associated with previous spontaneous abortion (Rocchetti et al., 2010).
### 1.2.2 Neonatal disease

Neonatal disease is subdivided into early onset disease (EOD), in which presentation occurs within the first week of life, and late onset disease (LOD) for cases presenting between one week and three months of life (90 days). 60-70% of neonatal GBS disease is early onset.

Neonatal disease is preceded by the asymptomatic colonisation of the female genital tract during pregnancy and 30-70% of babies born to colonised mothers will be colonised themselves. 1-3% of babies born to colonised mothers will develop disease (Schuchat, 1999, Melin, 2011, Daniels et al., 2011, Heath and Schuchat, 2007).

Density of vaginal colonisation is thought to be the most significant risk factor for early onset neonatal disease (Hansen et al., 2004, Van Der Mee-Marquet et al., 2009), although additional maternal and obstetric risk factors for the development of EOD include (Poyart et al., 2008, Melin, 2011):

- Premature birth or low birth weight
- Prolonged rupture of amniotic membranes
- Maternal fever during labour or chorioamnionitis,
- GBS isolated from urine samples during pregnancy
- A previous baby being born with GBS disease.

The route of infection in early onset neonatal disease is thought to be fetal aspiration of the vaginal contents during passage through the birth canal, or by ascending infection. The organism may travel through intact or ruptured amniotic membranes into the amniotic fluid, where it is able to replicate and be aspirated. Following
aspiration by the fetus the organism can remain localised in the lungs, causing pneumonia, or progress to sepsis (72%) and meningitis (2.5%) (Poyart et al., 2008, Doran and Nizet, 2004). Mortality rates for early onset disease have decreased in recent years from 50% to 4-10%, due to the implementation of prevention measures. Despite improvements in treatment, however, 50% of infants that survive neonatal disease will have long term neurological sequelae (Daniels et al. 2011, CDC, 2010).

Late onset disease commonly presents as meningitis or bacteraemia. The route of infection is yet to be proven but exposure is likely to be via horizontal transmission from the mother or a contaminated environment (Melin, 2011). The organism may then gain accesses to the blood stream by adhering to and translocation of the gut epithelium (Tazi et al., 2010). LOD carries a lower mortality rate than early onset disease at 2-6% (Schuchat, 1999, Johri et al., 2006), however deaths attributable to meningitis are higher at 14.5% of all LOD fatalities, compared to 2.5% in EOD (Poyart et al., 2008, Tazi et al., 2012).

**1.2.3 Adult disease**

Group B *Streptococcus* can cause maternal infection during pregnancy or labour, being associated with approximately 12-25% of puerperal fever (infection during childbirth). In pregnant women, GBS cause endometritis, chorioamnionitis and bacteremia (Rodriguez-Granger et al., 2012). Women colonised with GBS during pregnancy are at increased risk of stillbirths and premature delivery (Melin, 2011).
In non-pregnant adults GBS can cause skin and soft tissue infections, urinary tract infections, infection of the joints and a wide range of other presentations. The incidence in the US of invasive GBS disease in adults has been reported as 2.4-4.4 cases per 100,000; this rate is rising, which could be a reflection of the increasing population at risk. In the non-pregnant adult population risk factors for GBS disease include co-morbidities such as diabetes mellitus, cardiovascular disease, liver disease and cancer (Edwards and Baker, 2005).

Recently cases of GBS causing necrotising fasciitis and toxic shock syndrome have been reported in both adults and neonates (Sendi et al., 2009b). Relapsing infection has been seen in adults, especially presenting as cellulitis, this could be due to a reservoir in the GI tract, repeated exposure through a close contact or insufficient tissue clearance due to poor lymphatic drainage. However there is the possibility that the organism inhabits a privileged niche such as within host cells, preventing clearance (Sendi et al., 2008).

1.2.4 Epidemiology of disease and serotype distribution

Five serotypes (Ia, Ib, II, III and V) are responsible for 85% of neonatal GBS disease worldwide and the distribution of these serotypes is the same across all WHO regions (Edmond et al., 2012). Type III is the most prevalent serotype, being isolated from 37% of EOD and 67% of LOD (Weisner et al., 2004). A similar serotype distribution is also seen within isolates causing adult disease and is representative of the colonisation frequency of the different serotypes (Sendi et al., 2008).
1.2.5 Prevention of Group B Streptococcal disease

Current prevention of GBS disease in the UK is based on the administration of antibacterial prophylaxis, immediately before and during labour (Intrapartum prophylaxis or IAP) to women who have been identified to have risk factors. If the antibiotics are able to be administered four hours before birth it has been shown to significantly reduce the chance of babies born to colonised mothers becoming themselves colonised (from 40% to 25%) (Daniels et al., 2011). The implementation of IAP in the UK to at risk groups in 2000-2003 reduced the rate of culture-proven early onset disease by 83% (Brocklehurst and Kenyon, 2008, CDC 2010, Heath and Schuchat, 2007).

Guidelines introduced in 2002 in the USA advised screening all pregnant women at 35-37 weeks gestation, followed by IAP if a positive result was obtained. Screening has reduced the rate of EOD by a further 20-40% (CDC, 2010). Screening of all pregnant women at 35-37 weeks gestation has also been implemented in other European countries. Current United Kingdom National Institute of Clinical excellence (NICE) guidelines state ‘Pregnant women should not be offered routine antenatal screening for Group B *Streptococcus* because evidence of its clinical and cost effectiveness remains uncertain.’ (NICE, 2010). This has been further supported by a modelling study which suggested that the safest and most cost effective way to further reduce GBS disease in the UK is to give all women IAP without screening, a practice which would not be practical or well received (Kaambwa et al., 2010, Brocklehurst and Kenyon, 2008). One of the problems with the screening protocol is
the sensitivity of detection and the fact that the absence or presence of the organism at the time of screening is not necessarily predictive of the presence or absence of the organism at the time of birth. Trials have been carried out looking at rapid screening methods at the time of labour using nucleic acid amplification based techniques, but the conclusions from these have not supported changing current guidelines (Daniels et al., 2011, Brocklehurst and Kenyon, 2008, CDC, 2010). In the USA, approximately 30% of all women now receive antibiotics during labour (Stoll et al., 2011).

One study has suggested that the increased use of antibiotics has increased the incidence of neonatal sepsis with *E.coli* (Johri et al., 2006). However, generally the concern that IAP would increase the rate of sepsis with Gram negative resistant organisms has not been proven and GBS remains the leading causative organism of neonatal sepsis despite preventative measures (Stoll et al., 2011).

IAP has reduced the incidence of EOD but has not changed the incidence of LOD which has remained steady over a 20 year period at about 0.4 cases per 1000 live birth (CDC, 2010, Melin, 2011).

1.2.6 Treatment and antibiotic resistance

Treatment of GBS disease is with Penicillin or other β-lactam antibiotics with resistance being very rare. In cases where penicillin is counter indicated Erythromycin or Clindamycin would be the second choice. Resistance to macrolides and clindamycin is reported at 14% and 10% respectively. Resistance is thought to
be brought about by methylation of ribosomal RNA, thus altering the target site of the drug, or due to the presence of an efflux pump (HPA, 2011). An intriguing treatment possibility is to increase the ability of the host immune system to remove the organism by giving passive immunity (Senn et al., 2011).

1.2.7 Vaccine development

Currently there is not a vaccine available for Group B Streptococcus, although vaccination is an attractive preventative strategy. It is known that high levels of capsular type specific antibodies are protective against invasive neonatal disease (Lin et al., 2004). It could be predicted that vaccination, unlike IAP, could also reduce the incidence of LOD and adult disease. Capsular polysaccharide vaccines with tetanus toxoid or CRM conjugates are in human clinical trials, and protective levels of antibodies have been seen for up to two years following vaccination (Heath, 2011). Antibodies raised against the capsule have been shown to be protective but only against homologous serotypes, thus a successful vaccine would need to be multivalent. Vaccination against serotypes III, Ia and V could theoretically provide protection against 88% of GBS disease in the UK (Weisner et al., 2004). The disadvantages of capsular polysaccharide vaccines, notably the poor immunogenicity of polysaccharide and the serotype specificity, have led to research looking at GBS surface expressed proteins as potential vaccine candidates. Out of the proteins being investigated only two, the C5a peptidase and Sip are conserved at the gene level in most isolates, highlighting the challenges encountered with this approach (Johri et al., 2006).
1.3 OVERCOMING BARRIERS TO INFECTION

1.3.1 Physical barriers

GBS is able to colonise the mucus membranes lining the gastrointestinal and genitourinary tract of humans. If the organism is able to breach these barriers and gain access to the soft tissue and bloodstream it can lead to serious disease. Multiplication of the organism in the bloodstream can lead to bacteraemia and sepsis, whilst adherence to endothelial cells can lead to penetration of the blood brain barrier and the development of meningitis (Figure 3).
Figure 3: Barriers to infection

Group B *Streptococcus* is able to adhere to the mucus membranes of the gastrointestinal and genitourinary tract (1). In pregnant women the organism can ascend, invade the chorioamniotic membranes and replicate in the amniotic fluid (2). In EOD the organism enters the body through the lungs (3) in LOD the route of entry may be the gut epithelial cells (4). Within the mucosal tissue and the lung tissue resident macrophages will be the first cells of the immune system to encounter invading GBS. The organism is able to cross lung endothelial and epithelial cells and gut epithelial layers to gain access to the blood stream (5). The host prevents invading organisms from surviving in the blood by the presence of soluble factors such as antibodies or complement and the antimicrobial cells of the innate immune system namely the professional phagocytes, monocytes and polymorphonucleocytes (PMN’s). From the blood stream the organism can adhere to and invade brain microvessel endothelial cells (BMEC) then cross the blood brain barrier (6). In adults the organism has been associated with skin and soft tissue infections, urinary tract infections and joint and bone infections (7).
1.3.1.1 Adhesion and invasion into cells

GBS has been shown to adhere to and invade vaginal epithelial cells (Sheen et al., 2011), lung epithelial and endothelial cells, human brain microvessel endothelial cells and gut epithelial cells (Rubens et al., 1991, Rubens et al., 1992, Tazi et al., 2010). Adherence and invasion has been shown both in tissue culture and in whole organism infection. Adherence to the extracellular matrix is an important factor in the establishment of disease and it is noteworthy that the highly virulent ST-17 clones show enhanced adherence properties, due to a combination of a unique surface protein HgvA (Tazi et al., 2010), regulation of fibrinogen binding through the two component system (TCS) RgfA/C (Safadi et al., 2011) and a novel keratin binding Srr-2 protein (Seifert et al., 2006). Cell adherence may confer host specificity; bovine strains do not contain the genetically linked fibronectin binding ScpB protein and the laminin binding Lmb protein (Lindahl et al., 2005).

Successful colonisation by GBS of mucus membranes may be initially dependent upon an organism’s ability to interact with the extracellular matrix (ECM). The ECM is a structural component of epithelial and endothelial cell layers consisting of glycoproteins such as collagen, laminin, fibronectin and fibrinogen. Initial low avidity interactions between lipoteichoic acid in the organisms cell wall and ECM allows for surface expressed proteins to form stronger bonds with ECM components. GBS has numerous cell surface proteins implicated in binding epithelial cells, endothelial cells and ECM components; two of these currently attracting interest in the field are the pilus protein and HvgA. These are highlighted later in this section. Binding to the
ECM is multifactorial, since proteins that show isolated binding properties are dispensable for binding in the whole organism (Dramsi et al., 2012). In addition many of the proteins appear to have multiple ligands, allowing the organism to attach to a diverse range of cell types.

Regulation of adherence

GBS has a number of regulatory systems allowing the organism to respond and adapt to different environments encountered in the host. Proteins implicated in adherence appear to be under considerable, often negative, regulation. The transcriptional regulator RovS and the two component system CovS/R are both repressors of multiple genes whose products confer adherence to different ECM and cell types.

Strains deleted in components of the CovS/CovR two component system show hyper-adherence (Lamy et al., 2004, Jiang et al., 2005). CovS/R is a repressor of the Rga transcription factor which is a positive regulator of the pilus and the Srr-1 proteins (Dramsi et al., 2012). However overexpression of the pilus protein PI-1 in CovS/R deleted strains does not appear to contribute to the hyper-adherence of the mutant (Jiang et al., 2012). CovS/R mutant strains also show overexpression of cell wall proteins, such as BibA and ScpB which have been indicated to have roles in adherence (Lamy et al., 2004, Jiang et al., 2005).

Strains deleted in RovS show increased expression of fsbB a fibrinogen binding protein and demonstrate enhanced binding to fibrinogen and epithelial cells (Samen et al., 2006). As mentioned previously regulation of fibrinogen binding through the
TCS RgfA/C has been implicated as a factor leading to hyperadherence in the highly virulent ST-17 strain (Safadi et al., 2011).

The Role of the Pilus protein in adhesion and invasion

GBS displays a pilus protein which has been shown to be important for the organism’s ability to adhere to and invade multiple cell types (Maisey et al., 2008b, Lauer et al., 2005, Dramsi et al., 2006). In Gram positive organisms the pilus is predicted to have a role in cell adherence and biofilm formation. All clinical isolates of GBS encode a pilus from at least one of the two loci, PI-1 and PI-2 (which has two allelic variants PI2a and PI2b). Group B streptococcal pili are trimeric; a minor subunit of pilin PilC forms the base, covalently bound subunits of the major pilin PilB form the backbone and PilA (the adhesin) is located along the backbone and forms the tip. PilB was identified as a protein required for invasion but not for adhesion before the pilus structure was identified (Adderson et al., 2003) and is thought to be important in paracellular translocation of the organism (Pezzicoli et al., 2008). PilA is able to bind collagen by a Von Willebrand type A factor domain (Gilmore et al., 2009). The pilus is not always essential for cell binding (Jiang et al., 2012), mostly likely due to the numerous other adherence factors on the surface of the organism. However, there is more support for the role of the pilus in cell invasion, maybe through the binding of collagen in focal adhesion kinases as shown on hBMEC (Banerjee et al., 2011, Tazi et al., 2012).
BibA, HvgA and hypervirulent ST-17

BibA is a surface expressed GBS adhesin which aids binding to epithelial cells (Santi et al., 2007). A variant in the BibA locus is seen specifically in the hypervirulent MLST type 17, this has been described as the Hypervirulent GBS adhesin (HvgA). HvgA increases the adherence and invasion of ST-17 clones to intestinal epithelial cells and cells of the blood brain barrier, which could explain the increased association of this sequence type with late onset neonatal disease (Tazi et al., 2012, Tazi et al., 2010).

1.3.1.2 Invasion and tissue destruction

During systemic infection GBS traverses lung epithelial and endothelial cell barriers, the blood brain barrier (BBB) and the intestinal epithelium, giving the organism access to body compartments such as the amniotic compartment, the blood stream and the central nervous system. Bacterial translocation of cellular barriers can be through transcytosis, paracellular translocation or cell and tissue damage. GBS has been reported to use all three of these mechanisms. Epithelial cell layers may be broken down by internalised organisms causing apoptosis or necrosis of infected epithelial cells (Da Costa et al., 2011) or crossed by paracellular translocation (Soriano et al., 2006). The blood brain barrier is breached by cellular injury or transcytosis (Doran et al., 2003). The organism has shown to be able to trancytose through intact chorion cell monolayers (Winram et al., 1998).
Tissue damage and destruction; pore forming toxins and enzymes

One of the most important known virulence factors in Group B Streptococcus is the β-haemolysin/cytolysin (β-h/c), a surface associated pore forming toxin which is responsible for the characteristic zone of haemolysis on blood agar plates (Nizet, 2002, Nizet et al., 1996). The β-h/c toxin is expressed by cylE, a single open reading frame on the cyl operon (Forquin et al., 2007). cylE deletion mutants have been shown to be less virulent than wild type GBS in animal models (Liu et al., 2004). Four main roles in pathogenicity have been ascribed to the cyl operon, (i) apoptosis in macrophages (Fettucciari et al., 2000, Ulett and Adderson, 2005) (ii) induction of a pro-inflammatory host response (Lembo et al., 2010), (iii) increased resistance to phagocytic killing (Liu et al., 2004) and (iv) induction of cytolysis. The first three are discussed elsewhere in this chapter. The cytolytic action of the β-h/c toxin, can lead to cell necrosis, promoting bacterial invasion of epithelial and endothelial barriers and the production of pro inflammatory cytokines (Nizet et al., 1996). β-h/c has been implicated in cytolytic injury to lung endothelial cells, brain endothelial cells and at sub-cytolytic concentrations can promote the organism’s invasion into cells (Nizet et al., 1996, Nizet, 2002, Doran et al., 2002).

In addition to β-H/C, GBS also produces CAMP factor, a second pore forming toxin whose activity is seen mainly on red blood cells pre-treated with sphingomyelinase (Sterzik and Fehrenbach, 1985). Purified CAMP factor encoded by the cfb gene has been shown to be toxic when injected into rats (Lang and Palmer, 2003), although its role in virulence in the whole organism is uncertain (Hensler et al., 2008).
Group B Streptococcus also produces a hyaluronate lyase (Lin et al., 1994), which may aid tissue dissemination of the organism by breaking down hyaluron, a major component of the ECM found in high concentrations in the placenta, amniotic fluid and lung (Rajagopal, 2009).

1.3.1.3 Crossing the blood brain barrier

The blood brain barrier (BBB) comprises of choroid plexus epithelial cells and specialized brain microvessel endothelial cells (BMEC). The ability of GBS to cross the blood brain barrier has been suggested to be through a combination of mechanisms; direct invasion of the BMEC’s, toxin mediated damage and through the induction of a host inflammatory response which can compromise BBB integrity.

Initial interaction of GBS with the BBB endothelium is likely to be through the attractive properties of LTA (Doran et al., 2005). Stronger attraction may then be mediated by the Srr-1 protein (Van Sorge et al., 2009), the organism’s ability to bind glycosaminoglycan (Gilmore et al., 2011) and the pilus protein (Maisey et al., 2007). Subsequent breakdown of the BBB seen in GBS meningitis is due to the production of inflammatory mediators and cytokines (Barichello et al., 2011). The production of pro-inflammatory cytokines and chemokines in the brain microvessel cells has been attributed to collagen binding by the pilus protein and the presence of β-haemolysin (Doran et al., 2003, Banerjee et al., 2011, Lembo et al., 2010). Both the pilus and the β-h/c are repressed by CovR, the response element of the CovS/R TCS. A strain deleted in CovR (and therefore overexpressing β-h/c) shows an increased ability to incite a pro-inflammatory response and penetrate the blood brain barrier (Lembo et al., 2010).
1.3.2 Adaptation to the host environment

During the course of GBS infection, the organism is able to detect and rapidly respond to different environments and challenges encountered within the host. The organism can grow to a high density in the vagina, amniotic fluid, lung and bloodstream. In such sites, conditions will be varied, ranging from the low pH and micro-aerophillic environment of the vagina to the neutral pH and aerobic conditions of the blood stream. Growth rate and resistance to immune clearance will determine the ability of the organism to establish infection (Johri et al., 2003). In addition to the benefit to the organism of having increased biomass within the host, increased growth rate also promotes invasion into cells and enhances resistance to immune clearance, this is thought to be due to growth phase gene regulation (Malin and Paoletti, 2001, Johri et al., 2003, Shelver et al., 2003).

Adaptation

The GBS transcriptome shows considerable plasticity and remodelling in response to growth phase, exposure to human blood or growth in amniotic fluid (Ahmed et al., 2008, Mereghetti et al., 2008, Sitkiewicz et al., 2009). Regulation changes are complex, with global transcriptional regulators being both up and down regulated. The CovS/R TCS, for example, shows down regulation after 30 minutes incubation in blood but is up-regulated at 90 minutes (Mereghetti et al., 2008). Similarly, CovR is downregulated at late logarithmic phase in amniotic fluid (Sitkiewicz et al., 2009). In both blood and amniotic fluid, the strongest differential gene expression is seen in genes mediating metabolism (Ahmed et al., 2008, Mereghetti et al., 2008, Sitkiewicz
et al., 2009). In amniotic fluid, in which the organism can grow, GBS is not seen to up regulate a stress response but there is an increase in transport of amino acids (Sitkiewicz et al., 2009). In contrast, after 90 minutes incubation in blood (Mereghetti et al., 2008), in which GBS cannot grow, stress response genes and cell surface factors that bind or activate fibrinogen are strongly activated (Mereghetti et al., 2008, Yang et al.). The cylE operon is up regulated in amniotic fluid and also at fever temperature, this may have implications for pathogenesis due to the destructive and immunomodulatory properties of the toxin it produces (Mereghetti et al., 2008, Sitkiewicz et al., 2009).

Regulation of gene expression through two component systems

Signalling in bacteria is frequently achieved through two component systems (TCS), which sense environmental signals such as changes in chemical gradients, Mg\(^{2+}\) concentration, osmolarity and the presence of auto-inducing or anti-microbial peptides (Beier and Gross, 2006). A two component system generally comprises of a sensor histidine kinase on the bacterial membrane which becomes auto phosphorylated and will phosphorylate a cognate DNA binding protein. Group B Streptococcus has 17-20 identified two component systems (Tettelin et al., 2002, Tettelin et al., 2005, Glaser et al., 2002) and six standalone transcriptional regulators (Glaser et al., 2002). Compared to closely related streptococcal species, GBS has a high number of TCS: S.pneumoniae has 14, S.pyogenes 13 and L.lactis 8. This may reflect the lifestyle of GBS, which requires the organism being able to adapt to and thrive in different host environments (Glaser et al., 2002). A eukaryotic-like serine/threonine kinase regulator (Stk1) has been described in GBS, which can alternatively phosphorylate the DNA binding protein CovR, lifting repression. Finally,
a tyrosine kinase and phosphatase system has been implicated in regulation of capsule expression by GBS. A summary of these regulatory systems can be seen in Table 2 and they are discussed in the relevant sections of this chapter.
<table>
<thead>
<tr>
<th>Name (response element/histidine kinase)</th>
<th>Regulates</th>
<th>Role in host adaptation</th>
<th>Details</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DltR/DltS</td>
<td>DltR binds to promoter DNA of the dlt operon</td>
<td>Activates expression of the dltRSA-BCD operon increasing the D-alanine content of lipotechoic acid</td>
<td>Increasing the D-alanine content of LTA increases the resistance of the organism to AMP’s</td>
<td>(Poyart et al., 2001a)</td>
</tr>
<tr>
<td>RgfA/RgfC</td>
<td>Down regulation of ScpB (C5a peptidase) fsbB gene activation, fsbA gene inhibition. Regulation of fsbA independent of RovS</td>
<td>Regulation of cell surface proteins implicated in adhesion.</td>
<td>Differential regulation through this TCS is suggested to be important in the increased adherence seen in the ST-17 strains.</td>
<td>(Spellerberg et al., 2002, Safadi et al., 2011)</td>
</tr>
<tr>
<td>CiaR/CiaH</td>
<td>Regulation of purQ. Positive regulation of CiaR. Positive regulation of peptidases and proteins of unknown function.</td>
<td>Resistance to antimicrobial peptides. Plays a role in the organism’s ability to survive within multiple cell type. Promotes resistance to Antimicrobial peptides, oxidants and lysosome</td>
<td>Actions postulated to be due to cell wall stability or restructuring.</td>
<td>(Quach et al., 2008)</td>
</tr>
<tr>
<td>Stand alone regulators</td>
<td>Regulates</td>
<td>Role in host adaptation</td>
<td>Details</td>
<td>Reference</td>
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<tr>
<td>RovS</td>
<td>Binds to and activates cysE, and sodA, expression. Negative regulator of fsbA</td>
<td>Adherence to epithelial cell surfaces, expression of virulence factors which may facilitate intracellular survival.</td>
<td></td>
<td>(Samen et al., 2006)</td>
</tr>
<tr>
<td>RogB</td>
<td>Repressor or negative regulator of cpsA, the capsule operon. Positive regulation of pilus (PI-2b) and fsbA expression</td>
<td>Adherence and capsule production.</td>
<td>Not present in all strains</td>
<td>(Gutkeunst et al., 2003, Dramsi et al., 2006)</td>
</tr>
<tr>
<td>RgfB</td>
<td>ScpB (C5a peptidase)</td>
<td>Adherence</td>
<td></td>
<td>(Spellerberg et al., 2002)</td>
</tr>
<tr>
<td>Rga</td>
<td>Global regulation, 60 genes downregulated 36 upregulated in a deletion mutant. Includes proteins of unrelated functions including the Pilus and Srr-1.</td>
<td>Adherence. Possible antimicrobial peptide resistance due to pilus regulation.</td>
<td></td>
<td>(Rajagopal, 2009, Samen et al., 2006, Dramsi et al., 2012)</td>
</tr>
<tr>
<td>MtaR</td>
<td>Methionine uptake and transport, genes involved in arginine biosynthesis and cspA.</td>
<td>Mutant strains show attenuation in a mouse model. Does not regulate capsule genes.</td>
<td></td>
<td>(Rajagopal, 2009)</td>
</tr>
<tr>
<td>CpsA</td>
<td>Transcriptional regulator of the capsule locus, and possibly cell wall.</td>
<td>Cell wall integrity</td>
<td>Aids whole blood survival and virulence in zebrafish model</td>
<td>(Hanson et al., 2012)</td>
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<tr>
<td>Serine/threonine signalling</td>
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<tr>
<td>Stk1/Stp1</td>
<td>Phosphorylates CovR, decreasing promoter binding and repression, consequently acts as a positive regulator of the β-h/c. Negative regulation of the CAMP factor.</td>
<td>Resistance to reactive oxygen species and intracellular killing</td>
<td>Stk1 is membrane associated</td>
<td>(Rajagopal et al., 2006)</td>
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<tr>
<td>Tyrosine phosphorylation</td>
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<tr>
<td>CpsD/CpsB</td>
<td>Polysaccharide length</td>
<td>Capsule synthesis</td>
<td></td>
<td>(Whitmore and Lamont, 2012, Rubens et al., 1993)</td>
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</table>
**Energy and metabolism**

The majority of bacteria are heterotrophic, that is they require a carbon source for energy and substrates available for catabolism of macromolecules. Analysis of the GBS genome has predicted that the organism is auxotrophic for multiple amino acids, identified by the absence of biosynthetic pathways (Glaser et al., 2002). It is thought therefore that the organism must obtain most nutrients from the host. This hypothesis is supported by the presence in the genome of a large number of transporters suggesting a broad catabolic capacity (Glaser et al., 2002). Additionally, regulation of methionine utilisation through the MtaR transcription factor facilitates the organism’s growth in human plasma (Shelver et al., 2003). Small molecule transporters are proven to be important for the virulence of the organism - in their absence the organism is unable to take in peptides and scavenge residues for amino acid synthesis (Jones et al., 2000).

GBS is generally regarded as a strict fermentative organism. It is however not only aerotolerent, but can undergo oxidative respiration if supplied with exogenous haem or quinone. In the presence of these co-factors and oxygen, the organism shows improved survival in human plasma and increased biomass (Yamamoto et al., 2006, Lechardeur et al., 2010). Growth of the organism in aerobic conditions has been shown to increase growth rate, invasiveness of the organism to epithelial cells and virulence (Johri et al., 2003). Accordingly, inactivating aspects of the oxidative respiration pathway reduces virulence in an animal model (Yamamoto et al., 2006).
**1.3.3 Systemic response to Group B Streptococcus**

Having breached the physical barriers of the host and adapted to the new environment, the next challenge an invading organism faces is the host immune system.

**1.3.3.1 Immunity to Group B Streptococcal infection**

The presence of type specific antibodies against capsular polysaccharide has been shown play a role in protecting individuals against invasive GBS disease (Edwards *et al.*, 1979, Lin *et al.*, 2004). Capsule specific antibodies improve opsonophagocytic killing of the organism in both neonates and adults (Santi *et al.*, 2009b, Sendi *et al.*, 2008, Cheng *et al.*, 2001), and can offer protection through neutralising properties (Wessels *et al.*, 2011). Individuals who do not have high levels of antibodies, for example the elderly and the very young, are at a greater risk of GBS disease (Amaya *et al.*, 2004). The cells of the adaptive immune system are developed and able to start responding to challenges at birth. However levels of immunoglobulin are low in neonates, with the exception of IgG transferred placentally from the mother during the third trimester. Due to the poor immunogenicity of the GBS capsule only 10-20% of mothers have protective antibody levels (Melin, 2011), leaving neonates at risk of infection. During birth the fetus travels from the sterile intrauterine environment through the vagina where it will encounter commensal bacteria such as GBS. The innate immune response will be the main defence mechanism against these potentially pathogenic bacteria (Delves *et al.*, 2011, Kenzel and Henneke, 2006).
1.3.3.2 The innate immune system

The innate immune system has an important role in being able to distinguish self from non self. In comparison to the adaptive immune system which recognises specific epitopes the innate immune system has no epitope specificity and is often described as a non-specific system. It has a special role in protecting neonates against infection as it does not require pre-exposure to a pathogen.

The innate immune system has two arms; soluble molecules in blood such as complement and antimicrobial enzymes, and cells of the myeloid lineage. Cells of the innate immune system function to ingest and kill micro-organisms, present antigen and signal to other immune cells. They include the 'professional' phagocytes, macrophages and monocytes, neutrophils, and dendritic cells. Macrophages are tissue resident phagocytes which mature from circulating monocytes. They act as the first line of defence against invading microbes and orchestrate the subsequent inflammatory response. Macrophages are a major source of pro-inflammatory cytokines and immunomodulatory factors such as tumour necrosis factor α (TNFα), interleukin (IL) -1 and IL-12; they also function to resolve inflammation through the production of anti-inflammatory cytokines such as IL-10. If, when a tissue resident macrophage encounters microbial attack, they are not able to kill the organism, they produce cytokines and inflammatory mediators which lead to the recruitment of neutrophils and monocytes. Indeed this may be the primary role for this cell type. Neutrophils are granulocytes, they circulate in the blood and are recruited to sites of inflammation. Granulocytes, also called polymorphonuclear leukocytes (PMN’s) contain pre-formed granules containing antimicrobial enzymes and toxic molecules. They are highly antimicrobial but short lived. The main role for dendritic cells is
phagocytosis and then antigen presentation (Kenzel and Henneke, 2006, Murphy et al., 2008).

The neonatal innate immune system

Neonatal serum is generally poor at killing microorganisms, since levels of complement and other soluble enzymes are lower than in adult serum. However, neutrophils and monocytes are present in the developing foetus from the end of the first trimester and by birth are present at adult levels.

Despite adult levels of neutrophils, the most important immune deficiency in neonates is the reduced neutrophil response to infection, which is especially apparent in pre-term babies. The inability of neutrophils to respond to infection is thought to be due to a limited pool of neutrophils, poor tissue penetration and poor acceleration of production from bone marrow. Neonatal polymorphonuclear leukocytes (PMN’s) that are not activated have been suggested to have defects in production of reactive oxygen species and decreased levels of lactoferrin, lysozyme and other degradative enzymes (Henneke and Berner, 2006).

The distribution of tissue resident macrophages in neonates is the same as in the adult, with the exception of alveolar macrophages which are low until just before term, although this rises in the first 24-48 hours after birth (Remington et al., 2010). Phagocytosis and antimicrobial functions of neonatal macrophages are similar to that seen in adults, with the exception that they are insensitive to IFNγ activation. The ability of macrophages to recruit monocytes and neutrophils to a site of infection is delayed in neonates (Remington et al., 2010).
Innate immune system response to GBS.

The humoral arm of the innate immune system is probably ineffective at removing Group B Streptococcus. Along with host deficiencies in complement levels, GBS surface components BibA and the β protein GBS prevent activation of complement and hinder the formation of the membrane attack complex (Santi et al., 2007, Maisey et al., 2008a).

Both neonates (especially preterm newborns) and another high risk group, diabetics, have been shown to have defects in neutrophil function (Bjorkqvist et al., 2004). In addition, GBS produces two serine proteases, C5a peptidase (ScpB), which proteolytically cleaves the complement derived chemoattractant C5a, and CspA, which is able to cleave chemokines leading to reduced neutrophil chemotaxis (Bryan and Shelver, 2009, Cheng et al., 2002).

The reduced neutrophil function and recruitment to the site of invasion will leave resident macrophages as the key phagocytic and signalling cells of the neonatal innate immune response.

Recognition of GBS by the innate immune system can induce a pro-inflammatory response

An infectious agent may cause pathogenesis through its own virulence factors or it can be the host immune response to infection that causes the physiopathology of disease. The immune response to an invading organism must be carefully controlled to ensure that host damage is minimised whilst the organism is removed; macrophages and monocytes are important effector cells in this context. In neonatal
GBS disease a major pathogenic feature is the dysregulation of the immune response leading to sepsis and multi organ failure.

Macrophage and monocytes in neonatal blood and in the urinary tract respond to GBS with a pro-inflammatory cytokine release, involving Interleukin (IL) IL-1α, tumour necrosis factor (TNF) and IL6 (Currie et al., 2011, Kline et al., 2011). Elevated levels of TNF-α are characteristic of GBS sepsis. Monocytes can produce TNFα in response to GBS following deposition of complement factors on the organism’s surface (Levy et al., 2003). Similarly, macrophages activated in the liver and spleen secrete TNF-α into the blood stream, which acts on blood vessels to cause systemic oedema and can progress to disseminated intravascular coagulation leading to multiple organ failure (Kenzel and Henneke, 2006). The high level of monocyte derived TNFα in neonatal GBS sepsis could be due to monocytes being the most abundant cell type of the innate immune system in newborns, or due to the altered cytokine profile seen in neonatal cells (Remington et al., 2010, Levy et al., 2003). TNF-α does also have a positive effect, since it is important in activating macrophages to produce a protective response to GBS (Mancuso et al., 2009). However it is known that neonatal macrophages cannot be activated by TNF-α (Remington et al., 2010), so in this situation the high levels of TNFα are unlikely to be protective.

Cells of the immune system recognise invading pathogens through pattern recognition receptors such as the Toll-like and cytosolic NOD receptors, which then activate signalling pathways leading to cytokine production. GBS has been shown to activate a pro-inflammatory cytokine response through the interaction of the cell wall lipoteichoic acid (LTA) and lipoproteins with Toll like receptor 2 and 6 (TLR2/6),
involving the MyD88 and c-Jun kinase pathways (Draper et al., 2006, Henneke and Berner, 2006, Henneke et al., 2002). However production of TNF-α, a critical inflammatory mediator, occurs not through interactions with the organism’s polysaccharide capsule, peptidoglycan, lipoteichoic acid or lipoproteins but through the recognition of ssRNA by endosomal TLR-7 (Mancuso et al., 2009, Deshmukh et al., 2011a) and DNA through TLR-9 (Talati et al., 2008).

Recognition of GBS single stranded RNA activates host cells to increase production of Nitric oxide. Nitric oxide (NO) is an important inflammatory mediator which activates macrophages, aids phagosome acidification and subsequent processing of phagosome contents. Activation of macrophages in this way gives positive feedback, releasing more GBS nucleic acids which can then be detected to further activate the macrophage and generate more NO (Deshmukh et al., 2011b).

The β-h/c has immunomodulatory properties, it stimulates the detrimental production of pro-inflammatory cytokine production (IL-1 and IL-6) and can activate macrophages through the nitric oxide pathway (Maisey et al., 2008a), both contributing to sepsis. However, recognition of β-h/c in a dose dependent manner is required for inflammasome formation and subsequent beneficial pro-inflammatory cytokine release providing the host with a protective immune response (Costa et al., 2012).

Toxic shock syndrome has been seen infrequently in conjunction with invasive GBS infection. Toxic shock syndrome is an overproduction of polyclonal T cells in response to inappropriate antigen presentation, leading to massive cytokine release and multisystem disease. Isolates recovered from a patient with toxic shock
syndrome appeared phenotypically hyperhaemolytic, which was attributed to derepression of cylE due to mutations in CovR, a β-h/c repressor (Sendi et al., 2009a).

Together, the recognition of GBS by phagocytic pattern recognition receptors induces a pro-inflammatory response which, when correctly regulated, will aid the host removal of the organism. If incorrectly regulated or in the absence of responding cells, however, such responses can lead to immune dysregulation and sepsis. Thus, the interaction between GBS and macrophages warrants further investigation.

**1.3.4 Phagocytosis and killing of GBS by cells of the innate immune system.**

Phagocytosis is the receptor mediated endocytosis of particles into a new cytoplasmic compartment - the phagosome. This is a feature of professional phagocytic cells namely the neutrophils, dendritic cells and macrophages. The study of the interaction of cells of the innate immune system and bacterial pathogens has mainly focused on macrophages, and macrophages are also the main focus of the work in this thesis. Recognition and phagocytosis of an organism can be due to the direct interaction (‘non-opsonic’) of bacterial substrates with host cell receptors such as scavenger receptors, complement receptor 3 (CR3), lectins for example Dectin-1 and DC SIGN. Phagocytosis can be enhanced by host opsonins, which bind to surface components of the microorganism leading to opsonophagocytosis. Opsonins are primarily antibodies and complement but also other proteins such as surfactant protein A (Gil et al., 2009) or mannan binding lectin. Opsonisation of the organism
targets the bacteria to receptors such as the Fc receptor and complement receptors. Uptake by this method is efficient and generally leads to killing of invading pathogens by the host cell (Plüddemann et al., 2011).

1.3.4.1 GBS resists phagocytosis

GBS has mechanisms to prevent the deposition of opsonin on the surface of the organism. Many of the GBS components that prevent opsonophagocytosis (discussed in more detail below) are required for virulence, supporting the importance of opsonophagocytosis in allowing the host to raise a protective immune response. Host cells are unable to respond to GBS through signalling via cell surface expressed Toll like receptors, so phagocytosis and subsequent lysosomal processing is an important step in mounting a protective immune response to this organism (Deshmukh et al., 2011b).

Sialylated polysaccharide capsule

Group B Streptococcus possess a polysaccharide capsule, which is considered to be one of the major virulence factors of the organism since unencapsulated strains show reduced virulence in animal models (Rubens et al., 1987).

Group B Streptococcus is unusual among bacteria in that all serotypes display a terminal sialic acid (N-acetylneuramininc acid) on the capsular polysaccharide. Streptococcus suis is the only other Gram positive organism where this has been observed (Maisey et al., 2008a, Segura, 2012). Sialic acid is also present on the glycan of vertebrate cells, and GBS is therefore decorated with sugars that mimic the host cell, a form of molecular mimicry that has been suggested to be a major contributor to GBS virulence (Wessels et al., 1989).
The most important role of the capsule is probably in inhibiting opsonophagocytosis by preventing complement factor C3b deposition on the surface of the organism. Preventing activation of C3b has the added effect of reducing the production of the chemokine C5a (Marques et al., 1992, Maisey et al., 2008a). Additionally, and unrelated to phagocytosis, the sialyated residues on the capsule increase the affinity of the organism for factor H, an inhibitor of complement activation, thus preventing direct complement mediated killing (Maruvada et al., 2008). An unencapsulated serotype III GBS mutant was shown to be internalised almost twice as much during initial infection than its corresponding wild type strain, reinforcing the importance of capsule for evading opsonin-dependent uptake. Similarly, a serotype Ia strain with higher capsule content was phagocytosed less well than a serotype III strain (Cornacchione et al., 1998). However, we and others have shown that unencapsulated strains are phagocytosed to the same extent as those with capsule under non-opsonic conditions (Segura et al., 1998). Thus, the capsule may be important in protecting the organism against opsonophagocytic complement killing but does not affect internalisation or intracellular survival in the absence of opsonin.

(Areschoug et al., 2008) have suggested an additional role for the capsule to hide surface components, such as the lipoprotein Blr which can function as a ligand for the nonopsonic macrophage scavenger receptor (SR-A). However, this was drawn from experiments showing reduced uptake of the organism in the absence of both the scavenger receptor from the host cell and the capsule, and it is not clear if in the presence of the scavenger receptor they see increased uptake of the unencapsulated mutant (Areschoug et al., 2008).

**GBS surface components implicated in resisting phagocytosis**
In addition to the sialic acid capsule the organism resists opsonophagocytosis through surface components. The organism can be coated in an adherent fibrin like substance, a breakdown product of host fibrinogen through the action of the CspA protein (Harris et al., 2003). This layer helps resist complement deposition as it resembles host protein.

1.3.4.2 Phagocytosis of GBS in the presence of opsonising serum

Despite the efforts of the capsule, in the presence of opsonising serotype specific antibodies GBS can be phagocytosed and rapidly killed by the phagocytic cells of the innate immune system. Uptake is mediated by Fc receptors FcγRII and III and the engagement of these receptors leads to efficient intracellular killing of the organism (Henneke and Berner, 2006, Valenti-Weigand et al., 1996, Cheng et al., 2001). GBS has been visualised by electron microscopy residing in membrane bound compartments irrespective of uptake being in the presence or absence of opsonic factors (Marodi et al., 2000). More efficient killing of antibody opsonised organisms in phagosomes could be due to the observed increase in lysosomal vesicles (Valenti-Weigand et al., 1996) and a more frequent and intense oxidative burst (Cheng et al., 2001). In the absence of type specific antibodies, but in the presence of other opsonins such as lectins and L-ficolin, the competent receptor CR3 is very important in internalising and killing GBS (Fujieda et al., 2012). In the presence of complement the Fcγ receptor is not required for internalisation and killing (Albanyan and Edwards, 2000) and thus it is thought that opsonophagocytosis through the complement receptor may explain the low incidence of neonatal disease despite the high number
of mothers with insufficient IgG (Henneke et al., 2002, Henneke and Berner, 2006, Fujieda et al., 2012).

1.3.4.3 Opsonin independent phagocytosis of GBS

Opsonin independent phagocytosis is likely to be the major uptake mechanism of GBS due to host and pathogen factors. As previously discussed the organism produces a poor antibody response and levels of antibody are likely to be insufficient for opsonisation in the at-risk groups. Neonates have low levels of complement and, especially in preterm babies, lung surfactant may be low. Opsonin independent uptake of GBS is thought to involve direct recognition of the organism by the integrin, complement receptor 3 (CR3) (Antal et al., 1992), a receptor that shows decreased expression on preterm neonate cells (Remington et al., 2010). Internalisation is independent of TLR activation (Henneke and Berner, 2006) and engagement of the scavenger receptor A (Areschoug et al., 2008). Uptake requires actin (but not microtubules) suggesting it is a phagocytic event (Valenti-Weigand et al., 1996). CR3 is an integrin which in addition to a complement binding domain contains a lectin domain that is thought to interact directly with the GBS capsule (Albanyan and Edwards, 2000). Generally CR3 is thought to require additional signals to trigger phagocytosis such as complement deposition, TNFα, microbial products or fibronectin (Underhill and Ozinsky, 2002). Given that antibody blocking the CR3 receptor only reduced internalisation by 50% (Antal et al., 1992), and in neutrophils lectin-mediated GBS interaction with CR3 is insufficient for internalisation (Albanyan and Edwards, 2000) the mechanism for non opsonised GBS uptake is still unclear.
1.4 WHAT HAPPENS AFTER PHAGOCYTOSIS: LIFE ON THE INSIDE

1.4.1 The intracellular fate of phagocytosed microorganisms.

After phagocytosis the organism is internalised into a membrane bound vacuole, the phagosome. The phagosome undergoes a series of fission and fusion events with the endocytic pathway cumulating in the formation of a mature phagolysosome (see Figure 3 for a schematic of the phagosome maturation pathway). The phagolysosome is a hostile environment for micro-organisms, not only is it nutrient limiting, it is highly acidic and contains reactive oxygen species (ROS), reactive nitrogen species (RNS) and antimicrobial proteins and peptides (Flannagan et al., 2009, Basset et al., 2003, Soldati and Neyrolles, 2012, Haas, 2007, Plüddemann et al., 2011, Kinchen and Ravichandran, 2008).
Figure 4: Phagosome maturation
After phagocytosis the organism is internalised into a membrane bound vacuole, the phagosome (1). Phagosomes mature through fusion with membrane bound endosomes (2) and lysosomes (3) to form the phagolysosome (4). Very quickly (within 15 minutes) after engulfment the environment in the lumen of the phagosome becomes acidified through the fusion of early endosomes (acidified endocytic vesicles) and the recruitment and activity of V-type ATPases. The plasma membrane derived NADPH oxidase gives rise to reactive oxygen species and reactive nitrogen species are generated by inducible nitric oxide synthase (iNOS). The phagosome is a nutrient limiting environment with host transferrin sequestering available iron and the NRAMP (natural resistance-associated macrophage protein) transporter protein pumps divalent cations out of the vacuole. Maturation to the late phagosome is characterised by an increasingly acidic lumenal pH due to the increased recruitment of the vATPase pump, and the acquisition of late endocytic markers such as lyosomal associated membrane protein (LAMP1/2) and cathepsin D. The fusion of the phagosome with lysosomes, organelles containing hydrolytic enzymes, results in the formation of a phagolysosome and the culmination of the maturation process.
1.4.2 GBS can persist within macrophages

(Valenti-Weigand et al., 1996) and (Cornacchione et al., 1998) demonstrated that after infection of J774 macrophage-like cells or thioglycolate-elicited peritoneal murine macrophages, viable intracellular GBS can be recovered for up to eight hours at levels similar to those internalized and at reduced levels for up to 48 hours post infection (Valenti-Weigand et al., 1996, Cornacchione et al., 1998). Internalised organisms can be seen within a membrane-bound vacuole presumed to be a phagosome (Cornacchione et al., 1998, Valenti-Weigand et al., 1996). Survival of GBS in murine cells is higher than that of *Streptococcus suis* despite similarities in the capsule between the organisms (Segura et al., 1998), accordingly the presence of a capsule is not important for intracellular survival (Cornacchione et al., 1998). Importantly, persistence is also observed in primary human monocytes and monocyte derived macrophages (Peotta et al., 2001). Activation of macrophages with recombinant murine IFNγ, GM-CSF and LPS enhanced the ability of cells to remove GBS (Cornacchione et al., 1998, Marodi et al., 2000). In the context of neonatal disease, intracellular survival may be particularly relevant, since cord blood derived monocytes show a reduced ability to clear the organism compared to adult monocytes (Marodi et al., 2000). This is likely due to a combination of low levels of complement factors (Gille et al., 2009) and the insensitivity of cord blood monocytes to activation by IFNγ (Marodi et al., 2000).

Survival inside cells of the innate immune system is relevant in terms of disease pathogenesis, since many factors that are required for intracellular survival are also required for virulence in whole organisms (Poyart et al., 2001b, Maisey et al., 2008b).
The intracellular localisation of GBS in macrophages may protect the organism from neutrophils, which are the most antimicrobial and abundant phagocytic cells in blood. The potential importance of the survival of bacteria in cells of the innate immune system has been demonstrated in the GBS related bacteria *Streptococcus pyogenes*, since persistence of *S. pyogenes* in PMN cells can transfer infection to previously uninfected mice. This suggests that persistence inside cells could be a mechanism for systemic spread in the host (Medina *et al.*, 2003). Many antibiotics show poor eukaryotic cell penetration, hence residing within host cells protects organisms from the action of these antibiotics which may lead to recurrent infections and persistent colonisation (Thwaites and Gant, 2011, Kaplan *et al.*, 2006, Sendi *et al.*, 2008).

### 1.4.3 How does GBS avoid killing by phagocytic cells?

In addition to avoidance of phagocytosis as discussed in the previous section several pathogens can evade the host immune response by being able to survive within phagocytic cells. Organisms can modify the host response by being poorly immunogenic, thus producing a weak phagocyte response, or by fighting back and initiating programmed cell death. Avoidance of phagosomal killing is a commonly employed strategy and can be mediated through a number of mechanisms; arresting phagosome maturation in an early and non-antimicrobial stage, manipulating phagosome maturation to lead the vacuole to fuse with endosomal vesicles that are not highly antimicrobial, escape from the phagosome or resistance to the antimicrobial arsenal of the phagosome. The interaction of GBS with phagocytic cells is known to be weakly activating and the organism can initiate immune cell
death. There is little evidence for manipulation of phagosome maturation or escape from the phagosome but the organism has several factors which will enable it to cope with intra-phagosomal conditions. The following section will review the antimicrobial features of the phagocytic cells and mechanisms GBS may use to avoid killing. These mechanisms are summarised in Figure 5.
Figure 5: GBS factors that aid immune avoidance

GBS has multiple mechanisms which may aid the organism overcome the host immune response. The organism can prevent recognition, uptake and activation of cells and humoral components of the innate immune system. Additionally, the organism encodes gene products which can protect it from the phagosome environment, providing protection against antimicrobial peptides, reactive oxygen species and the limited availability of nutrients.
1.4.3.1 Modification of host response

To survive within phagocytic cells, pathogenic organisms can alter or prevent activation of the cell.

The sialic acid residue present on the capsule of all serotypes of GBS is similar to the sialylated glycans seen on host cells. Sialic acids (Sia) are recognised by Siglec receptors present on leukocytes. Siglec receptors are Sia-recognizing Ig superfamily lectins, thought to have a role in distinguishing self from non self and preventing inappropriate immune cell activation. GBS type III can interact with siglec receptors on neutrophils through the lectin domain and depress the activation of the cell leading to a reduced oxidative burst, NETS and increased bacterial survival (Carlin et al., 2006). GBS has also been shown to interact with Siglecs and depress immune cell function through interaction of the receptor with a surface protein (β-protein, Bac or β C) that is expressed in some strains of GBS (Nordström et al., 2011, Carlin et al., 2009). Additionally the surface expressed β protein, also known as Bac or β C is able to bind the Fc region of IgA. This prevents IgA from binding CD89 and therefore prevents downstream immunostimulatory signalling through this receptor (Pleass et al., 2001, Sendi et al., 2008, Nordström et al., 2011).

Once internalised there is some data to support GBS interacting with protein kinase C (PKC) an important mediator in signalling pathways leading to the activation of immune cells. In cells infected with GBS, PKC cannot be activated by exogenous stimuli (Valenti-Weigand et al., 1996). GBS is also able to interact with the host cell cytoskeleton; cytoskeletal modification can lead to signalling and cytoskeletal
dependant antimicrobial mechanisms. A decrease in intracellular survival of GBS is observed when the ability of the organism to induce calpain dependent cytoskeletal changes is inhibited (Fettucciari et al., 2011).

**GBS does not stimulate host cells to produce a large oxidative burst.**

The method of uptake into phagocytic cells can have effects on the subsequent activation state of the cell and the production of an oxidative burst. In the presence of antibodies and subsequent phagocytosis of GBS through the Fc receptor, levels of ROS induced are higher than those seen in non-opsonising conditions (Cheng et al., 2001, Poyart et al., 2001b) and correspond with improved removal of the organism.

In the absence of antibodies GBS is preferentially phagocytosed by the complement receptor (Antal et al., 1992). Unlike antibody-mediated uptake, it is not thought that phagocytosis through the complement receptor leads to a high level of superoxide production (Underhill and Ozinsky, 2002). Recognition of GBS through unspecified pattern recognition receptors, independent of uptake, is important in the generation of ROS (Henneke et al., 2002). Henneke et al. 2002 observed that deletion of the CR3 receptor did not alter ROS production in response to GBS phagocytosis, despite a decrease in uptake.

**1.4.4 GBS induces apoptosis in macrophages**

A strategy to circumvent the host immune response by Group B *Streptococcus* is to induce apoptosis in macrophages. The role of apoptosis of phagocytic cells in disease progression is unclear; on one hand loss of effector cells may reduce the
host’s ability to clear infection, however it has been suggested that early in sepsis apoptosis of phagocytic cells is advantageous (Gille et al., 2009). Host cells may use apoptosis to remove intracellular organisms and many pathogens have methods to prevent apoptosis in the cells they infect. Apoptosis is programmed cell death, tightly regulated by a signalling cascade of cytosolic cysteine proteases, the caspases or calpains. In contrast to necrosis or pyroptosis, apoptosis does not induce a host pro-inflammatory response. Pathogen induced apoptosis is seen in GBS infected host cells, but to a lesser extent in cord blood monocytes than adult monocytes (Gille et al., 2009).

Initially, GBS induced apoptosis is a β-h/c dependant process which does not require internalisation of the organism (Fettucciari et al., 2000). β-h/c dose dependent membrane permeability leads to a calcium influx and calpain initiated modification of the host cell cytoskeleton. Modification of host cell cytoskeleton can act to signal proapoptotic or survival signals to the cell (Fettucciari et al., 2011). GBS apoptosis is a calpain dependent process, so could be triggered by cytoskeletal rearrangements, or an alternative yet to be described mechanism (Fettucciari et al., 2011).

Post phagocytosis of GBS, apoptosis is also seen to involve the caspase pathway and is β-h/c independent. In murine cells caspase dependent apoptosis is related to increasing levels of Nitric oxide through iNOS activity (Ulett and Adderson, 2005) and can also be induced by GAPDH, a protein which is detected on the surface and in the supernatant of lysed organisms (Oliveira et al., 2012).
1.4.5 Resistance to the antimicrobial conditions within the phagosome

1.4.5.1 Low pH

A drop in bacterial cytoplasmic pH, caused by acidic environmental conditions such as those within the phagosome, can lead to inactivation of acid sensitive enzymes important for metabolism, as well as structural damage to membranes and DNA. Initially phagosomal pH is lowered by the fusion of the phagosome with early endosomal vesicles. The pH of the phagosome is then further lowered through recruitment of the vacuolar type ATPase (vATPase) proton pump and the prevention of leakage of H⁺ ions out of the organelle (Huynh and Grinstein, 2007, Kinchen and Ravichandran, 2008). Low pH is required for membrane fusion events which are integral to phagosome maturation; this is likely to be due to the recruitment of effectors at the cytoplasmic face of the phagosome membrane (Huss and Wieczorek, 2009, Huynh and Grinstein, 2007). The antimicrobial capacity of the phagosome is increased at low pH due to this being an optimal condition for activity of the degradative proteins contained within the phagolysosome.

Group B Streptococcus can produce lactic acid as a by product of carbohydrate fermentation and is therefore expected to have mechanisms to cope with conditions of low pH. Lactic acid bacteria regulate cytoplasmic pH primarily through the activity of a proton pump, pumping H⁺ ions out of the cytoplasm, and additionally the consumption of H⁺ ions by amino acid decarboxylases. Other protective mechanisms employed are general cellular protection and repair systems and changes to the membrane composition. The induction of an inducible acid tolerance response (ATR)
can lead to up regulation of general heat shock proteins, proteins involved in pH homeostasis and repair mechanisms (Cotter and Hill, 2003).

Group B Streptococcus regulates approximately 18% of its genome on adaptation to pH5.5 from pH7.0 (Santi et al., 2009a). Genes that are upregulated include those that would enhance the organism’s ability to cope with low pH including proteins involved in osmotic stress, cell wall and membrane composition and transport proteins (Santi et al., 2009a). The cell wall composition also offers some pH tolerance as strains that are not able to correctly D-alanylate lipoteichoic acid show reduced ability to replicate at low pH (Poyart et al., 2003).

Within the Group B Streptococcus genome there are genes encoding enzymes involved in the arginine deiminase pathway (Glaser et al., 2002), which will lead to an increase in cytoplasmic ammonia and thus neutralise protons (Cotter and Hill, 2003). Interestingly, these genes are up regulated at pH5.5 compared to pH7.0, suggesting that GBS may use this mechanism to regulate pH homeostasis (Santi et al., 2009a).

Some organisms use the low pH of the cytoplasm as an environmental cue to regulate genes which aid intracellular survival (Arpaia et al., 2011), although this response needs to be tightly regulated to prevent inappropriate antigen expression. Accordingly, GBS is seen to down regulate ‘virulence factors’ β-h/c, BibA and C5a peptidase in low pH. However the pilus island-1 and CAMP factor are up regulated in low pH (Santi et al., 2009a). The majority of gene regulation changes induced when GBS is exposed to low pH are mediated by the two component system CovS/CovR also called CsrS/CsrR (throughout this thesis it is referred to as CovS/R with the exception of strains that have been obtained from laboratories that use the
CsrR/S nomenclature) (Santi et al., 2009a, Lamy et al., 2004, Jiang et al., 2005).
The mechanism of activation of the CovS/CovR TCS in GBS has not yet been
described but given that it does mediate transcription changes in response to pH it
will be discussed in this context.

**CovS/CovR (CsrS/CsrR)**

The CovS/CovR (Control of virulence) two component system (TCS) is of particular
interest in the field, since this TCS regulates multiple virulence factors required for
host adaptation and intracellular survival. CovS is a sensor, in GAS known to be
membrane associated with extracellular domains which phosphorylates CovR the
response regulator increasing its DNA binding affinity (Churchward, 2007). CovR
can, however, be alternatively phosphorylated by the activity of a serine threonine
kinase (Stk1) which is also a membrane associated sensor (Rajagopal et al., 2006),
leading to a reduction in DNA binding affinity at certain promoter sequences. CovS/R
in GBS was identified by homology with the CsrR (Capsule synthesis regulator) gene
in Group A Streptococcus (GAS) (Lamy et al., 2004, Jiang et al., 2005, Churchward,
2007). In GAS and also in Streptococcus suis deletions in the TCS lead to increased
intracellular survival and virulence in animal models (Tran-Winkler et al., 2011, Pan et
al., 2009). Indeed in GAS, natural mutations in this TCS have led to the emergence
of a highly successful virulent clone M1TI causing outbreaks in the pacific (Tran-
Winkler et al., 2011, Churchward, 2007). In GBS the importance of this regulator in
virulence is not as clear cut; mutant strains have been shown to display reduced
virulence in mice and rat models (Lamy et al., 2004, Jiang et al., 2005). Conversely,
however, strains deleted in CovR have also shown increased sepsis and blood brain
barrier penetration in animal models, potentially explained by the lack of repression
of β-h/c in these strains (Lembo et al., 2010). As seen in GAS, virulence of mutant strains of GBS is variable depending on the strain background and there is considerable diversity among regulons from different strains (Jiang et al., 2008). Despite the role of this TCS in mediating global gene expression changes in response to change in pH, the exact stimulus for the GBS CovS phosphorylation has not been identified. In GAS, Mg$^{2+}$ and the antimicrobial peptide LL-37 can stimulate the membrane associated CovS probably by inducing general membrane stress (Tran-Winkler et al., 2011, Churchward, 2007). In contrast to GAS, where CovR acts only as a repressor, the CovS/R system in GBS acts both as a repressor and an activator. However in its role of pH adaptation CovR appears to act predominantly as a repressor (Santi et al., 2009a).

GBS strains deleted in CovS/R are hyperpigmented, hyperadherent and unable to grow in nutrient limited media. Accordingly, genes that are down regulated through this system include cylE (encoding the β-h/c and being required for carotenoid pigment synthesis), through direct binding of CovR to regulatory elements, several surface expressed proteins including FsbA and FsbB and many genes required for metabolism and transport. Strains deleted in the CovS/R system show an up regulation of genes associated with stress response, which may be due to regulation of these processes through the TCS but could also be explained by the mutant being generally stressed due to cell wall and metabolic defects. Table 3 details genes that are differentially regulated in ΔcovS/R strains.

In the low pH of the phagosome, data indicate that virulence factors such as the β-h/c, BibA, and C5a peptidase would be down regulated due to CovS mediated CovR gene repression. CovS/R associated changes in metabolism and transport proteins
would also be expected. The pilus protein is up regulated in low pH independently of its observed CovR repression and is therefore likely to be abundant (Jiang et al., 2012).
Table 2: Gene expression changes seen in $\Delta$covS/R ($\Delta$csrS/R) or $\Delta$covR ($\Delta$csrR) strains

<table>
<thead>
<tr>
<th>Modification</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\uparrow\downarrow$</td>
<td>Secretory pathway</td>
<td>(Lamy et al., 2004)</td>
</tr>
<tr>
<td></td>
<td>C5a peptidase</td>
<td>(Santi et al., 2009a)</td>
</tr>
<tr>
<td>$\uparrow\downarrow$</td>
<td>Iron transport</td>
<td>(Jiang et al., 2008)</td>
</tr>
<tr>
<td>$\uparrow\downarrow$</td>
<td>Stress responses</td>
<td>(Jiang et al., 2008)</td>
</tr>
<tr>
<td>$\uparrow\downarrow$</td>
<td>Metabolic pathways*</td>
<td>(Lamy et al., 2004, Santi et al., 2009a)</td>
</tr>
<tr>
<td>$\downarrow$</td>
<td>Trehalose pathway</td>
<td>(Lamy et al., 2004)</td>
</tr>
<tr>
<td>$\downarrow$</td>
<td>$F_{o}F_{1}$ ATPase</td>
<td>(Lamy et al., 2004)</td>
</tr>
<tr>
<td>$\downarrow$</td>
<td>Amino acid synthesis</td>
<td></td>
</tr>
<tr>
<td>$\uparrow$</td>
<td>Glycerol and glycerol phosphate uptake</td>
<td></td>
</tr>
<tr>
<td>$\uparrow\downarrow$</td>
<td>ABC Transporter proteins* Transporters of amino acids, peptides and amines.</td>
<td>(Jiang et al., 2008, Santi et al., 2009a, Lamy et al., 2004)</td>
</tr>
<tr>
<td>$\uparrow$</td>
<td>cy/E, $\beta$-haemolysin/cytolysin *</td>
<td>(Lamy et al., 2004, Jiang et al., 2008, Jiang et al., 2005, Santi et al., 2009a)</td>
</tr>
<tr>
<td>$\uparrow$</td>
<td>Surface proteins with LPXTG motif</td>
<td>(Lamy et al., 2004)</td>
</tr>
<tr>
<td>$\uparrow$</td>
<td>Aminopeptidase</td>
<td></td>
</tr>
<tr>
<td>$\uparrow$</td>
<td>Protease</td>
<td></td>
</tr>
<tr>
<td>$\uparrow$</td>
<td>Endopeptidase</td>
<td></td>
</tr>
<tr>
<td>$\uparrow$</td>
<td>General stress protein in <em>Enterococcus and Lactococcus</em></td>
<td>(Jiang et al., 2008)</td>
</tr>
<tr>
<td>$\uparrow$</td>
<td>Glycine/betaine osmoregulation system</td>
<td>(Jiang et al., 2008)</td>
</tr>
<tr>
<td>$\uparrow$</td>
<td>Immunogenic surface proteins Rib (strain 2603) and Alpha-like protein1 (Strain 515)</td>
<td>(Jiang et al., 2005)</td>
</tr>
<tr>
<td>$\uparrow$</td>
<td>BibA*</td>
<td>(Jiang et al., 2005, Santi et al., 2009a)</td>
</tr>
<tr>
<td>$\uparrow$</td>
<td>Pilus* (but up regulation in low pH, independent of CsrR)</td>
<td>(Jiang et al., 2012, Santi et al., 2009a)</td>
</tr>
<tr>
<td>$\uparrow$</td>
<td>fbsA and fbsB Fibrinogen binding proteins</td>
<td>(Lamy et al., 2004)</td>
</tr>
<tr>
<td>$\uparrow$</td>
<td>C5a peptidase</td>
<td>(Santi et al., 2009a)</td>
</tr>
<tr>
<td>$\downarrow$</td>
<td>Capsule and sialic acid content (moderate, not seen in all strains)</td>
<td>(Lamy et al., 2004, Jiang et al., 2005)</td>
</tr>
<tr>
<td>$\downarrow$</td>
<td>Homologue of alkyl hydroperoxide reductase, Gis24</td>
<td>(Jiang et al., 2008)</td>
</tr>
<tr>
<td>$\downarrow$</td>
<td>CAMP factor *</td>
<td>(Jiang et al., 2005, Santi et al., 2009a)</td>
</tr>
</tbody>
</table>

* indicates that CovS/R is thought to mediate expression of this gene in response to environmental pH (Santi et al., 2009a)
1.4.5.2 Oxidative killing

Micro-organisms can evade the damaging effects of reactive oxygen and nitrogen species by evasion, suppression, enzyme inactivation, scavenging and up regulation of stress response and repair mechanisms. There is evidence to support GBS using all of these mechanisms with the exception of evasion.

Reactive oxygen species

The non-mitochondrial generation of reactive oxygen species upon engulfment of bacteria known as the oxidative burst, involves the membrane bound NADPH oxidase which converts oxygen (O₂) to superoxide (O₂⁻) which in turn is a precursor for hydrogen peroxide (H₂O₂). Hydrogen peroxide is membrane diffusible and within the phagosomal lumen or the bacterial cytoplasm exerts antimicrobial effects in combination with iron or copper ions forming highly damaging superoxide and hydroxyl radicals (OH⁻). Hydroxyl radicals can cause lipid peroxidation, protein oxidation and DNA damage. The subunits of NADPH oxidase are dissociated from each other and inactive in resting cells; components are translocated to the phagosome membrane upon cell activation. Concentrations of hydrogen peroxide in an activated cryptococcal phagosome have been suggested to be as high as 14mM (Brown et al., 2007). However, Slauch 2011. calculated the steady state concentration of hydrogen peroxide to be in the region of 1-4μM based on the rate of superoxide production, the rate of spontaneous dismutation, the diffusion of hydrogen peroxide and the phagosome volume (Slauch, 2011, Diacovich and Gorvel, 2010, Fang, 2004). As previously discussed the oxidative burst of GBS-infected cells
may be suppressed, either through active dampening of the immune response, uptake mechanisms or deficiencies in neonatal phagocytes. Proteomic changes in cells infected with GBS suggest that the organism is actively reducing the amount of NADPH oxidase and iNOS that can be assembled. Such changes are not seen in macrophages infected with heat inactivated GBS (Susta et al., 2010).

Group B *Streptococcus* is able to inactivate reactive oxygen species through the activity of the enzyme superoxide dismutase (SodA). Despite the generally low levels of ROS generated in un-opsonised GBS infected cells (Cheng et al., 2001), SodA has been shown to be important for virulence in mouse models and to aid intracellular survival in murine bone marrow derived macrophages (Poyart et al., 2001b). Bacterial SodA offers resistance to oxidative killing by converting superoxide ions into oxygen and hydrogen peroxide. GBS is a catalase negative organism but can potentially detoxify hydrogen peroxide by the enzymes NADH peroxidase, NADH oxidase, a thiol peroxidase and an alkylhydroperoxide reductase (Glaser et al., 2002) (Jiang et al., 2008). The GBS encoded NADH oxidase *nox2*, is able to generate H$_2$O$_2$ from oxygen, which again is then detoxified by enzymes mentioned above. *In vitro* this is protective against the superoxide generating agent paraquat (Yamamoto et al., 2006). Resistance to ROS through SodA is hypothesised to protect the organism against phagocytic killing in the blood, where the GBS will encounter polymorphonuclear leukocytes (PMNS) which have a higher oxidative burst than tissue macrophages (Poyart et al., 2001b). SodA may also play a role in protecting the organism against endogenous ROS; mutant strains show higher mutation rates when treated with rifampicin (Poyart et al., 2001b).
There are two proteins that have been identified in GBS that have the ability to scavenge oxygen metabolites; glutathione which is reported to be present in high levels within the cell and a carotenoid pigment produced in concert with the $\beta$-heamolysin/cytolysin (Hamilton et al., 2006, Glaser et al., 2002, Liu et al., 2004).

**CylE, $\beta$-h/c and the carotenoid pigment**

As previously discussed, one of the most important known virulence factors in Group B *Streptococcus* is the pore forming $\beta$-haemolysin/cytolysin ($\beta$-h/c). The $\beta$-h/c toxin is expressed by *cylE*, a single open reading frame on the *cyl* operon (Forquin et al., 2007). Along with the 78kD $\beta$-h/c toxin, this open reading frame is associated, through yet to be identified mechanisms, with production of an orange carotenoid pigment (Pritzlaff et al., 2001). Strains deleted in *CylE* are less virulent in animal models and are more susceptible to phagocyte killing than the wild type organism (Liu et al., 2004). The role of *CylE* in intracellular survival and virulence at a high bacterial inoculum is thought to be due to the cytolytic activity of the toxin (Fettucciari et al., 2000, Liu et al., 2004). However, at a low bacterial inoculum cell death is not observed and survival is attributed to the production of the carotenoid pigment. Carotenoid pigments from plants and animals are known to be free radical scavengers and have antioxidant properties. Strains deleted in the *CylE* gene show increased sensitivity to hydrogen peroxide, hypochlorite, superoxide and singlet oxygen and a wildtype phenotype can be recovered by the addition of filtered pigment extract (Liu et al., 2004).

*CylE* is repressed by CovR - when CovR is phosphorylated by CovS or small molecule phosphodonor activated CovR binds directly to the *CylE* promoter.
sequence *CylX* (Jiang *et al.*, 2008). CovR mediated repression of *CylE* can be lifted by the action of a serine threonine kinase (Stk1), which phosphorylates CovR at an alternative residue, preventing promoter binding (Lin *et al.*, 2009). Strains deleted in Stk1 show increased sensitivity to phagocyte killing and oxidative stress hypothesised to be due to an inability to regulate *cylE* expression (Rajagopal, 2009). In addition to *cylE* regulation, the CovS/R TCS may promote resistance to ROS by up-regulating expression of homologs of the *S.pyogenes* alkyl hydroperoxide reductase system, AphC and AphF. The alkyl hydroperoxide reductase system contributes to hydrogen peroxide scavenging and has been linked to virulence in a murine model (Jiang *et al.*, 2008). Thus sensing the intracellular environment and converting this into an appropriate gene expression pattern through TCS such as CovS/R may be crucial for the organism’s ability to deal with ROS.

**Reactive nitrogen species**

Nitric oxide (NO\(^\cdot\)) is generated through the actions of the inducible nitric oxide synthase (iNOS) on the cytosolic side of the phagosome membrane. NO\(^\cdot\) can pass through the membrane and into the phagosome where it can react with oxygen radicals generated by the NADPH oxidase and form damaging reactive nitrogen species (RNS) such as peroxynitrite. Nitric oxide (NO\(^\cdot\)) can reversibly inhibit bacterial DNA replication and prevent respiration, potentially pushing the organism into a non-replicating state. Peroxynitrite (ONOO\(^-\)) is a highly reactive species which can interact with metal centres, lipids, thiols, amino acids and DNA (Fang, 2004). Expression of iNOS is upregulated during GBS infection in mouse cells - it is thought that peak RNS levels are achieved at approximately eight hours post phagocytosis (Slauch, 2011). RNS are thought to play a larger contribution to the antimicrobial function of
macrophages than in neutrophils, and are more important in murine cells in comparison to human cells (Fang, 2004).

As previously discussed, NO also has a role as a signalling molecule in cells infected with GBS in response to the recognition of ssRNA and bacterial DNA. Accumulation of NO can lead to apoptosis and provides a pro-inflammatory signal to the host cell to aid removal of the invading organism. (Ulett and Adderson, 2005) suggest that in vitro and in vivo GBS survival is not affected by nitric oxide (Ulett and Adderson, 2005), whereas Deshmukh et al. 2011 show that iNOS is important for the host’s ability to control infection both at a cellular and whole organism level (Deshmukh et al., 2011b).

In other microorganisms, resistance to RNS generally occurs through detoxification, suppression of iNOS and general stress response and repair. To date, however, no specific gene products have been associated with GBS resistance to RNS.

Thus the role of both ROS and RNS as antimicrobial effectors is controversial and they may be more important in signalling pathways to effect host clearance of invading microorganisms than in directly acting against invading GBS (Fang, 2004).

1.4.5.3 Nutrient limiting conditions

The phagosome is not rich in nutrients (Flannagan et al., 2009) and as previously discussed GBS encodes a large number of small molecule transporters which are required for virulence (Jones et al., 2000). Transporters are one of the classes of genes seen up regulated in low pH (Santi et al., 2009a), thus within the phagosome the organism may adapt and increase its scavenging capacity promoting survival.
Low Iron

Bacteria are estimated to require $10^{-8}$M of iron for metabolic processes, DNA synthesis and defence against ROS. Levels of available ferric iron can be as low as $10^{-18}$M within the host cell, due to the high affinity iron binding proteins transferrin and lactoferrin, both of which are present in the phagosome. Additionally, the membrane protein NRAMP (Natural Resistance-Associated Macrophage Protein) pumps divalent cations including Fe$^{2+}$ out of the acidified phagosome (Soldati and Neyrolles, 2012).

GBS require iron for growth, when iron rich media is depleted using NTA the organism is unable to grow. Growth is restored in a concentration dependant manor by the addition of FeCitrate above 2mM (Clancy et al., 2006). The organism has a surface expressed siderophore-dependent iron transporter FhuD. Siderophores are iron chelating proteins expressed and secreted by organisms to bind Fe$^{3+}$ which can compete with and remove iron from host iron binding proteins. Despite the presence of FhuD, which has demonstrated an ability to bind siderophores, GBS expressed siderophores are yet to be identified (Clancy et al., 2006) therefore the organism may utilise siderophores produced by other organisms.

The exact mechanism by which GBS is able to grow in limited iron is still to be characterised, since strains with FhuD deleted can still grow in low iron conditions and retain adequate levels of cytoplasmic iron. However, genome analysis of GBS reveals two NRAMP transporters which pump manganese or iron into the bacterial cell (Tettelin et al., 2002) as well as homologues of iron transport and uptake
systems from *S. pyogenes* and *S. mutans* (Ahmed *et al.*, 2008), all of which may contribute to iron homeostasis during intracellular persistence.

### 1.4.5.4 Antimicrobial peptides and hydrolases

Direct antimicrobial activity of the phagosome is mediated by the presence of antimicrobial peptides and hydrolytic enzymes. Hydrolytic enzymes include those that will break down carbohydrate, lipids and protein.

Cathepsins are a diverse class of proteases, seen in high abundance in the late phagosome and phagolysosome. At different points during phagosome maturation, different classes of cathepsins are present. The cathelicidins and defensins are preformed cationic antimicrobial peptides (CAMP) that are both present in the macrophage late phagosome and phagolysosome and are secreted by human epithelial cells and cells of the innate immune system (Peschel, 2002, Maisey *et al.*, 2008a) and, at least in the case of cathelicidins, increase in expression following invasion by GBS (Quach *et al.*, 2008).

Cationic antimicrobial peptides are positively charged, which allows them to interact with negatively charged bacterial membranes. Once the peptides have gained accesses to their target they can compromise the integrity of the membrane (Peschel, 2002). There are two ways for a microbe to increase resistance to AMPs and antimicrobial enzymes - firstly, by changing the surface of the bacteria, thus preventing the interaction with the peptide, or by production of proteins that can inactivate or degrade AMPs. In both cases, resistance to AMPs *in vitro* corresponds with increased intracellular survival and whole animal virulence (Peschel, 2002).
The cell wall composition of GBS is crucial to the organism’s ability to prevent damage caused by AMPs. Lipoteichoic acid of GBS has a reduced negative charge due to the incorporation of a basic amino acid through D-ala esterification, by the DltA enzyme. The subsequent reduced negative charge will electrostatically repel AMPs, preventing them from reaching their targets. Consequently, a strain deleted in the \textit{dltA} gene shows increased susceptibility to CAMPs and consistent with this reduced intracellular survival (Poyart \textit{et al.}, 2003).

Resistance to CAMPs is also dependent on correct cell wall biosynthesis, independent of charge. The penicillin binding protein (PBP1a), encoded by the gene \textit{ponA}, offers the organism increased resistance to CAMPs (Hamilton \textit{et al.}, 2006). Genes regulated through the TCS CiaR/H have homology to genes involved in regulation of proteolysis which, in other organisms, are critical for correct cell wall formation. Strains deleted in CiaR show increased sensitivity to CAMPs compared to wild type strains (Quach \textit{et al.}, 2008). Another surface expressed enzyme, phosphoglycerate kinase, plays a role in CAMP resistance as shown by the increased sensitivity of a deletion mutant, although the function of this enzyme outside of the cytoplasm is unknown (Boone and Tyrrell, 2012, Boone \textit{et al.}, 2011).

Thus, correct cell wall stabilisation and restructuring is important for the ability of GBS to display resistance to CAMP’s.

In addition, the PilB subunit of the pilus protein of GBS has been suggested to have a role in sequestering AMPs, therefore preventing them from reaching the membrane. Deletion of the PilB subunit increases the organism’s sensitivity to AMP \textit{in vitro} and corresponds with reduced intracellular survival. When the PilB subunit was
expressed in *L. lactis* it conferred resistance to CAMP mediated killing in this normally sensitive organism (Maisey et al., 2008b). The pilus has been shown to be a multifactorial virulence factor of GBS and, in contrast to work mentioned above showing that the PI-1 plus and PilB are required for intracellular survival and AMP resistance (Maisey et al., 2008a, Jiang et al., 2012), the PI-2a pilus did not play a role in resistance to AMP, nor did a deletion mutant show reduced ability to survive within phagocytic cells (Papasergi et al., 2011). Differing reports on the importance of the pilus protein in virulence between strains may reflect variations in the pilus backbone (Chattopadhyay et al., 2011).
1.5 SUMMARY

Despite the implementation of prophylaxis to prevent early onset neonatal infection GBS still remains the major cause of neonatal bacteremia in the UK.

Pathogenesis of Group B Streptococcus infection is multifactorial and the organism is a successful coloniser of mucous membranes by means of a multitude of cell surface proteins. The organism is then able to invade these membranes through a combination of cell invasion and tissue damage, potentially mediated by the expression of a β-haemolysin-cytolysin. When in the host GBS is able to adapt to the host environment through diverse signalling mechanisms and resist opsonophagocytosis, principally through the action of the polysaccharide capsule. GBS is an opportunistic pathogen, infecting the very young and those with underlying co-morbidities. Such individuals may be immunocompromised with low levels of antibody, opsonin and reduced neutrophil function. Therefore macrophages are important effector cells in the context of this disease. Tissue resident macrophages are the first cells to encounter invading organisms and if they are unable to kill the organism they will produce cytokines to activate other immune effector cells. In neonatal GBS disease this signalling cascade can be potentially fatal, since high levels of cytokine release can lead to devastating sepsis and multisystem organ failure.
Thus, the interaction between macrophages and GBS is an important aspect of the disease which warrants further investigation. There are several explanations for the ability of GBS to survive within macrophages, ranging from stimulating a weak host response to mechanisms promoting survival against oxidative stress and degradative proteins.
1.5 **SCOPE OF THIS THESIS**

Macrophages are important effector cells in the recognition, destruction and pathogenesis of GBS. It has been established that the organism is able to survive within these cells and mechanisms that promote this persistence have been proposed. This work aims to further characterise the resistance of GBS to macrophage killing. Whilst most of the current research interest in GBS is in identifying vaccine candidates, the fundamental basis of this host-pathogen interaction remains un investigated.

As introduced in section 1.4.3 there are various mechanisms by which pathogenic microorganisms are able to survive killing by phagocytic cells. In this study several questions were asked in order to improve the understanding of the mechanism by which GBS is able to persist within macrophages:

- Is the organism able to modify the phagosome maturation pathway?
- What is the contribution of GBS genes previously described to have a role in pathogenesis to the organism's ability to persist within cells?
- Can the organism tolerate the physiological stressors found in the phagosome?
- How does the host response, specifically the generation of reactive oxygen species and the decreasing phagosome pH act to control the growth of intracellular Group B *Streptococcus*?
The first results chapter of this thesis (Chapter Three) focuses on the tools the organism has to be able to resist host cell killing. Using mutants deleted in characterised virulence factors the contribution of each gene product to intracellular survival was evaluated. The second results chapter (Chapter Four) investigates the intracellular environment which GBS will be exposed to when internalised within macrophages, looking at the role of reactive oxygen species, phagosome acidification and maturation of the phagosome. The results of these two chapters should be considered together (Chapter Five) to get an overall picture of the interactions between macrophages and GBS.

To study host-pathogen interactions it is desirable to be able to visualise the organism within the eukaryotic cell, since this will allow the fate of the organism to be monitored and facilitate investigation into the subcellular localisation. Chapter Six therefore describes the evaluation of green fluorescent protein (GFP) expressing constructs for use in streptococci, specifically in relation to levels of fluorescence and fitness cost to the *Streptococcus* in which it is expressed.
CHAPTER 2: MATERIALS AND METHODS
2.1. **BACTERIAL STRAINS AND CULTURE CONDITIONS**

2.1.1 *Streptococcus*

For streptococcal strains and mutants used in this study see Table 3

*Streptococcus agalactiae* strains were grown in Todd Hewitt broth\(^1\) with the addition of 5\% yeast extract (MPbio, UK) subsequently referred to as THY, or on THY agar plates (THY broth with 1.2\% agar). When appropriate spectinomycin was used at 150\(\mu\)g/ml. Bacteria were always grown at 37\(^\circ\)C in air, when grown in broth streptococci were grown in standing cultures with head space. For long term storage strains were frozen at -80\(^\circ\)C in THY with 30\% glycerol. Strains recovered from frozen stocks were replaced every seven days and stored on THY plates at room temperature for this time.

The three strains used for survival, A909, COH1 and NEM316 were chosen as they represent the major serotypes associated with colonisation and disease and have whole genome sequence data available. To measure the contribution of streptococcal gene products, *cylE, cspE, sodA* and *CovS/R* to intracellular survival, deleted strains were compared in viability to their isogenic parent.

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\(^1\) Unless specified all reagents are from Sigma (Poole, UK)
### Table 3: Group B *Streptococcus* strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Serotype</th>
<th>Details</th>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEM316</td>
<td>GBS Wild</td>
<td>III</td>
<td>Isolated from neonatal blood culture (early onset disease).</td>
<td>ATCC 12403, CIP82.45</td>
<td></td>
</tr>
<tr>
<td>A909</td>
<td>GBS Wild</td>
<td>Ia</td>
<td>Mouse-passaged prototype strain 090. Isolated from neonatal blood culture.</td>
<td>M. Anthony, University of Birmingham/Oxford John Radcliffe Hospital</td>
<td>(Martins <em>et al.</em>, 2011)</td>
</tr>
<tr>
<td>COH1</td>
<td>GBS Wild</td>
<td>III (ST-17)</td>
<td>Highly encapsulated, isolated from neonatal blood culture. A member of the clonal complex 17, associated with hypervirulence.</td>
<td>M. Anthony, University of Birmingham/Oxford John Radcliffe Hospital</td>
<td>(Rubens <em>et al.</em>, 1993)</td>
</tr>
<tr>
<td>2603vr</td>
<td>GBS Wild</td>
<td>V</td>
<td>Clinical isolate</td>
<td>M. Anthony, University of Birmingham/Oxford John Radcliffe Hospital</td>
<td>(Tettelin <em>et al.</em>, 2002)</td>
</tr>
</tbody>
</table>

### Group B *Streptococcus* mutant strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Serotype</th>
<th>Details</th>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>COH-13</td>
<td>COH1 ΔcpsE</td>
<td>III</td>
<td>Unencapsulated strain. COH1 with Tn917deltaE insertion in cpsE</td>
<td>C. Rubens, Seattle children’s hospital.</td>
<td>(Rubens <em>et al.</em>, 1993)</td>
</tr>
<tr>
<td>A909 ΔcpsE</td>
<td>A909 ΔcpsE</td>
<td>Ia</td>
<td>Unencapsulated strain. Strain A909 with deletion in cpsE capsule gene.</td>
<td>C. Rubens, Seattle children’s hospital.</td>
<td>Described in (Jones <em>et al.</em>, 2003b)</td>
</tr>
<tr>
<td>NEM2456</td>
<td>NEM316 ΔacyIE</td>
<td>III</td>
<td>Non haemolytic strain, cylE is the structural gene for the haemolysin/cytolysin.</td>
<td>S. Dramsi, Institut Pasteur.</td>
<td>(Forquin <em>et al.</em>, 2007)</td>
</tr>
<tr>
<td>NEM1641</td>
<td>NEM316 ΔsodA</td>
<td>III</td>
<td>SodA deleted, sodA encodes a superoxide dismutase enzyme.</td>
<td>S. Dramsi, Institut Pasteur.</td>
<td>(Poyart <em>et al.</em>, 2001b)</td>
</tr>
<tr>
<td>2603 csrRΔ</td>
<td>2603ΔcsrR</td>
<td>V</td>
<td>2603 with the response regulator of the CsrRS (CovRS) CsrR deleted</td>
<td>M. Wessels, Childrens Hospital, Boston</td>
<td>(Jiang <em>et al.</em>, 2005)</td>
</tr>
<tr>
<td>2603 csrRΔrep</td>
<td>2603 csrRΔ repaired</td>
<td>V</td>
<td>2603 csrRΔ transformed with pJRS233csrRΔ, this plasmid contained a genetically labelled copy of csrR which was inserted into the chromosome of csrRΔ strains.</td>
<td>M. Wessels, Childrens Hospital, Boston</td>
<td>(Jiang <em>et al.</em>, 2005)</td>
</tr>
</tbody>
</table>
2.1.2 *Escherichia coli*

Please see table 4 and 5 for strains used.

*E.coli* was cultured in Luria Broth (LB) broth or LB plates (1.2% agar) Growth was at 37°C in air, if grown in broth the culture was shaken at approximately 200rpm. spectinomycin was used at 100μg/ml. Strains were stored at -80°C in LB broth with the addition of 10% glycerol. DH5α strain was chosen as a control in survival assays as it is not associated with disease.

2.1.3 *Lactococcus*

Please see table 4 for strains used.

*Lactococcus* was grown either on M17 (Oxoid, Basingstoke, UK) plates with the addition of 0.5% glucose (M17G) at 30°C, or at 37°C in standing cultures of M17G broth. Strains were stored at -80°C in M17G broth with the addition of 30% glycerol. Organisms to be used in experiments were recovered from frozen at 30°C on M17G plates, and stored at room temperature for no longer than seven days.

*Lactococcus lactis* sub species *cremoris* was used in intracellular survival assays as a non-pathogenic control. This type strain was chosen as it has been classified as generally recognized as safe (GRAS) by the US Food and Drug Administration and has been used as a control in similar assays (Gentry-Weeks et al., 1999, Maisey et al., 2007). *Lactococcus* are Gram positive, lactic acid bacteria and part of the Streptococcaceae family. The organism is therefore a good representative non-pathogenic relative of *Streptococcus*. The natural source of *cremoris* has not been identified however other lactic acid bacteria can be isolated from green plants and
dairy products. *Lactococcus* is not part of the normal human microbiota but can survive for 3-4 days in the human gastrointestinal tract (Michelsen *et al*., 2010).

### 2.1.4 Sensitivity of GBS strains to physiological stress

Sodium phosphate buffers of specified pH were made by mixing appropriate volumes of 0.2 M NaH$_2$PO$_4$ and 0.2 M Na$_2$HPO$_4$ and the pH adjusted with HCl when necessary.

**Oxidative and nitrosative stress**

To assess the organisms resistance to the conditions of physiological stress that would be experienced in the phagosome, an overnight culture of GBS was washed in PBS then diluted 1/500 into buffer containing hydrogen peroxide or sodium nitrite. This gave an inoculum of GBS of approximately 1x10$^6$ organisms/ml. To investigate nitrosative stress, sodium nitrite (NaNO$_2$) was added at concentrations from 1 to

### Table 4: *E.coli* and *Lactococcus* strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Serotype</th>
<th>Details</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E.coli</em> DH5α</td>
<td>K12 derivative</td>
<td></td>
<td>This is a strain of <em>E.coli</em> commonly used for molecular biology techniques. Chromosomal mutations in DNA repair genes allow for stable acceptance of nucleic acid. It is not associated with pathology.</td>
<td></td>
</tr>
<tr>
<td><em>Lactococcus lactis</em></td>
<td>MG1363, type strain</td>
<td></td>
<td>Plant and dairy bacterium, generally recognized as safe.</td>
<td>H. Jenkinson, University of Bristol</td>
</tr>
</tbody>
</table>

H. Jenkinson, University of Bristol
20mM (Voelz et al., 2010) in 0.1M sodium phosphate buffer at pH4.4. Sensitivity to reactive oxygen intermediates was measured by adding hydrogen peroxide (H$_2$O$_2$) to 0.1M sodium phosphate buffer at pH7.4 and at pH4.4 at concentrations from 0.125 mM to 14 mM (Voelz et al., 2010). In each experiment a buffer only control tube was included.

After one hour incubation at 37°C, cultures were serially diluted tenfold and plated onto THY plates to give an estimated viable count in colony forming units per ml (CFU/ml). A survival ratio was estimated for each condition by dividing the number of viable organism recovered after exposure to each stressor with the number of viable organisms recovered from the control tube with buffer only.

**Low pH**

To test acid tolerance, approximately 1x10$^6$ organisms/ml were added to 0.1 M sodium phosphate buffers of varying pH and incubated at 37°C for one hour. Viable counts were estimated by a serial dilution and plating at the end of this incubation period. To estimate a survival ratio the number of recovered organisms from each pH buffer was divided by the starting inoculum.

### 2.2 J774 MACROPHAGE-LIKE CELL LINE

#### 2.2.1 J774 murine macrophage-like cell line

The J774.16 mouse monocyte derived macrophage-like cell line was originally isolated from a murine reticulum cell sarcoma arising in a female BALB/c mouse (Ralph et al., 1975). A reticulum cell sarcoma is a lymphoma comprising of large
tumour cells resembling histocytes, these are large immobile macrophages found in connective tissues. The cell line is semi adherent and expresses Fc and complement receptors.

### 2.2.2 Culture of J774 cells

Cells were cultured in Dulbecco`s modified Eagle`s medium (DMEM) with the addition of 2mM L-glutamine, 100 units/ml penicillin, 0.1mg/ml streptomycin and 10% Foetal Bovine serum (FBS). Cells were incubated at 37°C in 5% CO₂. For maintenance culture, cells were grown to semi confluence in T75cm² tissue culture flasks with 15mls of media and split approximately every two days by scraping the cells off the plastic and diluting cells to between 1/3 and 1/8 in fresh complete DMEM. Cells were grown for no more than 15 passages and used in experiments between passages 5-15.

### 2.2.3 Storage of J774 cells

For long term storage, J774 cells were frozen in liquid nitrogen as follows. Four T75cm² flasks with confluent growth of J774 macrophages at passage two were harvested and pooled into 10ml of complete DMEM in a centrifuge tube. The cells were pelleted by centrifugation for 10mins at 1000 x g at room temperature. The medium was discarded and the pellet re-suspended in 10ml of filter sterilised freezing medium consisting of 50% FBS, 40% complete DMEM and 10% dimethyl sulfoxide (DMSO). Re-suspended cells were then quickly dispensed into 1ml cryovials and slowly frozen at -80°C. The freezing process is carried out slowly in the presence of

---

2 This media will now be referred to as complete DMEM
DMSO to reduce the formation of ice crystals. After 24 hours cryovials containing cells were transferred to liquid nitrogen.

To recover cells from frozen stocks, cryovials were removed from liquid nitrogen and quickly defrosted at 37°C. DMSO is cytotoxic to defrosted cells so the time that cells are exposed to this solvent must be minimised. The defrosted cell suspension was immediately added to 10ml of complete DMEM and spun at 1000 x g, room temperature for four minutes. The media was discarded and the cells re-suspended in 15ml of complete DMEM and transferred into a T75cm² tissue culture flask.

2.3 ISOLATION OF HUMAN MONOCYTE DERIVED MACROPHAGES (HMDMs) FROM BUFFY COAT

Intracellular survival of GBS was measured in human macrophages, this cell type may represent a more clinical relevant model system than the murine cell line J774.

Monocytes were isolated from buffy coat. Buffy coat is the layer of lymphocytes that can be segregated when whole blood is separated by centrifugation into plasma and red blood cells. To isolate monocytes, buffy coat was separated by density centrifugation using Ficoll-plaque PLUS (Ficoll, GE healthcare, Bucks, UK). Ficoll is a synthetic high molecular weight polymer of sucrose and the cross linker epichlorohydrin which allows for differential migration of cells during centrifugation. Monocytes were selected for by their adherent properties and differentiated into macrophages using human recombinant granulocyte macrophage stimulating factor (GM-CSF, Immunotools, Germany).
25ml of buffy coat was gently transferred into 50ml tubes and diluted in an equal volume of sterile PBS (Oxoid, Basingstoke, UK. Tablets dissolved in dH₂O and sterilised by autoclaving at 121°C for 15 minutes). Approximately 30mls of diluted buffy coat was layered on top of 20ml of Ficoll in a fresh centrifuge tube without mixing. The layered solution was centrifuged for 40 minutes at 2000 x g, room temperature with the brake off. Erythrocytes will aggregate and therefore sink to the bottom of the tube. Granulocytes will remain with Ficoll in the bottom layer. Mononuclear cells and other cells that are slow to sediment such as platelets are found in a layer on top of the Ficoll-Paque media (figure 6)
Figure 6: Isolation of Mononuclear cells by density centrifugation.

Ficoll allows the differential migration of cells. Mononuclear cells are slow to sediment so form a layer on top of the Ficoll media (arrowed).
The mononuclear layer (arrowed on Figure 6) was carefully removed using a Pasteur pipette into a new centrifuge tube and washed in PBS at 680 x g for five minutes, the supernatant was discarded and the pellet re-suspended in the residual liquid. The washing step was repeated until the supernatant appeared clear, this is required to remove platelets from the preparation. The cell pellet was then re-suspended in RPMI media with 2% FBS, counted by haemocytometer and plated into T75 or T25 tissue culture flasks at a cell density of 1-6x10^6/ml. Flasks were incubated at 37°C and 5% CO2 for one hour, this allowed the separation of monocytes by the adherent nature of the cells to plastic. After one hour the flasks were washed twice in warm PBS to remove non adherent cells and the media replaced with RPMI containing 10% FBS, 100 units/ml penicillin, 0.1 mg/ml streptomycin (complete RPMI) and 100 units/ml of recombinant Human Granulocyte Macrophage Colony Stimulating Factor (GM-CSF). GM-CSF is a cytokine that controls the production, differentiation and function of granulocytes and macrophages and will stimulate the differentiation of monocytes into macrophages (hMDMs). After overnight incubation hMDMs were detached from the flasks by incubation on ice, counted and seeded in complete RPMI with additional 100 units/ml of rhGM-CSF at a density of 1-0.5x10^6/well in 24 well plates and 1-2x10^5/well in 96 well plates. After a further 24 hours of incubation the media was changed for complete RPMI (without GM-CSF). The media was changed every three days from this point. hMDMs were used in each assay at day five post isolation. Each buffy coat yielded between 6x10^6 and 12x10^6 hMDMs.

Infection of hMDMs was carried out following the same survival assay protocol as for J774 macrophages using RPMI in the place of DMEM.
2.4 **INTRACELLULAR SURVIVAL ASSAY**

An intracellular survival assay was developed in the J774 macrophage like cell line to measure intracellular survival of range of GBS strains and mutants. The assay was used in conjunction with drug treatments to investigate the role of phagosome acidification and IFN$\gamma$ activation on intracellular survival of the organism (Figure 7). The same infection method was used to look for recruitment of the LAMP protein to streptococcal containing phagosomes and to monitor the level of reactive oxygen species.
Figure 7: Intracellular survival assay.

The survival of GBS inside macrophages was measured by infecting macrophages with bacteria, then lysing cells and counting viable organisms at subsequent time points. The same method was used to investigate the effect of treating macrophages with concanamycin A, iron supplementation and IFNγ,
2.4.1 Preparation of macrophages

J774 macrophages at between 5 and 15 passages were subcultured into a 24 well tissue culture dish at a concentration of 1x10^5 macrophages/well in 1ml of complete DMEM. The dish was incubated at 37°C in 5% CO₂ for approximately 18-24 hours to allow the macrophages to settle, hMDM were plated at 1-0.5x10^6/well as described earlier. The higher inoculum of hMDM is required as these cells, in contrast to the J774 cells, are terminally differentiated and therefore do not undergo cell division. The only change to the infection protocol for hMDM was the use of RPMI in the place of DMEM.

2.4.2 Preparation of bacteria

To prepare bacteria for infection 1ml of overnight broth culture was centrifuged for 5 minutes at 7000 x g, the bacterial cell pellet washed twice in PBS and re-suspended in PBS to an OD₆₀₀ of 1.0. The bacterial suspension was diluted 1/500 into warm serum free DMEM (DMEM containing 2mM of L-glutamine but no FBS or antibiotics). This dilution gave approximately 10x10^5 bacteria/ml which gives a multiplicity of infection (MOI) of 10 GBS to one macrophage (MOI of 10). The bacterial suspension was mixed vigorously by vortex to try to reduce clumping and to break up long chains.

2.4.3 Infection procedure

Before infection macrophages were washed 3 times in warm PBS, 1ml of the bacteria diluted in DMEM as described above was added to each well, dishes were
incubated for 30 minutes at 37°C in 5% CO₂. To end infection, after 30 minutes the medium was removed, macrophages washed three times in PBS and serum free DMEM containing 5μg/ml penicillin and 100μg/ml gentamicin was added. This media remained on the cells for the remainder of the assay. Time point 0 in the assay was taken 30 minutes after the replacement of the media with antibiotics, this ensured that only intracellular bacteria were counted at this time point. Uptake for each strain was estimated as the viable count/well at time point 0.

2.4.4 Measurement of intracellular survival

At time 0, 3, 6, 9, 13 16, 19 and 21 hours post infection the media was removed from the well, the macrophages washed 3 times in PBS then 1ml of 0.02% triton-X added to the well. The macrophages were lysed for 10 minutes at 37°C before being scraped into a 1.5ml micro-centrifuge tube. The macrophage lysate was centrifuged in a micro-centrifuge at 7000 x g for 5 minutes to pellet the bacteria then re-suspended in 100μl of PBS. The lysate was serially diluted 10 fold in PBS and 10μl of each dilution plated out on THY, LB or M17G plates. Bacterial colonies were counted after approximately 18 hours growth and the number of viable bacteria/well estimated.

2.4.5 Analysis

To analyse the number of bacteria surviving over time compared to the initial number of intracellular bacteria the relative colony forming units (rCFU) was calculated.
The assay was repeated at least five times and the data analysed using SPSS 16.0 software. The Mann Whitney U test or Kruskall Wallis test were used with a cut off of \( p<0.05 \) to establish significant differences between strains or conditions. Due to the biological variability of the assay it was decided that the box and whisker plot was the most appropriate way to display the data (see section 2.8 for a description of these graphs).

2.5 THE GROUP B STREPTOCOCCUS-CONTAINING PHAGOSOME

2.5.1 Concanamycin A treatment

To look at the importance of the acidification of the phagosome in control of intracellular streptococci the compound concanamycin A was added to GBS or Lactococcus infected macrophages. Concanamycin A is a macrolide antibiotic structurally related to the bafilomycins. First isolated from Streptomyces species, concanamycin A is biologically active against many fungi but not bacterial species and is an inhibitor of the membrane bound vacuolar type ATPase (vATPase) (Drose and Altendorf, 1997). The compound inhibits the Vo, membrane bound subunit of the complex, this is involved in iron translocation (Huss et al., 2002).

To measure intracellular survival in the absence of phagosome acidification, 0.1\( \mu \text{M} \) of concanamycin A dissolved in DMSO was added to serum free DMEM along with antibiotics after a 30 minute infection period (Figure 7) and remained with the cells for
the duration of the assay. Macrophages were lysed, viable intracellular organisms enumerated and relative CFU calculated as previously described.

2.5.2 Iron Supplementation

One consequence of preventing phagosome acidification is the requirement of low pH for intracellular transport and mobilisation of iron. Within the host cell iron is bound in iron storage proteins such as ferritin and transferrin. Ferric irons are released from storage proteins at the low pH of the phagosome and the ferrous iron is available to the organism. In neutral conditions ferric iron remains protein bound and survival of the intracellular organisms may be compromised (Byrd and Horwitz, 1991). To rule out the effects of iron limitation in conjunction with concanamycin A, GBS infected macrophages were supplemented with ferric nitrilotriacetate (FeNTA) an iron chelator that releases iron independently of pH.

Compounds were made up as previously described (Bates and Wernicke, 1971). Ferric chloride (FeCl) was dissolved in distilled H$_2$O (dH$_2$O) with 2% HCl to a concentration of 6 mg/ml, nitrilotriacetate (NTA) was dissolved in dH$_2$O and the pH adjusted to neutral using NaOH. Both preparations were filter sterilised. To make up ferric nitrilotriacetate (FeNTA) FeCl and NTA were mixed in equal volumes (3 mg/ml) and the pH adjusted to neutral by the addition of 0.9 M NaOH. The volume of NaOH added was recorded to allow for an estimation of the concentration of FeNTA. The solution was then filter sterilised through a 0.22 $\mu$M filter. All solutions were made fresh for each experiment. Iron solutions were then added to GBS infected J774 cells, with or without concanamycin A treatment, at time 0 (T0) at concentrations of
5μM, and 50μM of FeNTA and 50μg/ml of NTA or FeCl and remained in the media for the duration of the experiment.

2.5.3 IFN\(\gamma\) activation of macrophages

Macrophages were plated as described for the survival assay (2.4.1). Before infection with bacteria the cells were washed and the media replaced with serum free media containing 10 U/ml IFN\(\gamma\) (Immunotools). After 30 minutes incubation at 37°C cells were washed and infection and survival measured as described above (from 2.4.3).

2.6 Reactive Oxygen Species

2.6.1 Measurement of hydrogen peroxide production using Amplex® UltraRed

Hydrogen peroxide generated through the activity of the NADPH oxidase complex can pass through cell membranes and therefore can be detected in the culture supernatant. Hydrogen peroxide was measured by the horse radish peroxidase (HRP) catalysed oxidation of the non coloured Amplex® UltraRed reagent (amplex red, Invitrogen, life technologies, Paisley), to a fluorescent derivative resorufin. Using this method, hydrogen peroxide production from cells infected with GBS in 96 well plates was measured using a plate reader to detect fluorescence, therefore allowing a quick and convenient method of screening multiple strains of GBS.

J774 cells and hMDM were settled in a 96 well plate at 0.4x10^5 J447 cells/well and 1-2x10^5 hMDM/well. Organisms were prepared as previously described (2.4.2) and
added to cells at MOI 10 in filter sterilised Krebs-Ringer phosphate solution (145mM NaCl, 5.7mM Sodium phosphate, 4.86mM KCl, 0.54mM CaCl₂, 1.22 mM MgSO₄, 5.5mM glucose, 10mM HEPES, pH adjusted to 7.35) with 50μM of Amplex® UltraRed reagent and 0.1 U/ml of HRP. A well containing cells with no stimulus was included with each run. To one well 150ng/ml of Phorbol-12-myristate 13-acetate (PMA) was added as a positive control. PMA is a stimulator of the protein kinase C (PKC) signalling transduction pathway.

Amplex red was prepared by dissolving one, 1mg vial in 100μl of DMSO to give a stock solution of 10mg/ml, this was frozen at -20°C in single use aliquots. A 10mg/ml stock solution was prepared of HRP in 0.1M phosphate buffer (0.2 M NaH₂PO₄, 0.2 M Na₂HPO₄) this has an activity of 2520U/ml, to make a working stock solution this was diluted 1/1000 in Krebs-Ringer phosphate solution.

Post infection with organisms plates were sealed using a breathe easy membrane (Breathe-Easy™ sealing membrane, Fisher) and immediately placed within the BMG FLUOstar omega plate reader (BMG labtech, Basingstoke, UK). Fluorescence measurements were taken at an excitation of 544nm and an emission of 580nm, immediately post-infection, then every subsequent 15 minutes for eight hours. For the duration of the assay the plate was incubated within the plate reader at 37°C in normal atmospheric conditions.

Results were analysed using the BMG labtech analysis software, Mars. To compare the rate of hydrogen peroxide production in infected cells, the maximum slope was calculated using the kinetic calculation wizard function.
2.6.2 Measurement of ROS using H$_2$DCFDA and flow cytometry

To measure generation of reactive oxygen intermediates in infected and non-infected J774 cells the cell-permanent fluorescent dye 2',7'-dichlorodihydrofluorescein diacetate (H$_2$DCFDA, Invitrogen) was used. H$_2$DCFDA is a chemically reduced form of the fluorescent compound fluorescein. H$_2$DCFDA is non-fluorescent until cleavage by intracellular esterases and oxidation within the cell, making it a specific marker of intracellular ROS.

J774 cells were infected with GBS as previously described for the survival assay (2.4). At individual time points cells were removed from plastic using accutase treatment.

Accutase treatment

Accutase is a mix of proteolytic and collagenolytic enzymes which will break the bonds between the macrophages and ECM components. J774 cells can adhere to plastic, this is mediated through cellular interaction with extracellular matrix (ECM) proteins, present in the culture dish either from FBS or produced by macrophages themselves. Cells in a 24 well plate were washed twice in PBS before being incubated for 10 minutes at 37°C in 200μl of accutase. Cells were removed from the plastic by adding 400μl of PBS and using this to ‘flush’ round the plate, cells were then washed in PBS at 1500 x g for two minutes in a micro centrifuge and re-suspended in 200μl of DMEM containing 5μM H$_2$DCFDA.
CHAPTER 2: Materials and Methods

ROS staining and flow cytometry

H$_2$DCFDA powder was dissolved to 10mM in DMSO and stored at -20°C in 10μl aliquots under nitrogen. Cells were incubated with the dye for 45 minutes at 37°C before immediate analysis. Fluorescence was measured by flow cytometry using a FACSCaliber instrument and analysed with CellQuestPro (BD Biosciences). 10000 events were counted and the percentage of positive cells identified by gate analysis.

2.6.3 Blocking ROS generation through inhibition of the NADPH oxidase

To investigate the role of ROS generated through the NADPH oxidase for the control of intracellular streptococci, the activity of the enzyme was blocked using Apocynin (4-hydroxy-3-methoxyacetophenone). Apocynin is a prodrug which upon activation by H$_2$O$_2$ or myloperoxidase oxidises thiols in the NADPH oxidase complex thus preventing subunit assembly. Apocynin, made fresh for each experiment was dissolved in serum free DMEM overnight at 50°C to make a stock solution of 15mM this was added to cells at the time of infection at a final concentration of 0.5mM (Hu et al., 2011). The survival assay was performed as previously described (2.4). Apocynin remained in the media for the duration of the experiment. In parallel apocynin treated cells were detached, stained for ROS using H$_2$DCFDA, and analysed using flow cytometry as described above (2.6.2).
2.7 MICROSCOPY AND IMMUNOSTAINING

2.7.1 General techniques

Preparation of macrophages for microscopy

Macrophages at the density required for the assay were seeded into 24 well dishes which contained a 13mm prepared glass coverslip in each well. Coverslips were prepared by washing the coverslips for 10 minutes in 1M Nitric acid, followed by repeated washing in large volumes of water and a final wash in 100% ethanol. Coverslips were then left at room temperature to allow residual ethanol to evaporate. Macrophages were allowed to settle onto the coverslip for 18 hours and infected as described previously (2.4).

Paraformaldehyde fixation of cells for microscopy.

Before microscopy, macrophages adhered to glass coverslips were fixed to ensure preservation of the cell structure. Paraformaldehyde (PFA) forms cross links between cellular proteins via basic amino groups. PFA powder was added (in a fume hood) to a concentration of 4% weight to volume in PBS. To completely dissolve the powder the solution was incubated overnight at 37°C. PFA was frozen at -20°C for long term storage and kept at 4°C when in use. To fix cells; after washing three times in PBS, the coverslips were removed, and placed into a well of a 24 well plate containing 0.5ml of 4% PFA. Fixation was carried out for 10 minutes at room temperature. Following this coverslip’s were removed from PFA, washed three times in PBS and stored in PBS at 4°C for a maximum of 24 hours. Fixed cells were either mounted directly and used for microscopy or used for immunostaining.
Antibody labelling

For long term storage, antibodies were diluted in equal volume of 100% glycerol, aliquoted and stored at -80°C. In use aliquots were kept at 4°C. All labelling incubations were carried out at room temperature by placing the coverslip cell side down on a 100µl drop of the reagent on laboratory sealing film.

Immunostaining was carried out as follows: Fixed coverslips were washed three times in PBS and incubated for 10 minutes in 50 mM ammonium chloride. Ammonium chloride inhibits the activity of PFA by quenching free aldehyde groups; this reduces the background fluorescence. Following three washes in PBS Cells were permeabilised for four minutes using a weak detergent such as 0.1% Triton X 100 or 0.1% Saponin, this allows antibodies to access intracellular targets. Coverslips were then immediately transferred to the primary antibody for 20 minutes (for specific antibody concentrations and buffers see later sections), washed three times and then incubated with secondary antibody for 20 minutes. Human IgG at 1mg/ml was added to both primary and secondary antibody reactions at five times the volume of the staining antibody. The presence of human IgG will block Fc receptors on the macrophages thus preventing non specific binding of the secondary antibody to these receptors which would lead to background fluorescence. Following incubation with the secondary antibody coverslips were washed three times in PBS, three times in water and mounted in 6µl of mowiol mounting media on a glass slide as described below.
CHAPTER 2: Materials and Methods

Mounting of coverslips

Mowiol mounting media (mowiol) contains 9% Mowiol (Calbiochem), 25% glycerol and an antifade reagent p-phenylene diamine (Sigma) in 100mM TRIS-HCl pH8.5. Mowiol is a polyvinyl alcohol solution with hardening properties, it is non absorbent and non light scattering. To prepare mowiol media, Mowiol powder is initially mixed by rotation with glycerol and water for two hours, after which time Tris-Cl is added and the mixture is left to dissolve overnight at 50°C. Once dissolved the solution is filtered through a 0.45μm filter, anti fade is added and the mowiol is aliquoted for storage at -80°C. Once defrosted mowiol can not be stored.

Mounted coverslips were left either at 37°C for one hour or at room temperature overnight to allow the mowiol to harden.

2.7.2 Immunostaining and scoring for LAMP acquisition

Synchronised infection

J774 cells were seeded overnight on 12mm coverslips then infected with GBS, heat killed GBS or latex beads. Infection was synchronised by incubating GBS or beads with J774 cells on ice for 30 minutes. During this time, under these conditions adherence will take place but internalisation will be inhibited. The assay plates were moved to 37°C to stimulate uptake and the experiment was carried out as described above for the survival assay (2.4.3) except for the procedure at each time point.

LAMP-1 antibody labelling

At each time point coverslips were removed, fixed and immunostained as described previously (2.7.1). For optimal LAMP staining, cells were permeabilised with 0.1%
Saponin, this and all antibody dilutions were made up in blocking buffer, comprising of PBS with the addition of 3% normal goat serum (Invitrogen). To stain for lysosomal associated membrane protein-1 (LAMP-1) the supernatant of the rat monoclonal antibody ID4B was used at a 1/25 dilution (Developed by J. Thomas August, ID4B was obtained from the Developmental Studies Hybridoma Bank and developed under the auspices of the NICHD and maintained by the University of Iowa.). To stain for GBS, the mouse anti-*Streptococcus agalactiae* monoclonal antibody 1.B.501 was used diluted 1/50 (Santa Cruz Biotechnology). The anti-GBS antibody was raised in mice against UV irradiated non-typable bovine isolate, ATCC27956. The target of this antibody is not identified, but when visualised by microscopy it localises round the edge of the organism so is presumed to be capsule or cell wall. Secondary antibodies used were anti-rat TRITC conjugate for the detection of LAMP-1 and anti-mouse FITC conjugate for the detection of GBS, both at a concentration of 1/100 (Invitrogen).

### 2.7.3 Microscopy

**Fluorescent microscopy; LAMP-1 scoring**

For visualisation of LAMP-1 and streptococcal staining cells were viewed using the 100x differential interference contrast (DIC) oil immersion objective on a Nikon Eclipse Ti microscope with TRITC and FITC filter set. Images were captured using an ORCA-R² camera (Hamamatsu) and NIS elements software (Nikon). Images were processed in Adobe Photoshop (Adobe) and each streptococcal containing phagosome was scored by eye for LAMP acquisition. At least 50 streptococcal containing phagosomes were scored for each time point in three independent
repeats. Acquisition is presented as a percentage of GBS containing phagosomes that could be seen having a ring of LAMP surrounding the contents. Statistical analysis was carried out using the raw data by $\chi^2$ test (section 2.9).

**Fluorescent microscopy: GFP expression**

To visualise intracellular GFP expressing GBS, macrophages infected with GFP expressing organisms were fixed and visualised using phase contrast and a GFP filter set under 63x oil immersion objective on a Zeiss Axiovert 135TV. Images were captured using a Qimaging 12-bit QICAM with a 0.63x camera lens and Qimage pro software (Qimaging).

**Stereo microscopy**

GFP expressing colonies were visualised for fluorescence using a 1x objective and zoom (range 0.75 to 7.5) on a stereo microscope (Nikon, SMZ-U). Colonies were viewed using brightfield and then for GFP expression using a GFP filter set. Images were captured using a Qimaging 12-bit QICAM with a 0.63x camera lens and Qimage pro software (Qimaging).

**Live cell imaging**

To visualise the intracellular location of GBS and to follow the course of infection in real time J774 macrophages were infected with GFP expressing streptococci and imaged.

J774 macrophages were infected with the GFP expressing plasmid NEM:pMA2 (for plasmid details please see Table 5) as previously described (2.3) but with the addition of spectinomycin 150μg/ml in the media during infection for plasmid
maintenance. Following infection the medium was replaced with DMEM without phenol red, containing spectinomycin 150\(\mu\)g/ml, penicillin 5\(\mu\)g/ml and gentamycin 100\(\mu\)g/m. Cells were cultured in a controlled chamber maintaining the normal culture conditions of 37\(^\circ\)C and 5% CO\(_2\) and visualised on a Nikon Eclipse TE2000-U microscope using a 20x objective with phase contrast and a GFP filter set. Images were captured every two minutes for 16 hours with a Nikon Digital Sight DS-QiLMC camera and compiled into time lapse movies using the software NIS-Elements AR3.0 (Nikon).

For presentation images were processed in Image J (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2012) and Adobe Photoshop.

### 2.8 Development and Evaluation of Strains Expressing Green Fluorescent Protein (GFP)

#### 2.8.1 Transformation of *E. coli*

To increase plasmid yield GBS plasmids pBSU98, 100 and 101 (Table 5) were shuttled through *E. coli* and plasmid prepped. Transformation was carried out in accordance with the protocol supplied with the competent cells. XL-blue *E. coli* cells treated with \(\beta\)-mercaptoethanol were incubated on ice with 0.1-50ng of plasmid DNA then heat-shocked at 42\(^\circ\)C for 45-50 seconds. Cells were returned briefly to ice before an hour incubation, shaking at 37\(^\circ\)C in SOC medium (2% Tryptone, 0.5% Yeast Extract, 10mM NaCl, 2.5M KCL, 10mM MgCl\(_2\).6H\(_2\)O, ...
10mM MgSO4.7H2O, 20mM Glucose [anhydrous]). Transformed cells were plated onto LB agar with appropriate antibiotics, cultured overnight and antibiotic resistant colonies isolated. Confirmation of transformation was by PCR.
<table>
<thead>
<tr>
<th>plasmid</th>
<th>Backbone</th>
<th>Antibiotic resistance</th>
<th>Promoter</th>
<th>Properties</th>
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<th>Reference</th>
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<td>pMA02</td>
<td>pDL277</td>
<td>Spectinomycin</td>
<td>PhppA (Streptococcus gordonii)</td>
<td>GFP cassette from pBJ169</td>
<td>A. Nobbs, University of Bristol, UK</td>
<td>(Aspiras, Kazmerzak et al. 2000)</td>
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<tr>
<td>pBSU98</td>
<td>pAT28 (Trieu-Cuot et al., 1990)</td>
<td>Spectinomycin</td>
<td>Cfb (Streptococcus agalactiae CAMP factor)</td>
<td>Plasmid from strain BSU98</td>
<td>C. Gille, University Hospital Tübingen, Germany</td>
<td>(Gille et al., 2009)</td>
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<tr>
<td>pBSU101</td>
<td>pAT28</td>
<td>Spectinomycin</td>
<td>Cfb</td>
<td>GFP from clonetech EGFP</td>
<td>B. Spellerberg, University of Ulm, Germany</td>
<td>(Aymanns et al., 2011)</td>
</tr>
<tr>
<td>pBSU100</td>
<td>pAT28</td>
<td>Spectinomycin</td>
<td>None</td>
<td>GFP from clonetech EGFP</td>
<td>B. Spellerberg, University of Ulm, Germany</td>
<td>(Aymanns et al., 2011)</td>
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### Table 6: *E.coli* strains used for molecular biology

<table>
<thead>
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<th>Strain</th>
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<td>HB101:pMA2</td>
<td>HB101</td>
<td><em>E.coli</em> strains with pMA2 <em>E.coli</em>-streptococcal shuttle vector</td>
<td>A. Nobbs, University of Bristol, UK</td>
<td>(Aspiras et al., 2000)</td>
</tr>
</tbody>
</table>
| XL-blue cells | recA1 recombination deficient  
endA1 Endonuclease deficient  
hsdR17 Prevents cleavage by EcoK endonuclease system | XL-blue chemically competent cells. Used for subcloning Streptococcal plasmids | Agilent technologies          |                                |

#### 2.8.2 Plasmid preparation from *E.coli*

Plasmid pMA2 was received in *E. coli* strain HB101, plasmids PBSU98, 100 and 101 were transformed into *E. coli* for storage and amplification purposes. To obtain a sufficient level of high quality plasmid DNA for streptococcal transformation plasmid DNA was extracted using a Qiagen midi prep kit (Qiagen, Crawley, UK).

A single colony of HB101 or XL-Blue was inoculated into 5ml of LB broth with 150μg/ml spectinomycin and incubated shaking for six hours. This culture was then diluted 1/500 into a 500ml flask containing 100ml of LB broth with 150μg/ml spectinomycin and incubated overnight. The nucleic acid extraction for plasmid DNA was carried out according to manufacture’s instructions. Bacterial cells were pelleted and re-suspended in a Tris-EDTA buffer containing RNaseA, cells were then lysed by sodium dodecyl sulfate (SDS) with the addition of sodium hydroxide (NaOH) which
denatures genomic DNA, plasmid DNA and proteins. The solution was neutralised and protein and genomic DNA precipitated by the addition of acidic potassium acetate; plasmid DNA remained in solution. The lysate was cleared by centrifugation and the supernatant containing plasmid DNA added to a QIAGEN-tip. The QIAGEN Anion-Exchange resin can bind DNA under these salt and pH conditions, but RNA and protein are not able to bind. The QIAGEN-tip was washed using a medium salt buffer to remove any remaining RNA and proteins before elution of DNA with a high salt buffer. Plasmid DNA was precipitated from the eluate using an isopropanol and ethanol precipitation protocol. DNA was dried and re-suspended in Tris-EDTA (TE, 1mM EDTA, 10mM Tris). The concentration of plasmid DNA was measured using a spectrophotometer at 260nm, purity was checked using the OD_{280}/OD_{260} ratio.

2.8.3 Plasmid preparation from GBS

Plasmid pBSU98 was removed from strain BSU98 to allow transformation into NEM316 (see table 5 for plasmid details and table 7 for strain details). Plasmid DNA was purified using the QIAprep spin mini prep kit (Qiagen) with an initial enzymatic lysis step required to break down the gram positive cell wall.

A single colony of BSU98 was inoculated into 5ml of THY broth containing 150μg/ml spectinomycin and cultured overnight at 37°C. Cells were pelleted by centrifugation at 3000 x g for 10mins and re-suspended in 100μl of TG buffer (50mM Tris-HCL pH8 with the addition of 25% glucose). To lyse the streptococcal cells, 100μl of TES buffer (50mM Tris, 5mM EDTA, 0.15M NaCl) was added containing 0.42mg/ml of RNase A, 8.3mg/ml lysozyme and 1KU/ml mutanolysin. Lysosome and mutanolysin
are muramidase enzymes which break down the peptidoglycan cell wall structure. Following lysis at 37°C for one hour 250μl of buffer P2 from the QIAprep spin mini prep kit was added, this alkaline buffer will denature proteins and genomic DNA and allow the subsequent purification of plasmid DNA. The manufacture’s protocol was then followed. For a more detailed explanation of the process of plasmid isolation please see section 2.6.2. Plasmids were eluted in 50μl of buffer EB (10mM Tris-Cl, pH8.5) and stored at -20°C. The concentration of plasmid DNA was measured using a spectrophotometer at OD\textsubscript{260}, purity was checked using the OD\textsubscript{280}/OD\textsubscript{260} ratio.

2.8.4 Transformation of GBS

GBS strain NEM316 was transformed with plasmids pMA2, pBSU98, pBSU100 and pBSU101 by electroporation following a modified method from Framson \textit{et al} (Framson \textit{et al.}, 1997). For details of streptococcal strains used please see table 7.

Preparation of electrocompetent GBS

GBS is made electrocompetent by culturing the organism in minimal medium with glycine, which will modify the cell wall. When grown in these conditions the organism is sensitive to lysis by detergent, therefore single use plastic ware was used to avoid this occurring

One colony of streptococcus was inoculated into 10ml of M9/YE (1% M9 salts, 0.2% glucose, 0.3% Yeast extract, 1% casaminoacids, 2 mM MgSO\textsubscript{4}, 1mM CaCl\textsubscript{2}). After overnight culture 500μl was transferred to 50ml of M9/YE plus 0.6% glycine and grown overnight. The following morning, 10ml of this culture was added to 500ml
M9/YE+ 0.6% glycine and incubated until the culture reached early exponential phase OD<sub>600</sub> 0.13-0.2 (transformation efficiency is increased at this growth phase). The culture was split into two sterile, detergent free centrifuge tubes, and rapidly cooled on ice for 15 minutes. Cells were kept on ice throughout the procedure. Bacterial cells were centrifuged at 18000 x g for 10 minutes at 4°C, the supernatant was immediately discarded and two pellets pooled and resuspended in 150ml of 0.625M sucrose pH4. The cell suspension was centrifuged again at 18000 x g for 10 minutes, the supernatant removed and the pellet resuspended in 1ml of 0.625M sucrose pH4 in a 1.5ml centrifuge tube. The thick cell suspension was centrifuged at 16000 x g for 1 minute and cells were removed to a fresh 1.5ml centrifuge tube with 1ml of 0.625M sucrose. Cells were snap frozen, using liquid nitrogen then stored at -80°C.

**Electroporation of competent GBS**

Up to 5μg of plasmid DNA was added to 75μl of competent GBS on ice then electroporated in a 1mm cuvette (gene flow) at 1.25kV, 600 Ω and 25μF in a Bio-Rad Gene pulser electroporator. Immediately 2ml of THY with 0.5M sucrose, pH7, was added to the cuvette and the bacteria removed. The bacteria were incubated in this medium for two hours at 37°C before pelleting by centrifugation and plating out on THY agar plates containing 150μg/ml spectinomycin. Plates were incubated for 24-48 hours; resulting colonies were purified and presence of the plasmid confirmed using molecular techniques as described below.
### Table 7: Group B *Streptococcus* strains containing GFP expressing plasmids

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Details</th>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEM316:pMA2</td>
<td>NEM316</td>
<td>NEM316 carrying plasmid pMA2</td>
<td>This work</td>
<td>(Aspiras et al., 2000)</td>
</tr>
<tr>
<td>BSU98</td>
<td>090R</td>
<td>Serotype 1a strain carrying plasmid pBSU98</td>
<td>C. Gille, University Hospital Tübingen, Germany</td>
<td>(Gille et al., 2009)</td>
</tr>
<tr>
<td>BSU99</td>
<td>090R</td>
<td>Serotype 1a strain carrying control plasmid</td>
<td>C. Gille, University Hospital Tübingen, Germany</td>
<td>(Gille et al., 2009)</td>
</tr>
<tr>
<td>NEM316:pBSU100</td>
<td>NEM316</td>
<td>NEM316 carrying plasmid pBSU100</td>
<td>This work</td>
<td>(Aymanns et al., 2011)</td>
</tr>
<tr>
<td>NEM316:pBSU101</td>
<td>NEM316</td>
<td>NEM316 carrying plasmid pBSU101</td>
<td>This work</td>
<td>(Aymanns et al., 2011)</td>
</tr>
</tbody>
</table>

### 2.8.5 Molecular methods used to confirm GFP expressing strains.

To confirm positive transformants of GBS with GFP plasmid a crude DNA isolation method was used followed by PCR.

**Streptococcal DNA isolation**

DNA was extracted by mechanical cell lysis. 1ml of TE (10mM Tris-HCL, 1mMEDTA.Na₂) was added to a lysing matrix B tube (MPbio). Lysing matrix B consists of 0.1mm Silica spheres in a 2ml screw cap tube. A suspension of GBS was made in the TE buffer in the tube. Tubes were agitated for two cycles of 23 seconds at 6500rpm using a Precellys 24 lysis and homogeniser (Precellys). The cell lysate was centrifuged at 16000 x g for 3 minutes and the supernatant which contains nucleic acids removed to a fresh tube. This crude lysate was used immediately for PCR and not stored.
### Table 8: Primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'-3')</th>
<th>Details</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>16sfwb</td>
<td>GCTCAGGAYGAACGCTGG</td>
<td>Used to sequence for strain confirmation</td>
<td>(Harris and Hartley, 2003)</td>
</tr>
<tr>
<td>16srv</td>
<td>TACTGCTGCTCCCGTA</td>
<td>Used to sequence for strain confirmation</td>
<td>(Harris and Hartley, 2003)</td>
</tr>
<tr>
<td>RpoD5</td>
<td>GACTGATGAAGAGCTCATAGG</td>
<td>Positive control for GBS PCR. Major sigma factor of GBS</td>
<td>R.Whitehead, University of Birmingham (un published)</td>
</tr>
<tr>
<td>RpoD6</td>
<td>CTCAGGAGTTGGATCTTGACC</td>
<td>Positive control for GBS PCR. Major sigma factor of GBS</td>
<td>R.Whitehead, University of Birmingham (un published)</td>
</tr>
<tr>
<td>Pr6F</td>
<td>CGCGGATCCTAACCAGTAACGAAGAGATATCA</td>
<td>Used to sequence PhppA from plasmid pMA2</td>
<td>(Aspiras et al., 2000)</td>
</tr>
<tr>
<td>pAT28-3</td>
<td>GTTGTGTGGAATTGAGCGG</td>
<td>Primers for checking pAT28-GFP construct, plasmids pBSU98,100 and 101</td>
<td>(Gleich-Theurer et al., 2009)</td>
</tr>
<tr>
<td>pAT28-EGFP4</td>
<td>CCTTGAAGAAGATGGTGCGGC</td>
<td>Primers for checking pAT28-GFP construct plasmids, pBSU98,100 and 101</td>
<td>(Gleich-Theurer et al., 2009)</td>
</tr>
<tr>
<td>PBSU100-for1</td>
<td>GTTGTGTGGAATTGAGCGG</td>
<td>MCS pBSU100 and pBSU101</td>
<td>(Aymanns et al., 2011)</td>
</tr>
<tr>
<td>PBSU100-rev1</td>
<td>CCTTGAAGAAGATGGTGCGGC</td>
<td>MCS pBSU100 and pBSU101</td>
<td>(Aymanns et al., 2011)</td>
</tr>
</tbody>
</table>

---

3 Primers were obtained from Eurofins with the exception of RpoD5 and RpoD6 which were obtained from Altabiosciences.
Polymerase chain reaction (PCR)

PCR was used to confirm the presence of the plasmid in the transformed strains. Please see table 8 for primers used. Primers against RpoD the major sigma factor of GBS and the 16s rRNA gene were used as positive controls for PCR. The PCR reaction mix and cycling conditions were as follows (PCR reagents were obtained from Bioline, London, UK):

<table>
<thead>
<tr>
<th>Table 9: PCR reaction mix</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Stock concentration</strong></td>
</tr>
<tr>
<td>Forward primer</td>
</tr>
<tr>
<td>Reverse primer</td>
</tr>
<tr>
<td>NH₄ Buffer</td>
</tr>
<tr>
<td>MgCl₂</td>
</tr>
<tr>
<td>dNTP mix</td>
</tr>
<tr>
<td>Taq polymerase</td>
</tr>
<tr>
<td>Crude DNA extract</td>
</tr>
<tr>
<td>Water</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 10: PCR cycling conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Step</strong></td>
</tr>
<tr>
<td>Denaturation</td>
</tr>
<tr>
<td>Denaturation</td>
</tr>
<tr>
<td>Annealing</td>
</tr>
<tr>
<td>Extension</td>
</tr>
<tr>
<td>Extension</td>
</tr>
<tr>
<td>Hold</td>
</tr>
</tbody>
</table>

Agerose gel electrophoresis

Size of PCR products was confirmed on 1% agarose gel.
2.8.6 Sequencing the promoter region of pMA2

Sequencing was carried out by the Functional Genomics Proteomics and Metabolomics facility within the School of Biosciences at the University of Birmingham.

The sequencing reaction mix was prepared as recommended by the facility:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Volume/10μl reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer Pr6fw</td>
<td>(1pmol/μl)</td>
<td>3μl</td>
</tr>
<tr>
<td>Plasmid pMA2</td>
<td>(352ng/μl)</td>
<td>1.5μl</td>
</tr>
<tr>
<td>Water</td>
<td></td>
<td>15.5μl</td>
</tr>
</tbody>
</table>

The reaction was made up to 20μl by the addition of big dye terminator and labelled DNA run on an ABI3730 capillary sequencer.

Sequence analysis

Resulting sequences were downloaded as ABI files and opened using Chromas light software. Chromatograms were checked for base calling and exported as text or FASTA files. The sequence of the promoter region of the hppA gene that had been used to construct plasmid pMA2 (Aspiras et al., 2000) was identified from the genbank sequence L41358 using the primers Pr6F and HJ26. ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) was used to align the sequence obtained from the plasmid mini prep and the published phppA sequence.
2.8.7 Phenotypic analysis of GFP expressing NEM316

**Colony phenotype on solid media**

Organisms carrying the GFP plasmid were plated on THY agar with the addition of 150μg/ml spectinomycin. Colony fluorescence was viewed using a steromicroscope (section 2.7.3)

**Intracellular survival of GFP expressing organisms**

Intracellular survival of GFP expressing NEM316 in J774 macrophages was carried out as previously described in section 2.4. GFP expressing streptococci were allowed to infect macrophages settled on coverslips, these were fixed and viewed using fluorescent microscopy (section 2.7.3) to visualise intracellular organisms.

**Stability of GFP in killed microorganisms**

NEM316:pBSU98 was inactivated through heat, exposure to antibiotics and UV light. Killed organisms were used to infect J774 cells, cells were fixed and intracellular organisms visualised for fluorescence.

A suspension of approximately 1.4x10^6 cfu/ml NEM316:pBSU98 in PBS were heat killed by treatment at 60°C for 30 minutes or UV killed by exposure to UV 254nm at 0.16j/cm² for 10 minutes. Antibiotics were used at 100μg/ml gentamycin and 5μg/ml penicillin, organisms were incubated in PBS containing the antibiotics for 30 minutes at 37°C. Killing was confirmed by plating the PBS suspension onto THY plates and incubating overnight at 37°C.
Fitness of plasmid carrying strains and fluorescence intensity

Growth and fluorescence kinetics of NEM316:pMA2 and NEM316:pBSU98 were measured. Overnight cultures in THY broth were diluted 1/200 in 200μl of imaging RPMI in a 96 well plate. The plate was sealed using a breathable membrane (Breathe-Easy™ sealing membrane, Fisher) and incubated at 37°C in the BMG labtech FLUOstar omega microplate reader. Readings were taken at OD₆₂₀ for absorbance and excitation 485-12nm emission 520nm for green fluorescence. The plate was shaken in a linear direction for 5 seconds at 200 strokes per minute before each reading. Fluorescence and OD end point reads were combined using script mode and taken every 20 minutes for 11 hours to generate a growth curve. Growth curves were viewed and presented using the BMG labtech Mars data analysis software.

2.9 Statistical Methods

All statistical analysis was performed using SPSS software (IBM); graphs were made using this software or Microsoft excel. Further details of the main statistical methods used are below.

The $\chi^2$ test

The $\chi^2$ test is used to investigate if the observed data are different from what would be expected if the characteristics were distributed randomly among the population, taking into account the frequencies of each observation. This gives a probability of the observed value happening by chance in a population with no association.
Two sample T test

A two sample T test was used to calculate the significance of differences between GBS tolerance to phagosomal stress conditions in vitro. The two sample T test, tests the probability that the distribution of the sample means will overlap. Equal variances were not assumed, this was due to the no treatment control group being normalised to one so not having a standard deviation value.

ANOVA (Analysis of variance)

ANOVA calculates the chance of a set of observations being different based on within group and between group variability. To identify if organisms are responding differently to a set of physiological conditions such as low pH ANOVA was performed. ANOVA calculates the variability of the data obtained within each physiological condition, and can compare this with the variability of the data between each physiological condition.

Mann-Whitney U

The Mann-Whitney U test was used to establish if there was any statistical difference between the medium relative colony forming units (rCFU) count measuring intracellular survival in different strains and in different conditions. Each rCFU value obtained from the two sets of data to be compared were ranked together, the sum of the rank scores for each group calculated separately, and the test statistic calculated based on the probability of the two summed ranks being different.
**Kruskal Wallis**

To identify if treatment of infected J774 cells with iron compounds changed intracellular survival, the Kruskal Wallis test was used. The Kruskal Wallis test measures if a set of data has arisen from the same distribution. This test is the non-parametric version of ANOVA, it uses the median values.

**Box plot**

Results for intracellular survival are presented as box and whisker plots, the box represents the upper and lower quartiles with the line in the centre the median value of all repeats, the whiskers are drawn up to the maximum and minimum values excluding outliers (marked with open circles) which are between 1.5 and 3 box lengths away from the ends of the plot and extreme values (marked with asterisk) which are over three box lengths away from the end of the plot. This presentation best represents the variable nature of the assay whilst still showing the overall significance of findings.
CHAPTER 3: INTRACELLULAR SURVIVAL OF

GROUP B STREPTOCOCCUS

Major parts of this chapter have been published (Cumley et al., 2012):

INTRODUCTION

In neonatal GBS disease the cells of the innate immune system provide an important role in host defence. Tissue resident macrophages are likely to be among the first cells to encounter invading GBS. It is known that many pathogenic microorganisms are able to survive the killing mechanisms of macrophages and that this persistence may contribute to disease progression and dissemination (Flannagan et al., 2009, Medina et al., 2003, Thwaites and Gant, 2011). It has been previously published that GBS can survive inside macrophages (Valenti-Weigand et al., 1996, Cornacchione et al., 1998), but the mechanism for the observed persistence requires further characterisation.

GBS has several key gene products previously implicated in full virulence in animal models (Maisey et al., 2008a). In the following work, strains deleted in genes hypothesised to have a role in enabling the organism to persist within the antimicrobial environment of phagocytic cells have been assessed for a role in intracellular survival and tolerance to physiological stresses in vitro.

This chapter describes work carried out using an intracellular survival assay primarily in the murine macrophage- like cell line J774 and also in human monocyte derived macrophages (hMDM’s) to identify features of GBS which enable it to resist host cell killing and cope with physiological stress similar to that experienced within a phagosome.
RESULTS

3.1 Group B Streptococcus shows extended survival in J774 macrophages.

It has been previously reported that group B Streptococcus can survive inside phagocytic cells for longer than non-pathogenic organisms such as E. coli (Cornacchione et al., 1998, Valenti-Weigand et al., 1996).

To allow further investigation of the mechanism behind this phenotype an antibiotic-exclusion and survival assay was developed in the macrophage-like murine cell line J774. J774 cells are considered a useful model for macrophage activity and have previously been used for investigating intracellular survival of many pathogens, including GBS (Ralph and Nakoinz, 1975, Ralph et al., 1975, Liu et al., 2004, Valenti-Weigand et al., 1996). Although Lactococcus lactis, a non-pathogenic species that is closely related to the streptococci, was rapidly killed in J774 cells, GBS showed extended survival for up to 12 hours in macrophages (Figure 8). The survival difference is not attributable to a difference in uptake (Figure 9) and there was no significant difference in intracellular survival between three GBS strains, representing serotype III (NEM316), serotype 1a (A909) and the hypervirulent MLST type 17 clade (COH1).

In conclusion, Group B Streptococcus can persist inside macrophages for prolonged periods.
Figure 8: Intracellular survival of GBS in J774 cells

The intracellular survival of three strains of Group B Streptococcus in J774 macrophages, compared to the non-pathogenic organisms Lactococcus lactis subspecies cremoris (MG1363) and E. coli (dH5α), over a period of 24 hours (a) and (b) at six hours post infection (P.I.). The lysate of infected macrophages was plated out and the number of colony forming units (CFU) counted at each time point. rCFU is calculated as the relative/ratio difference between the initial number of intracellular bacteria (T0) and the number at each time point. Intracellular survival of three different serotypes of GBS is comparable and significantly higher than both control organisms (P<0.05, Mann-Whitney U, n>5 for each).
CHAPTER 3: Intracellular survival of Group B Streptococcus

All strains and mutants of GBS are internalised by J774 cells to the same extent with the exception of the strain deleted in the two component system CovS/CovR (CsrR). Uptake into J774 macrophages was enumerated by lysing infected J774 cells at the start of the intracellular survival assay (time point 0) and counting viable organisms. **a)** Strains derived from GBS NEM316 as the parent strain and *L. lactis*. Strains deleted in the CovS/CovR TCS show increased uptake in J774 macrophages. The intracellular burden of ΔcovS/R can be reduced to the same as wild type by reducing the inoculum 10 fold (low MOI). Strains deleted in the cylE gene and sodA are internalised to the same extent as the parental strain. **b)** Wild type strain GBS 2603 and mutants in CsrR. Strains deleted in CsrR show increased uptake which is reduced back to wild type levels upon repair of the strain. **c)** GBS wild type strain COH1 and COH1-13 a strain with the capsule gene disrupted. Bars represent the average number of colonies recovered at T0 (n=4, error bars represent standard deviation).
3.2 The polysaccharide capsule is not required for intracellular survival

Generally regarded as one of the most important virulence factors of GBS, the polysaccharide capsule prevents complement deposition and inhibits phagocytosis. The intracellular survival of two strains deleted in capsule genes A909ΔcspE (Jones et al., 2003a) and COH-13ΔcspE (Rubens et al., 1993) was measured.

In the absence of opsonin in this assay, the presence of a capsule does not significantly affect uptake (Figure 9). It is noteworthy, however, that while an unencapsulated MLST-17 strain (COH1-13) showed no intracellular survival defect (Figure 10a) the loss of capsule had a larger impact on intracellular survival in a serotype 1a strain (A909) (Figure 10b).

The observed role of the capsule in strain A909 may be due to an apparent increase in survival of the parent strain. However, serotype 1a has been previously shown to have an enlarged capsule, compared to other serotypes and in contrast to our data has been observed by others to be phagocytosed less well (Cornacchione et al., 1998). It should be considered that in highly encapsulated strains such as serotype 1a, which is the serotype of A909, the capsule may play a larger and yet to be identified role than in strains with less capsule.

In line with previous findings (Cornacchione et al., 1998), the presented data suggest that once internalised the capsule does not have a role in aiding the organism to survive within cells.
Figure 10: Intracellular survival of capsule mutants

The polysaccharide capsule does play a major role in intracellular survival of GBS in J774 macrophages. Intracellular survival in J774 cells was measured as described previously. (a) COH1-13, a strain disrupted in the capsule gene cpsE and (b) A909 ΔcspE were compared to the isogenic parent strains COH1 and A909 respectively. In both cases survival at three, six and nine hours post infection was not significantly different between the mutant strain and the wild type parent strain (Mann Whitney U, n>4, P>0.05, outliers marked by •).
3.3 Resistance to oxidative stress does not contribute to intracellular survival.

Host cells can produce highly antimicrobial reactive oxygen species (ROS) and reactive nitrogen species (RNS) in an attempt to kill intracellular organisms. In order to overcome oxidative stress GBS has two well-characterised mechanisms, the presence of a superoxide dismutase enzyme SodA (which can convert superoxide anions to molecular oxygen and hydrogen peroxide) and a carotenoid pigment associated with the cylE gene (which can scavenge free radicals).

When oxidative stress was mimicked in vitro by incubating strains in the presence of hydrogen peroxide, strains deleted in cylE and sodA showed, as expected, a slight increase in sensitivity to high concentrations of hydrogen peroxide (Figure 16 aiii and iv, b iii and iv). However, in contrast to previous studies, (Poyart et al., 2001b, Liu et al., 2004) strains lacking cylE and sodA did not show any significant difference in intracellular survival relative to isogenic wildtype strains at three, six and nine hours post infection (Figure11).

Therefore in this assay, mechanisms employed by GBS to cope with oxidative stress are dispensable for intracellular survival.
GBS proteins conferring resistance to oxidative stress are not crucial for prolonged intracellular survival of GBS in J774 macrophages. Intracellular survival in J774 cells was measured as described previously. Strains deficient in the Superoxide dismutase gene, \((sodA)\) and the \((cylE)\) gene, expression of which leads to production of a free radical scavenging carotenoid pigment, were compared to the isogenic parent strain NEM316. Neither mutant strain showed a dramatically reduced survival defect at three, six or nine hours post infection compared to strain NEM316 (Mann Whitney U, \(n>4\), \(P>0.05\), outliers marked by * extreme values **).
3.4 Survival of GBS in phagocytes is dependent on the presence of the CovS/R regulatory system

In order to survive in different host environments, including within cells, GBS must be able to detect and respond to external stimuli. The two component system (TCS) CovS/R is a global regulator, shown to mediate gene changes in response to pH (Santi et al., 2009a). Strains deleted in covS/R have been shown to be both hypervirulent, (Lembo et al., 2010) and also decreased in virulence (Jiang et al., 2005, Jiang et al., 2008, Lamy et al., 2004) in whole organism models.

In marked contrast to the other mutants tested, strain ΔcovS/covR, showed dramatically impaired intracellular survival and was no longer distinguishable from the non-pathogenic control organism at six and nine hours post infection (Figure 12). To confirm the role of the CovS/CovR (alternatively named CsrS/CsrR⁴) TCS in the organism’s ability to resist macrophage killing, the assay was repeated using a strain, csrR Δ, deleted in only the regulator element, in a different strain background 2603v/r. Strain 2603ΔcsrR has been repaired by the chromosomal integration of a genetically labelled copy of csrR to generate strain csrR Δrep the ability of this strain to survive in macrophages was measured (Figure 13a).

ΔcsrR also showed a significant decrease in intracellular survival at six hours PI when compared to the isogenic parent and the repaired strain, confirming the

---

⁴ In GBS this two component system has been annotated as both CovS/CovR (Lamy et al., 2004) and CsrS/CsrR (Jiang et al., 2005), the strain ΔcsrR was constructed in a laboratory which uses the csr nomenclature and for presentation of results using this strain the nomenclature is retained. Throughout the rest of the thesis the TCS is referred to using the CovS/R nomenclature.
importance of the CovS/R regulator across the species. Since the ΔcovS/R strain is phagocytosed more efficiently than wildtype GBS, (Figure 9) one possibility is that the ΔcovS/R survival defect may reflect ‘overloading’ of host macrophages. However, reducing the number of internalised bacteria at time point 0 to the same level as that of the wild type organism did not change the survival kinetics (Figure 13 and 9).

Thus, the survival defect of ΔcovS/R mutant occurs independently of bacterial burden and reflects a genuine defect in intracellular survival, which may explain the importance of this TCS in rodent models of virulence (Jiang et al., 2005, Lamy et al., 2004).
Figure 12: Intracellular survival of ΔcovS/R

The two component system CovS/CovR is required for prolonged intracellular survival in J774 macrophages. Intracellular survival in J774 cells was measured as described previously. A strain NEM2089 deleted in the CovS/CovR TCS shows decreased survival compared to NEM316 the isogenic parent. Viability is reduced in the mutant strain at six hours and nine hours post infection but not at three hours. The same survival pattern is seen for the control organism L. lactis (Mann-Whitney U, N>4, p<0.05).
The survival defect in CovS/R mutant strains is not species specific and cannot be explained by an increased intracellular burden of this strain. The survival defect of ΔcovS/R was confirmed by measuring intracellular survival with a strain deleted in csrR (covR) in a different parental background, 2603v/r (a). 2603v/r csrRΔ shows decreased intracellular survival at 6 hours post infection, the defect can be repaired by expressing the csrR gene in trans (2603 csrRΔ rep) in the mutant strain. Uptake by J774 cells of GBS with ΔcovS/R deleted is approximately 10 fold higher than the wildtype. The survival defect of the ΔcovS/R is not changed when the number of internalised organisms is adjusted to be comparable to the wild type strain (b) (Mann-Whitney U, n>4, p<0.05, outliers are marked by ● extreme values ●).
CHAPTER 3: Intracellular survival of Group B Streptococcus

3.5 Response of group B Streptococcus to physiological stresses

Within the phagosome, organisms encounter reactive oxygen species (ROS), reactive nitrogen species (RNS) and a decreasing pH (Flannagan et al., 2009). It can be hypothesised that increased tolerance to these damaging molecules could explain the intracellular persistence of GBS and perhaps the rapid intracellular killing of L. lactis and the covS/covR deleted strain. In vitro survival assays were undertaken with selected GBS mutants under a range of stressful conditions.

3.5.1 Low pH

To test the organisms ability to survive in low pH organisms were incubated in sodium phosphate buffers of decreasing pH. The viable count of each strain after one hour incubation was compared to the initial inoculum to give a relative survival ratio for each pH.

There was no difference in acid tolerance between wild type GBS, ΔcovS/covR and L. lactis (Figure 14). Viability did not decrease until the environmental pH reached 4.4 (Figure 14).

GBS and L. lactis have the ability to ferment carbohydrates to produce lactic acid and it is therefore not unexpected in agreement with previous published reports (Yang et al., 2012), that they are able to remain viable in acidic conditions. However, ΔcovS/R does not show decreased survival in low pH compared to the parental strain suggesting that the pH mediated response of this TCS is not crucial in the acid stress response of the organism. One caveat is that, under the conditions tested, the ability
of the organism to replicate was not measured, and it could be that, in the absence of CovS/R, GBS is able to survive low pH but not replicate efficiently.

Consequently, the CovS/R two component system does not appear to be critical for the organism’s survival in low pH and may instead utilise the environmental pH change to regulate genes required to tolerate phagosomal conditions.

### 3.5.2 Nitrosative stress

Intra-phagosomal nitrosative stress was mimicked by exposing GBS to increasing concentrations of sodium nitrite (NaNO₂) which, at low pH, can dissociate to yield nitric oxide (NO). It has previously been demonstrated that both in culture medium and within J774 cells, RNS exert limited antimicrobial effects on GBS (Ulett and Adderson, 2005), so could this explain the increased intracellular persistence of GBS relative to *L. lactis*?

Sensitivity to nitrosative stress, measured by survival in sodium nitrite was comparable across wild type GBS, GBS ΔcovS/R and *L. lactis* to NaNO₂ are comparable (Figure 15).

The intra-phagosomal concentration of nitric oxide is predicted to be around 57 μM (Brown *et al.*, 2007), hence this agent may be responsible for limiting intra-phagosomal growth of GBS and *L. lactis* but not for killing the organisms.
Both wild type GBS and a strain deleted in covS/R are able to tolerate acidic conditions. NEM316, NEM2089 (ΔcovS/R) and L. lactis were incubated in sodium phosphate buffer solutions at decreasing pH for one hour before plating to give a viable count. Relative to the input, there is no obvious difference in tolerance between the strains. Bars display the mean relative survival compared to starting CFU, error bars represent the standard deviation of at least four repeats (ANOVA, p>0.05, n≥4).
The sensitivity of GBS strain NEM316, NEM2089 (ΔcovS/R) and *L. lactis* to sodium nitrite (mimicking nitrosative stress) is comparable across all concentrations tested. GBS, GBS ΔcovS/R and *L. lactis* were incubated for one hour in increasing concentrations of sodium nitrite in a pH4.4 buffer solution and relative survival scored. Bars display the mean relative CFU compared to a no treatment control, error bars display one standard deviation. Viability of organisms is reduced with increasing concentrations of NaNO2. No difference in survival was observed between strains at any concentration (ANOVA, p>0.05, n =5).
3.5.3 Oxidative stress

To test the ability of the ΔcovS/R strain to respond to physiological stress, and to try to explain the survival differences seen between wild type GBS and L. lactis. Wild type GBS, ΔcovS/R and L. lactis were exposed to increasing concentrations of hydrogen peroxide, mimicking oxidative stress. Assays were undertaken at two different pH levels, representing the pH drop that occurs in the phagosome.

At pH7.4 (Figure 16a) wild type GBS (Figure 16ai) showed a marked increase in tolerance to hydrogen peroxide when compared to the non-pathogenic organism L. lactis (Figure 16av), which was highly sensitive to the chemical. At pH7.4 ΔcovS/R behaves as wildtype in its ability to handle the presence of hydrogen peroxide (Figure 16aii). However at pH4.4, survival of the ΔcovS/R strain in H2O2 was decreased (Figure 16bii). Increased sensitivity to H2O2 at low pH compared to neutral pH was not seen in the other strains tested. In contrast, L. lactis showed a strikingly improved survival when exposed to H2O2 at pH 4.4 compared to 7.4 and this survival improvement was not seen in any of the other strains tested.

Based on these data, GBS can clearly survive in moderate, but not high, concentrations of hydrogen peroxide. It has been suggested that in an activated phagosome the level of ROS can be equivalent to a hydrogen peroxide concentration of 14mM (Brown et al., 2007), but may be as low as 1-4μM (Fang, 2004). Based on our in vitro data, it is likely that phagocytosed GBS would be killed if hydrogen peroxide concentrations within the phagosome reach 14mM, in contrast to the observed intracellular persistence. This suggests either that the phagosomal concentration of reactive oxygen species is not this high, or that GBS engages
additional survival mechanisms within macrophages that are not triggered by our in vitro assay.

Together, these data indicate that the ΔcovS/R mutant may have an inability to regulate appropriate ROS protective genes in response to low pH, which should be considered as a possible explanation for the poor intracellular survival of this mutant. Similarly, the importance of being able to respond to environmental pH could be an explanation for the observation that *L. lactis* can respond better to hydrogen peroxide at low pH, since *L. lactis* may be able to induce an acid stress response when in contact with weak acid. This response would not only lead to the up regulation of genes involved in pH homeostasis but also genes involved in general cell protection and repair which may aid hydrogen peroxide resistance (Cotter and Hill, 2003). Within the phagosome, *Lactococcus* is quickly killed, potentially by reactive oxygen species generated at the early stages of phagosome maturation at neutral pH. It may therefore never experience the low pH of the mature phagosome and so may never be able to activate a protective response.
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Figure 16: Sensitivity of GBS strains to Hydrogen peroxide

**NEM316 shows increased tolerance to Hydrogen peroxide (H₂O₂) compared to L. lactis.** Strains of GBS were exposed to increasing concentrations of H₂O₂ in pH7.4 buffer (a) or pH4.4 buffer (b). Viable counts were taken after one hour and normalised to the control (buffer alone). Bars represent the mean relative CFU, error bars indicate one standard deviation. Hydrogen peroxide shows a dose dependant killing affect at both pH conditions. Paler bars indicate concentrations where mean rCFU is significantly different from the control. *L. lactis* is very sensitive to H₂O₂ at pH7.4 (16av) with no viable colonies recovered from H₂O₂ concentrations above 0.5mM. NEM316 (16ai) shows increased resistance to H₂O₂ compared to *L. lactis* at concentrations indicated with *. At pH 4.4 *L. lactis* (16bv) shows increased resistance to hydrogen peroxide killing and is no longer different in its sensitivity compared to wild type GBS. H₂O₂ concentrations where a difference in survival is seen between pH7.4 and pH4.4 are indicated with ** (n<6 for pH7.4, n<4 for pH 4.4, t-test, p<0.05).
3.6 The CovS/R two component system is not required for intracellular persistence in human monocyte derived macrophages (hMDMs).

In the murine cell line J774 the CovS/R two component system was identified as being important for intracellular survival. To see if these findings could be extended to human macrophages, GBS was used to infect human primary monocyte derived macrophages (hMDMs). Adherent monocytes isolated from pooled buffy coats were differentiated using Granulocyte macrophage colony stimulating factor (GM-CSF). GBS was incubated with hMDMs and intracellular survival measured using the same method as for J774 cells.

In agreement with the survival phenotype seen in J774 cells, GBS strain NEM316 was able to survive better in hMDM’s than L. lactis at six hours post infection (Figure 17a). Contradictory to data obtained in J774 cells, ΔcovS/R showed a similar or possibly improved intracellular survival capacity to the wild type strain. To confirm that this was not due to increased internalisation of the mutant, infection was carried out using a lower MOI of 1 which lead to a comparable internalisation to the wild type strain (Figure 17b). Survival of ΔcovS/R mutant at a lower MOI was enhanced compared to the wild type at a MOI of 10. Caution must be taken when analysing these data due to the low number of assay repeats especially with L. lactis and low MOI ΔcovS/R (n=1).

In contrast to findings in the J774 murine macrophage like cell line, the two component system CovS/R does not appear to play a role in the organism’s ability to
withstand intracellular killing by human macrophages. Potential reasons for this discrepancy are discussed below and in Chapter Five.
CovS/R is not required for persistence in human monocyte derived macrophages (hMDMs). Strain NEM316, NEM2089 (D covS/R) and L. lactis were used to infect primary monocyte derived macrophages. Figure 17a shows intracellular survival at three, six and nine hours post infection. Six hours post infection the number of viable intracellular Lactococci are greatly decreased (Yellow bars) however 50% of the internalised NEM316 (burgundy bars) and D covS/R (Orange bars) are able to be recovered. To confirm survival independent of intracellular burden a low MOI control was included (Orange square), this shows enhanced intracellular survival (NEM316 and D covS/R n=3, D covS/R low MOI n=1, L. lactis n=2). Figure 17b shows the number of organisms internalized at the start of the assay similar to the internalisation of the mutant in J774 cells, NEM2089 (D covS/R) shows a higher intracellular burden which can be reduced (low MOI).
**DISCUSSION**

The data presented confirms the ability of Group B *Streptococcus* to persist for up to eight hours within the macrophage like cell line J774 independent of serotype, MLST type and clinical presentation (Cumley *et al.*, 2012). The two component system CovS/CovR has been identified as being required for intracellular survival in J774 cells, but not the polysaccharide capsule or factors required for resistance of the organism to oxidative stress. Loss of CovS/CovR abrogates intracellular survival, in line with previous data that has shown this mutant to be altered in virulence in a rodent model (Jiang *et al.*, 2005, Jiang *et al.*, 2008, Lamy *et al.*, 2004, Lembo *et al.*, 2010). The CovS/CovR system is known to be an important transcriptional regulator in pathogenic streptococci, regulating approximately 7% of the GBS genome, particularly during adaptation to host conditions (Lamy *et al.*, 2004, Jiang *et al.*, 2008). Adaptation may be important in responding to the physiological stress of being in a host phagosome, such as induction of resistance to reactive oxygen species, reactive nitrogen species and low pH. *In vitro*, wild type GBS show similar tolerance levels to sodium nitrite (mimicking RNS) and low pH conditions as are seen in GBSΔcovS/R and *L. lactis*, suggesting that RNS and low pH within phagosomes are not sufficient to explain the survival differences between these strains. The CovS/R deletion mutant does, however, show reduced tolerance to hydrogen peroxide at pH4.4, as compared to pH7.4, whilst *L. lactis* shows the converse relationship. The CovS/CovR mutant may have an inability to regulate appropriate ROS protective genes in response to low pH which could be considered as an
explanation for the poor intracellular survival of this mutant. However the in vitro hydrogen peroxide sensitivity data taken together with the predicted hydrogen peroxide levels in an activated phagosome and the lack of a major role for the sodA and cylE gene products suggests that, within the phagosome, RNS and ROS are not playing a major role in controlling intracellular GBS.

Interestingly the CovS/R regulator is not required for survival within human monocyte derived macrophages (hMDMs). This may reflect differences in the composition of the mouse and human phagosome: These include levels of available nutrients in the two cell types, levels of ROS or RNS, acidification, or species-specific antimicrobial peptides and proteases (Schneemann and Schoeden, 2007). There are known differences in the inflammatory activation of mouse and human macrophages in response to bacterial products (Munford, 2010), one of the major differences being the activation of inducible nitric oxide synthase (iNOS) (Fairbairn et al., 2011). GBS strains deficient in aspects of the CovS/R system have been shown to be both hyper-virulent (which is associated with an increase in expression of the β-haemolysin/cytolysin, an inflammatory mediator, (Lembo et al., 2010)) and reduced in virulence (attributed to the mutant’s inability to adapt to and survive in nutrient limiting conditions (Lamy et al., 2004, Jiang et al., 2005, Jiang et al., 2008)). Thus, the role of this TCS is likely to be complicated in the context of intracellular survival and virulence.
Major parts of this chapter have been published (Cumley et al., 2012):

**INTRODUCTION**

In the previous chapter it was demonstrated that GBS can survive within macrophages. These cells of the innate immune system constitute the first line of host defence against invading microorganisms. Persistence within cells may be an important step in disease pathogenesis, facilitating dissemination of the organism and providing protection from other antimicrobial effectors (Thwaites and Gant, 2011). In order for an organism to survive within a macrophage, it must be able to either subvert or resist the antimicrobial effects of the phagosome.

Recognition of an organism by the phagocytic cells of the innate immune system leads to engulfment of the organism through a process called phagocytosis (Underhill and Ozinsky, 2002). The nascent phagosome matures through the sequential fusion of early endosomes, late endosomes and finally lysosomes, to form the phagolysosome. Reactive oxygen species (ROS) are generated through the membrane assembly of subunits of NADPH oxidase and reactive nitrogen species (RNS) can be generated by the inducible nitric oxide synthase (iNOS). This phagosomal compartment is characterised by the acquisition of lumenal proteases and lysosomal associated membrane proteins (LAMPs). Furthermore, this is an extremely hostile environment for the micro-organism, being highly acidic and containing reactive oxygen species (ROS), reactive nitrogen species (RNS) and antimicrobial proteins and peptides (Flannagan *et al.*, 2009, Basset *et al.*, 2003).

This chapter focuses on the GBS-containing phagosome, and asks the following important questions;
CHAPTER 4: The Group B Streptococcus-containing phagosome

- Does the organism modify phagosome maturation?
- What is the role of the generation of reactive oxygen species and phagosome acidification in the host’s attempt to kill the organism?
- Can the antimicrobial activity of the GBS-containing phagosome be enhanced?
RESULTS

4.1 Group B Streptococcus resides in a phagosome that recruits late endosomal markers

A mechanism by which pathogenic micro-organisms can survive phagocytic killing is to alter the maturation of the phagosome to create an environment that is more favourable for growth or survival (Flannagan et al., 2009, Huynh and Grinstein, 2007).

To assess whether GBS use this mechanism to survive within macrophages, the time-dependent acquisition of lysosomal associated membrane protein 1 (LAMP-1) was studied (Figure 18). This glycoprotein associates with lysosomal compartments and is an important marker of acidification (Kinchen and Ravichandran, 2008). The extent of LAMP-1 acquisition by GBS-containing phagosomes did not differ from that of phagosomes containing latex beads or heat killed GBS up to 180 minutes post infection (Figure 19). Peak LAMP acquisition is seen at 90 minutes post infection in live and heat killed streptococci and latex beads (Figure 19).

Although the bulk of GBS appear remain within an intact phagosome for the duration of the experiment, the possibility that GBS visualised in the absence of LAMP may represent bacteria that have escaped from the phagosome cannot be ruled out.

These data indicate that, in the majority of GBS containing phagosomes, the rate of LAMP acquisition is not being modified by an active GBS process.
CHAPTER 4: The Group B Streptococcus-containing phagosome

Figure 18: LAMP-1 recruitment

LAMP-1 is recruited to the Group B Streptococcus-containing phagosome. J774 macrophages grown on coverslips were infected synchronously with live wild-type GBS strain NEM316, or latex beads. Coverslips were removed at time points post infection, fixed and immunostained. Streptococci were visualised using an anti-streptococcal antibody and FITC conjugated secondary antibody (green). LAMP-1 acquisition was detected using an antibody directed against LAMP-1, probed using a TRITC conjugated secondary antibody (red). Coverslips were visualised using a Nikon Eclipse Ti microscope with 100x DIC objective. Representative images are shown from 90 minutes post infection. Panel A shows GBS (green) (i) and (iv) with LAMP, (red) (ii) and (v), acquisition. Merged image shown in (iii) and (vi). Panel B shows a representative image of a latex bead containing phagosome with corresponding DIC, (i) and (iv), LAMP, (iii) and (iv) and merged, (v) and (vi) images.
**Figure 19: Time dependent acquisition of LAMP-1**

**Group B Streptococcus containing phagosomes show no delay in LAMP acquisition.** The graph shows the average percentage of GBS or bead-containing vesicles which co-stained for LAMP. At least 50 phagosomes were scored in each independent repeat. Peak acquisition can be seen at 90 minutes post infection (P.I.) with a slight decrease at 360 minutes P.I. in the phagosomes containing live GBS. There is no difference observed between live organisms (black bars), heat killed organisms (light grey) and 3 μm latex beads (dark grey) ($\chi^2$ on raw data $P > 0.05$) with the exception of the live organisms and latex beads at 360 minutes P.I ($P = 0.004$). Error bars represent standard error of three repeats.
4.2 Infection of hMDM and J774 cells with GBS does not lead to an increase in ROS as measured by hydrogen peroxide production.

Although, as demonstrated in the previous chapter, the viability of GBS is reduced at a concentration of hydrogen peroxide comparable to that within an activated phagosome (Brown et al., 2007), J774 cells are still able to support survival of the organism. In Chapter Three, bacterial survival is also shown to be independent of the superoxide dismutase gene (sodA) and the cylE-encoded carotenoid pigment. Both of these mechanisms have previously been suggested to protect the organism against damaging ROS (Liu et al., 2004, Poyart et al., 2001b). The most likely explanation for this paradox is that GBS fails to induce a large oxidative burst in infected J774 cells.

To test this, the rate of hydrogen peroxide production was measured in GBS and Lactococcus infected hMDM (Figure 20a) and J774 cells (Figure 20b). The rate of hydrogen peroxide production was higher in hMDM (Figure 20a) than in J774 cells (Figure 20b). In support of the hypothesis that GBS fails to induce a large oxidative burst in our assay, neither hMDM or J774 cells produced significantly more hydrogen peroxide than uninfected macrophages when infected with GBS or L. lactis.

These data support previous findings that uptake of GBS through non-opsonic phagocytosis does not lead to a large oxidative burst (Carlin et al., 2009, Cheng et al., 2001)
**CHAPTER 4: The Group B Streptococcus-containing phagosome**

GBS does not stimulate macrophages to produce a large oxidative burst. Hydrogen peroxide production from hMDM (a) and J774 cells (b) was measured using Amplex Ultra Red reagent. Fluorescence was measured every 15 minutes post infection with wild type GBS NEM316, strains with covS/R or cylE deleted and *L. lactis*. Uninfected cells and cells stimulated with PMA were included as controls. Bars represent the average of the maximum rate of hydrogen peroxide production from three repeats. Error bars represent one standard deviation. Both cell types respond positively to PMA, however J774 cells produce a low response (note the Y axis scale difference). hMDM show a much larger rate of hydrogen peroxide production, but neither cell type responds to infection with an increased rate of hydrogen peroxide production.

**Figure 20: Rate of hydrogen peroxide production from infected macrophages**

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<tr>
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<th>Rate of Hydrogen peroxide production (max slope/second)</th>
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<tr>
<td></td>
<td>Control</td>
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<tr>
<td><strong>hMDM</strong></td>
<td></td>
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<td></td>
<td>0</td>
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<td><strong>J774</strong></td>
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**Control**

- Rate of Hydrogen peroxide production from infected macrophages (GBS does not stimulate macrophages to produce a large oxidative burst).

**Rate of Hydrogen peroxide production from uninfected macrophages**

- Both cell types respond positively to PMA, however J774 cells produce a low response (note the Y axis scale difference).

**Rate of Hydrogen peroxide production from infected macrophages**

- hMDM show a much larger rate of hydrogen peroxide production, but neither cell type responds to infection with an increased rate of hydrogen peroxide production.
4.3 The generation of reactive oxygen species through the NADPH oxidase does not contribute to killing of intracellular streptococci

Although weak, the oxidative burst generated through the NADPH oxidase may still contribute to the cellular host response to GBS infection. To further investigate the role of ROS in controlling survival of intracellular organisms, the NADPH oxidase was pharmacologically inhibited using 0.5mM apocynin, a compound that prevents proper formation and thus subsequent activity of the complex (Stefanska and Pawliczak, 2008).

When infected with GBS, the proportion of cells in the population that have measurable ROS is approximately 45%, not significantly different from uninfected cells (Figure 21). Apocynin inhibited the percentage of ROS positive cells by 10%, (Figure 21) but had no effect on the intracellular survival of wild type GBS (Figure 22a) or Lactococcus (Figure 22e). In support of my findings in chapter three (Figure 11), which show that GBS factors thought to play a role in resistance to oxidative stress are dispensable for intracellular survival, blocking the NADPH oxidase does not improve the capacity of ΔsodA or ΔcylE strains to survive (Figures 22c and 22d).

Apocynin requires activation by H₂O₂ or myeloperoxidase in order to give maximal inhibition of the NADPH oxidase (Stefanska and Pawliczak, 2008) and thus the relatively low level of inhibition observed most likely reflects weak induction of the NADPH oxidase by intracellular GBS and therefore low levels of H₂O₂.
In summary, the presented data suggest that GBS is not activating the NADPH oxidase complex and that the resultant low level of ROS does not play a role in the host cell control of the organism. This can explain the lack of requirement in our assay for mechanisms that can protect GBS against oxidative stress, in contrast to previously published work (Liu et al., 2004, Poyart et al., 2001a).
Chapter 4: The Group B Streptococcus-containing phagosome

Figure 21: Apocynin blocks ROS production in GBS infected cells

Apocynin can be used to reduce the oxidative burst stimulated by GBS. Reactive oxygen species (ROS) production in J774 macrophages was measured using the dye H$_2$DCFDA and flow cytometry. GBS infection does not significantly increase the proportion of cells exhibiting ROS production. The percentage of cells with detectable ROS levels can be inhibited by 0.5mM apocynin, a chemical that prevents assembly of the NADPH oxidase. Bars represent percentage of cells gated by flow cytometry as positive for ROS, averaged over three repeats, error bars indicate one standard deviation.
**Figure 22:** Intracellular survival of GBS in apocynin treated cells

Intracellular survival of GBS and *L. lactis* within J774 cells is not affected by blocking the assembly of NADPH oxidase. Intracellular survival was measured in J774 cells in which the action of the NADPH oxidase was blocked using the drug apocynin. In all strains tested, intracellular survival of GBS and *L. lactis* in cells treated at the point of infection with 0.5mM apocynin (dark bars) is comparable with survival in untreated cells (pale bars), at three, six and nine hours post infection (n=4, Mann-Whitney U p>0.05, outliers are marked by o extreme values by †).
4.4 CovS/R and the oxidative burst

In chapter three the strains 2089ΔcovS/R was shown to have a reduced ability to survive in hydrogen peroxide when combined with low pH. Could the increased sensitivity of ΔcovS/R to oxidative killing explain the decreased intracellular survival of the mutant strain in murine cells? Alternatively, it is possible that (as previously described in human brain microvascular endothelial cells), ΔcovS/R may stimulate murine cells to produce a larger, host beneficial pro-inflammatory response (Lembo et al., 2010, Rajagopal et al., 2006) and a correspondingly higher level of ROS. To investigate if infection with the ΔcovS/R strain triggers a higher level of ROS production in murine cells than in J774 cells, the rate of hydrogen peroxide was measured in both cell types. However, as with wild type organisms the rate of hydrogen peroxide production in cells infected with ΔcovS/R was not enhanced above the background rate of production (Figure 20). In addition, blocking the NADPH oxidase using apocynin did not improve intracellular survival of the CovS/R mutant (Figure 22b).

These data all suggest that the reduced intracellular survival of ΔcovS/R strain in murine cells is not due to the mutant inciting an increased oxidative burst or exhibiting increased sensitivity to intracellular ROS.

4.5 Acidification of the phagosome is important for GBS survival

Phagosome maturation is characterised by a decrease in luminal pH. A decrease in phagosomal pH is a required for the fusion events that lead to the development of the
mature phagolysosome and also provides an environment for optimal activity of the hydrolytic enzymes in the phagosome (Flannagan et al., 2009). Acidification of the phagosome can be blocked using concanamycin A, an inhibitor of the vATPase proton pump on the phagosomal membrane, which is largely responsible for acidification of the phagosome (Drose and Altendorf, 1997, Huss and Wieczorek, 2009).

After infecting J774 macrophages with GBS strain NEM316 or the control organism L. lactis, acidification of the macrophage phagosome was inhibited using 0.1μM concanamycin A (a concentration that has no effect on J774 survival or the survival of GBS in Todd Hewitt broth). Concanamycin A treatment significantly reduced the survival of wild type GBS at six hours post infection, but not L. lactis (Figure 23).

This suggests that GBS not only resides in a mature phagosome but also requires the normal phagosome acidification and subsequent maturation to survive.
Figure 23: Intracellular survival of GBS in concanamycin A treated cells

Blocking acidification of the phagosome reduces the intracellular survival of GBS. J774 macrophages infected with NEM316 and *L. lactis* were treated with the vATPase inhibitor concanamycin A (dark bars). Under these conditions, the number of viable intracellular streptococci isolated at 6 hours post infection is significantly reduced (Mann Whitney U, *P*<0.001, *n*>5, outliers are marked by *o*).
4.6 The requirement of phagosome acidification for intracellular survival of GBS is independent of the availability of iron.

The increased pH of the phagosome following concanamycin A treatment will prevent the dissociation of iron from transferrin, which only will occur at a pH below 7. Therefore the possibility that the reduced survival of GBS under these conditions may be an indirect result of iron deprivation was considered.

Addition of FeNTA (a non-physiological iron chelator that releases iron in a pH independent manner over a range of pH 5-8, used in previous similar studies (Levitz et al., 1997, Bates and Wernicke, 1971) did not alter the intracellular survival phenotype of GBS either in the presence (Figure 24b) or absence (Figure 24a) of concanamycin A.

Iron availability therefore appears to have limited impact on GBS survival, which could reflect the relatively short time period during which the organism resides within the host cell.
Reduced survival of GBS in concanamycin A treated cells is independent of iron availability. J774 macrophages infected with strain NEM316 incubated in the presence (panel B) or absence (panel A) of 0.1 μM concanamycin A. The graphs show rCFU at 6 hours post infection (PI) demonstrating the effect of the addition of FeCl, NTA and FeNTA. The non–physiological iron chelator complex FeNTA failed to recover the concanamycin A associated loss of viable intracellular streptococci. There is no significant difference between the untreated control and the infected cells that received iron supplementation in either concanamycin A treated (p=0.572) or untreated (p=0.428) cells (Kruskal Wallis test n=6).
4.7 IFNγ treatment of macrophages does not enhance streptococcal killing.

IFNγ activation of macrophages can increase the rate of phagosome fusion with endosomes and lysosomes, decrease the pH of the phagosome and lead to up-regulation of iNOS, resulting in higher levels of RNS and ROS (Torrado et al., 2010, Gilberthorpe et al., 2007).

To investigate if IFNγ activation of macrophages increased the anti-streptococcal properties of J774 cells, cells were pre-treated with IFNγ before infection with GBS. At six hours post infection IFNγ activated cells did not display any significant difference in their ability to remove GBS or Lactococcus than non-treated controls (Figure 25).

The inability of IFNγ activated macrophages to remove intracellular Streptococci was surprising. The concentration of IFNγ used in this assay, 10U/ml is felt to be a physiologically relevant concentration (Voelz et al., 2009), although other assays looking at the role of IFNγ activation have used higher concentrations for longer incubation periods (Gilberthorpe et al., 2007). Additionally, IFNγ activation is thought to require a second signal, which can be either endogenous TNFα, produced in response to recognition of the pathogen through a pathogen recognition receptor such as toll like receptors (Mosser, 2003), or exogenous TNFα. LPS is also known to be able to augment IFNγ activation, although it is not known if GBS can stimulate
macrophages to produce the necessary signals to achieve full IFNγ mediated phagosome activation.
**Figure 25: IFNγ activation**

IFNγ activation of J774 macrophages does not affect the intracellular survival of NEM316. J774 macrophages were incubated with 10U/ml IFNγ before infection with NEM316 and *L. lactis*. No significant difference is seen in the intracellular survival of organisms in activated (treated, pale bars) or not activated macrophages (dark bars) (Mann Whitney U, N=5, P>0.05).
DISCUSSION

As shown in Chapter Three, Group B *Streptococcus* can survive the killing mechanisms of phagocytic cells for a prolonged period of time. By staining for lysosomal associated membrane protein (LAMP), a marker of late endosomes or phagolysosomes it appears that the organism is not modifying the recruitment of late phagosomal markers. *In vitro*, the organism is sensitive to hydrogen peroxide killing at a physiologically relevant concentration (Brown *et al.*, 2007, Fang, 2004), which is discordant with its ability to survive the phagosome oxidative burst. It is widely accepted that cell lines have reduced levels of reactive oxygen species when compared to primary cells, however, measurable levels of reactive oxygen species in both hMDM and J774 cells are not raised above baseline levels when infected with GBS. This suggests an inadequate oxidative burst in both cell types which, when considered together with the pharmacological inhibition of the complex in this system, indicates that the NADPH oxidase does not play a major role in GBS killing.

The two component system CovS/R has a role in intracellular survival in murine cells, as demonstrated by poor survival of the deletion mutant. This strain shows an increased sensitivity to hydrogen peroxide killing at low pH (Chapter Three). However, the increased sensitivity of the mutant to intracellular killing cannot be explained by an increased sensitivity to the phagosome oxidative burst as cells infected with $\Delta$covS/R fail to show high levels of ROS or an involvement of the NADPH oxidase.
In order to try to improve the anti-streptococcal activity of infected J774 cells the cells were incubated with IFNγ. Surprisingly this did not improve the outcome of infection and GBS survival was unchanged. One aspect of IFNγ activation of phagocytic cells is increased phagosome and lysosome fusion which results in a decreased phagosomal pH (Torrado et al., 2010). A decrease in phagosomal pH is required for GBS to be able to survive within the phagosome, as blocking this leads to reduced intracellular survival. To this effect IFNγ activation may actually aid the organism’s intracellular lifestyle. Unlike some intracellular pathogens, such as Legionella pneumophila (Byrd and Horwitz, 1991), the dependence on low pH to aid phagosomal survival is not due to the lack of available iron in these conditions, since iron replacement does not improve the outcome. GBS is reported to be able to survive in low iron conditions due to the presence of the siderophore receptor FhuD and other, as-yet described, mechanisms (Clancy et al., 2006). As seen in the pathogenic fungus Cryptococcus (Levitz et al., 1997) it could be that GBS is using the decrease in pH to sense the environment and regulate genes that are required for resistance to the host cell killing mechanisms. In this context it is intriguing that the CovS/CovR regulator, know to respond to environmental pH (Santi et al., 2009a) is required for intracellular survival in murine cells.
CHAPTER 5: DISCUSSION

INTRACELLULAR SURVIVAL OF GBS IN MACROPHAGES
SUMMARY

The data presented in Chapter Three and Chapter Four of this thesis should be considered together to understand the interaction of GBS with host cells. Chapter Three examined the role of four genes previously characterised as having an involvement in the pathogenesis of the organism; csp, cylE, sodA and covS/R. Only covS/R was found to be required for the persistent survival phenotype of the organism seen in macrophages. Sensitivity of the organism in vitro to conditions expected to be found in the phagosome could not fully explain the capacity of the organism to survive macrophage killing. Chapter Four focused on the host response to GBS; data presented suggested that although GBS is not modifying the phagosome maturation pathway the organism is not exposed to high levels of oxidative killing. A major finding in this chapter is the importance of phagosomal pH for the organism's survival.

5.1 How important is intracellular survival in phagocytes in the context of GBS disease?

In this study we sought to further understand the interaction between GBS and host macrophages. Although Group B Streptococcus is not thought to be an intracellular pathogen per se, this study confirms previous accounts (Cornacchione et al., 1998, Valenti-Weigand et al., 1996, Segura et al., 1998) that demonstrate it is not efficiently killed by phagocytic cells, the first line of the host defence. In contrast to a close
relative Group A Streptococcus (GAS) (Hertzén et al., 2012) there is no evidence to suggest that the organism is replicating in macrophages, but this is an area that warrants further investigation. In clinical GBS disease, the organism achieves a high level of bacteraemia, which precedes life threatening septicaemia and meningitis. Intracellular survival within phagocytes may contribute to disease progression by reducing the effectiveness of phagocyte clearance. In addition, some pathogens, such as GAS, can actively exploit phagocytes to facilitate traffic between host tissues and it is therefore possible that a related process may underlie GBS dissemination (Thwaites and Gant, 2011, Medina et al., 2003).

Due to poor cellular penetrance of most antimicrobial compounds, intracellular survival of an organism will protect it from the activity of antibiotics (Kaplan et al., 2006). GBS colonises 30% of pregnant women and penicillin treatment of colonised pregnant women does not remove the organism (Rodriguez-Granger et al., 2012). This has major clinical implications as prophylactic treatment has to be given during labour. The first line treatment against GBS infection is penicillin, which shows very poor absorbance into eukaryotic cells, hence understanding the intracellular persistence of this organism may enable novel therapies to combat colonisation.

Macrophages not only have an important role in killing invading micro-organisms but also in mounting an immune response. Such signalling is potentially double-edged, since GBS infection can lead to an inappropriate cytokine release and potentially fatal sepsis syndrome. Improved knowledge of how macrophages are responding to GBS may therefore enable treatment options that prevent sepsis. For example dextromethorphan, which is commonly used as a cough suppressant but was recently described to have anti-inflammatory properties, (inhibition of the NADPH
oxidase and suppression of TNFα) prevented cytokine release and sepsis in GAS-infected mice (Li et al., 2011).

One of the interesting features of GBS disease is the serotype distribution; serotype III is more commonly associated with neonatal disease, whilst serotype Ia is often isolated from sites of colonisation (Weisner et al., 2004). Compounding this is the emergence of the hypervirulent clade ST-17, which is strongly associated with late onset neonatal meningitis (Manning et al., 2009, Poyart et al., 2008). GBS shows considerable interspecies variability at a genetic level and host adaptation appears to improve strain fitness (Sorensen et al., 2010, Weisner et al., 2004, Brochet et al., 2006). In addition to the two serotypes tested in this study, III (NEM316) and Ia (A909) and the COH1 strain representing the hypervirulent ST17 clade, a diverse collection of GBS isolates (Herbert et al., 2005) has been tested in the J774 survival assay by other members of our group (Cumley et al., 2012). No significant differences in their capacity to survive within macrophages were observed between strains, serotypes or MLST groups. These data suggest that epidemiological variation in disease progression and hypervirulence may result from differences in colonisation or extracellular growth, rather than differences in the interaction with phagocytes. Interestingly, such a conclusion is supported by recently published data indicating that one explanation for the increased virulence of ST-17 clones of GBS is the observation that they overexpress the cell wall surface protein HgvA, leading to increased adherence to host epithelia (Tazi et al., 2012).
5.2 How does GBS avoid killing by the innate immune system?

As mentioned in the Introduction (Chapter One) organisms can employ multiple strategies in order to avoid killing by the host immune response: Avoidance of phagocytosis, modification of the host cell or a systemic response, initiating programmed host cell death and avoidance of phagosome killing. The latter can occur by modifying phagosome maturation, escaping the phagosome or adapting to and resisting the antimicrobial features of the phagosome. The following section will discuss the ability of GBS to avoid immune killing by these mechanisms based on the data obtained in this study.

Avoidance of phagocytosis and modification of host response

The host response to an engulfed organism has been shown to be in part related to the receptor that mediates phagocytosis. Even though the polysaccharide capsule of GBS has been described to be important in this context, in our assay the presence or absence of a capsule did not influence the organism's ability to survive within cells, nor did it significantly prevent uptake of the organism. The capsule has been shown to be important in prevention of opsonophagocytic killing (Maisey et al., 2008a), by reducing deposition and activation of complement components (Marques et al., 1992, Wessels et al., 1989). However, in our study the absence of opsonin made this function of the capsule immaterial. It has been shown previously that when GBS is taken up through the Fc receptor, in the presence of specific antibodies, a strong oxidative burst is generated (Senn et al., 2011, Cheng et al., 2001). Uptake through this receptor leads to increased numbers of lysosomal vesicles seen and the
organism is cleared more effectively (Valenti-Weigand et al., 1996). It is not clear what receptor is being engaged to promote uptake in our assay but uptake did not induce a large oxidative burst. Therefore mechanisms that the organism employs to protect itself against oxidative stress, such as the superoxide dismutase and carotenoid pigment are not required for intracellular persistence. The Fc receptor signals directly to the NADPH oxidase complex, whereas phagocytosis through the complement receptor (the most likely uptake mechanism in opsonin independent GBS phagocytosis (Antal et al., 1992, Albanyan and Edwards, 2000)) does not lead to the production of superoxide (Underhill and Ozinsky, 2002).

Thus, intracellular survival of GBS is linked to the receptor involved in uptake and subsequent signalling pathways that it engages. This may be particularly relevant in neonatal disease, where antibody or opsonin levels may be low and consequently uptake may result in the organism residing in a weakly antimicrobial compartment. To confirm the role of the receptor in downstream signalling it would be interesting to look at phagocytosis of GBS through interaction with the complement receptor in the presence of complement, which provides a second signal to activate the oxidative burst (Russell et al., 2009). Further characterisation of the antimicrobial features of the GBS containing vacuole and identification of the receptor which the organism is taken up by would give insight into the environment which the organism is residing in.

**Host cell death**

GBS has been described to be able to cause apoptosis in macrophages through the action of the β-h/c (Fettucciari et al., 2006, Fettucciari et al., 2000, Ulett and Adderson, 2005). The cylE gene which encodes the structural β-h/c has multiple
roles in GBS virulence; not only does it produce the pore forming toxin, it is also linked to the expression of a carotenoid pigment which can be protective against oxidative stress (Liu et al., 2004). In the survival assay used for this study significant cell death was not observed because the assay conditions were optimised to reduce the likelihood of apoptosis, which has been shown to be a β-h/c dose-dependent response (Fettucciari et al., 2000, Liu et al., 2004). Host cell death is discussed further in relation to the survival of the ΔcovS/R strain, below.

**Avoidance of phagosome killing**

**Arrest or manipulation of phagosome maturation**

One method used by microorganisms to avoid being killed in the phagosome is to actively perturb phagosome maturation. For instance, in both *Burkholderia cenocepacia* and *Mycobacterium tuberculosis*, a delay in phagosome maturation is associated with intracellular survival (Lamothe and Valvano, 2008, Pethe et al., 2004). The close relative of GBS, Group A *Streptococcus*, has been shown to prevent phagosome maturation, since GAS-containing phagosomes do not recruit LAMP or become acidified (Hertzén et al., 2012). In contrast, GBS does not appear to modify the recruitment of LAMP to the phagosome membrane, since recruitment occurs at the same rate in live and heat killed organisms and latex beads. This suggests that the organism is not modifying the phagosome maturation pathway, although it should be noted that recruitment of LAMP is not necessarily indicative of a fully functional mature phagosome. For example *Salmonella enterica* resides within a LAMP positive vacuole, which is not acidified, and has diverged from the phagosome
maturation pathway (Eswarappa et al., 2009, Steele-Mortimer, 2008). Mature phagosomes can be distinguished by functional analysis, acidification, or the presence of active proteases (Russell et al., 2009). As part of the continuing work in our group, an acidotropic dye, lysotracker, was used by a member of the group to measure if GBS was residing within acidified phagosomes. Interestingly this data suggested that acidification of the GBS containing compartment may be actively modified, as the accumulation of dye was higher in vacuoles containing heat killed organisms than in vacuoles containing live organisms.

One significant barrier to further characterising the GBS containing vacuole is the lack of a reliable method to track live GBS within cells. Consequently most analyses must be carried out on fixed samples. The development of a GFP expressing strain (or alternative means of fluorescently labelling live organisms) would facilitate further investigations into the fate of intracellular GBS. Better markers of phagosome maturation include ratiometric pH imaging (Russell et al., 2009) and measurement of functional protease; for example, by using substrates that are fluorescent when degraded by an active enzyme (Russell et al., 2009).

**Phagosome escape**

A strategy employed by some organisms to avoid phagosomal killing is to escape from the antimicrobial phagocytic vacuole and replicate in the cytoplasm of the cell. *Listeria monocytogenes* has a well characterised mechanism to escape the membrane bound vacuole before it becomes acidified and can survive in the cytoplasm of cells. *Shigella flexneri, Burkholderia pseudomallei, Rickettsia prowazekii* and *Francisella tularensis* can also escape the phagosome and replicate successfully.
in the cytosol of the cell (Ray et al., 2009). The ability of GBS to use this strategy has not been tested in the work discussed above. However, the increasing presence of LAMP on the GBS containing phagosome membrane until late in infection suggests that the vast majority of intracellular organisms reside in a membrane bound compartment. Nonetheless, the availability of a GFP-expressing strain of GBS would allow for a much more detailed analysis via time lapse imaging.

**Adaptation or resistance to conditions inside the phagosome**

The mature phagosome of macrophages is an inhospitable environment for any ingested microorganisms, being highly acidic, high in reactive oxygen and nitrogen species and additionally containing degradative enzymes and antimicrobial peptides. GBS was shown *in vitro* to have limited resistance against ROS and RNS. However, as discussed previously, the levels of ROS generated in macrophages upon engulfment of GBS are low and therefore fail to efficiently control the organism.

GBS is clearly well adapted to growth in conditions of low pH, since it can ferment carbohydrates to lactic acid and, as a commensal, will reside in the vagina at a pH of 3.8-4.5. However, during a systemic infection the organism must be able to respond to the much higher pH of blood (7.4). Previous microarray analysis has demonstrated that expression of 18% of the GBS genome is altered in response to a change in environmental pH and the majority of these changes are controlled by the covS/covR operon (Santi et al., 2009a). This agrees well with our finding that normal acidification of the phagosome appears essential for GBS survival, since inhibiting acidification through the vATPase with concanamycin A reduces the survival of the organism. An analogous phenomenon occurs in the fungal pathogen *Cryptococcus*
neuroformans, in which a change in pH triggers transcriptional changes to promote intracellular survival (Levitz et al., 1997), and in Salmonella typhimurium, where a pH drop is a prerequisite for expression of SPI-2 secretion system and the consequential expression of effectors that modify the phagosome (Sanchez, 2011, Arpaia et al., 2011).

5.3 How might the CovS/R two component system be aiding the organism’s survival within murine cells?

In this work the CovS/R two component system has been demonstrated to be required for GBS to survive within the murine macrophage cell line J774, although seemingly not within human macrophages. The CovS/R (Control of virulence regulator) system in GBS was identified due to homology to the CsrR (capsule synthesis regulator) system in GAS (Glaser et al., 2002). Orthologs of the regulator element but not the cognate sensor have also been described in S. suis (Pan et al., 2009) and S. mutans (Idone et al., 2003).

On initial investigation, the GBS CovS/R TCS appears unusual in comparison to orthologous systems in closely related species. In GAS, S. suis and S. mutans, CovR acts chiefly as a repressor (Graham et al., 2002, Idone et al., 2003, Pan et al., 2009) but in GBS it up-regulates and down-regulates equal numbers of genes (Jiang et al., 2008). Additionally, deletion of CovR in S. suis (Pan et al., 2009) or CsrR/S in GAS (Graham et al., 2002) leads to increased virulence in animal models, and improved intracellular survival of the mutant strain in phagocytic cells compared to wild type strains. In support of the reduced intracellular survival of a ΔcovS/R strain, GBS
strains deleted in CovS/R or CovR have been shown to display decreased virulence in murine models (Lamy et al., 2004, Jiang et al., 2005). However, it is clear both from my own work and that of others (Jiang et al., 2012) that the contribution of the CovS/R system to GBS virulence is complicated and may be host variable. In support of this, one study has found the GBS ΔcovR strain to be increased in virulence in a murine model compared to the wild type (Lembo et al., 2010), due in part to its increased ability to cross the blood brain barrier (Lembo et al., 2010).

Interestingly, a very successful strain of GAS that is currently causing outbreaks in the Pacific region has been shown to have mutations in the CsrR system, leading to an increased invasive phenotype (Tran-Winkler et al., 2011). In GBS, a strain isolated from a patient with toxic shock syndrome was hyperhaemolytic and had mutations in the covR gene (Sendi et al., 2009a), confirming the clinical importance of studying this TCS.
The role of CovS/R in intracellular survival

In GAS the CovS/R TCS has been shown to be up regulated 6 hours post phagocytosis (Hertzén et al., 2012), whereas in GBS CovS is downregulated after 30 minutes incubation in blood but increased after 90 minutes (Mereghetti et al., 2008). Having identified phenotypically that the TCS is important for GBS survival in murine cells, it will be of great interest in the future to characterise the gene regulation that occurs through this system within the conditions of the phagosome.

Unsurprisingly, given that the CovS/R system regulates approximately 7% of the GBS genome, strains deleted in CovS/R show pleiotropic effects in both GBS and S. suis. Deleted strains are phenotypically hyperhaemolytic, hyperadherent (Pan et al., 2009, Jiang et al., 2005, Lamy et al., 2004) and show a reduced ability to replicate in nutrient limited media (Lamy et al., 2004).

An attractive model based on our data is therefore an extension of that postulated by Santi et al (Santi et al., 2009a) in which intracellular GBS within a phagosome require a drop in pH to stimulate CovS autokinase activity. This is required for phosphorylation of CovR, increasing its promoter binding activity and regulating genes that aid the organism’s survival in the phagosome. In the absence of this decreasing pH signal, either through inhibition of the phagocyte vATPase or due to the loss of the pathogen’s ability to sense the pH change, then the organism fails to adapt to the intraphagosomal conditions. Figure 26 summarises a potential model by which CovS/R aids the organisms ability to survive within the phagosome.
CHAPTER 5: Discussion: Intracellular survival of GBS in macrophages

Figure 26: Adaptation to the phagosome through CovS/R

Adaptation to phagosomal conditions through the action of the CovS/R two component system. In response to a drop in pH CovS may phosphorylate CovR and increase the DNA binding capacity of this regulator. Genes regulated by CovR include the immunomodulatory proteins cylE and bibA and proteins involved in metabolism, nutrient transport and protein synthesis. The pilus protein is upregulated in acidic conditions independently of CovR, while cell wall modifications may help the organism resist the antimicrobial phagosome in a CovR dependent manner.
As heterotrophs, GBS and other bacteria use transporters to bring nutrients into the cytoplasm and this is an important adaptive response to a nutrient limiting environment such as the phagosome. The largest group of functional genes dysregulated in the ΔcovS/R strain are those involved in small molecule transport, including the iron siderophore receptor FhuD and genes required for polyamine synthesis (Santi et al., 2009a). These genes are up-regulated in low pH in a CovS/R dependent manner. This suggests that in acidic conditions GBS enters a highly active metabolic state, and the low pH of the phagosome could be a favourable environment to trigger this. Without the pH regulated metabolic change, the organism may therefore not be able to remain viable in the nutrient limiting phagosome.

CovR has been shown to bind directly to cylX, the regulatory element of the cylE operon, and repress transcription of this operon (Lamy et al., 2004). CylE is down regulated at low pH in the presence of CovR (Santi et al., 2009a), which suggests that CovR will be repressing expression of β-h/c within an acidified phagosome. In our experimental conditions, ROS production was low and therefore the role of the cylE associated carotenoid pigment is thought to be minimal. Consequently the repression of cylE by CovR in acidic conditions such as the phagosome may be more important due to the decreased production of the β-h/c toxin (Santi et al., 2009a). The β-h/c has been shown to cause cell and tissue damage and increase membrane permeability (Liu et al., 2004, Pritzlaff et al., 2001), as well as stimulating cells to increase nitric oxide production and undergo cytoskeletal rearrangements (Talati et al., 2008, Fettucciari et al., 2006, Fettucciari et al., 2011, Ulett and Adderson, 2005). The immunomodulatory role of the toxin is discussed in more detail later in
connection with the differences seen in survival of the mutant in murine and human
cells. An interesting future line of work would be to look at the intracellular or even
specifically intraphagosomal expression of \textit{cylE}, either by RT-PCR or using a reporter
assay.

To further investigate the role of CovS/R and pH in resisting phagosomal killing it
would be interesting to analyse the sensitivity of the \textit{ΔcovS/R} mutant to antimicrobial
peptides and degradative enzymes. Within the CovS/R regulon are genes involved
in cell wall synthesis, and consequently \textit{ΔcovS/R} has an altered cell wall structure
(Lamy \textit{et al.}, 2004), which is likely to strongly impact upon the organism’s ability to
resist antimicrobial peptides (Boone and Tyrrell, 2012, Poyart \textit{et al.}, 2003, Quach \textit{et

5.4 How can the survival difference observed with the \textit{ΔcovS/R} strain
between J774 cells and hMDM be explained?

Despite the convincing killing of \textit{ΔcovS/R} strain by J774 murine macrophage-like
cells, in human monocyte derived macrophages (hMDM) the mutant shows similar if
not improved survival to the wild type. Potentially, this could be due to differences
between cell lines and primary cells and/or differences between mouse and human
cells. The increased survival of the \textit{ΔcovS/R} mutant in hMDM was surprising as it is
generally held that primary cells are more antimicrobial than cell lines. As shown in
this study, the rate of ROS production is higher in primary human cells than the J774
cell line.
One of the most striking differences between human and mouse macrophage biology is the role of the inducible nitric oxide synthase (iNOS) (Fairbairn et al., 2011, Mestas and Hughes, 2004). In mice iNOS is upregulated during macrophage infection and is important for control of intracellular organisms, but this has not been observed in human macrophages. This general difference is also true for GBS infection, which upregulates iNOS in murine macrophages (due to the combined action of the β-h/c and the cell wall (Ring et al., 2002) but not in human macrophages (Ulett and Adderson, 2005). Increased NO production in murine cells infected with GBS can also be achieved through TLR9 signalling. TLR9 is more abundant in murine cells than human cells and in GBS infection responds to the presence of ssRNA and DNA (Talati et al., 2008, Deshmukh et al., 2011a, Deshmukh et al., 2011b). The increased NO production in this context has been shown to enhance phagosomal processing of intracellular organisms in a positive feedback loop (Deshmukh et al., 2011b).

The experimental data obtained in hMDM taken with previous published reports on GBS and murine macrophage interactions leads to an alternative model to that proposed in section 5.4 and figure 25 to explain the decreased survival of ΔcovS/R strain observed in murine macrophages (Figure 27)
CHAPTER 5: Discussion: Intracellular survival of GBS in macrophages

Overexpression of β-h/c may stimulate murine macrophages to incite an increased antimicrobial attack. In the absence of CovR mediated repression, ΔcovS/R strains overexpress β-h/c. In murine cells but not in hMDM GBS infection, through recognition of β-h/c, the cell wall and signalling through TLR9, increases the transcription and activity of iNOS leading to elevated levels of nitric oxide. An increase in NO leads to apoptosis, increased cytokine production and increased phagosomal processing. β-h/c has also been shown to cause membrane damage with a subsequent influx of calcium ions activating calpain proteins. Calpain activation can modify the host cell cytoskeleton, leading to cytoskeleton dependent cell signalling events and also initiation of apoptosis.
Overexpression of the β-h/c pore forming toxin combined with murine cell host factors may be the key to the different survival within the cell types. β-h/c, in murine cells but, importantly, not in hMDM, has been shown to increase the levels of nitric oxide (and subsequently RNS) within the GBS infected cell (Ulett and Adderson, 2005). Whilst in vitro data presented in this thesis revealed that wild type and ΔcovS/R have similar sensitivities to RNS in terms of killing, others have proposed a more important role for these molecules in mediating host cell signalling (Ulett and Adderson, 2005, Bogdan et al., 2000). Indeed it has been shown that NO increase enhances phagosomal processing of ingested GBS (Deshmukh et al., 2011b). To further understand the differences seen between murine and human cells, and the role of NO as a signalling molecule, it would be interesting to measure the levels of NO produced by cells infected with both wild type and ΔcovS/R GBS and to look at subsequent changes in inflammatory signalling within these cells.

Bacterial pore forming toxins can impair membrane integrity, and trigger an influx of calcium ions which can act as cell signalling molecules (Gekara et al., 2007). GBS infection has been shown, through a β-h/c-dependent mechanism, to increase the activity of calpains; Ca²⁺ dependent cytosolic proteases that drive cytoskeletal re-modelling (Fettucciari et al., 2011). Therefore a change in host cell signalling due to the potential of β-h/c to cause membrane alterations should be considered when analysing any experimental data from a strain overexpressing β-h/c.

A further aspect of both calpain activation and NO increase by the GBS β-h/c is the induction of apoptosis in infected cells (Ulett and Adderson, 2005, Fettucciari et al.,
2006). It is known that the induction of apoptosis in murine macrophages by GBS is β-h/c dose dependent (Liu et al., 2004, Fettucciari et al., 2000), so in the ΔcovS/R strain (which is overproducing the cytolysin) there may be an increase in host cell death. Nonetheless, based on trypan blue counting there was no evidence for increased macrophage death in our assay, although it would be useful to use a more sensitive method for example a lactate dehydrogenase release assay to monitor cellular lysis or staining for cellular markers of apoptosis such as annexin 5 in order to confirm this in the future.

More generally, our data highlight the significance of species-specific differences and thus the importance of choosing appropriate model systems. At present, most published data on GBS pathogenesis and host pathogen interactions is from murine models and thus extrapolation to the human disease should be carried out with caution.
CONCLUSION

This study has focused on improving our understanding of the interaction of GBS with phagocytic cells of the innate immune system. Important findings are the reduced oxidative burst produced by GBS infection under non-opsonising conditions and the requirement for acidification of the phagosome to trigger intracellular survival of GBS. The role of the two component system CovS/R in intracellular survival is likely to be complex and multifactorial. Not only does it upregulate genes that may aid adaptation to phagosome conditions but it also downregulates the β-h/c, which (in murine cells at least) has previously been shown to play an important role in stimulating a host cell response.

A large amount of work still needs to be done to understand the true nature of the GBS-macrophage interaction. An organism which can be visualised within cells would allow for experiments that would greatly enhance this understanding and therefore the following chapter describes the evaluation of GFP expressing streptococci for this purpose.
CHAPTER 6: DEVELOPMENT AND EVALUATION OF GFP EXPRESSING-STREPTOCOCCI
INTRODUCTION

Many of the interesting questions concerning the ability of GBS to persist inside macrophages would benefit from being able to follow live organisms within cells.

Due to the small size of streptococci, resolving the organism inside cells by light microscopy is difficult. Visualisation requires a detectable marker such as a fluorescent dye. Fluorescent antibody labelling generally requires the cells to be fixed, therefore preventing any live imaging. Live organisms can be covalently labelled with fluorescein isothiocyanate (FITC) (Carlin et al., 2009) and followed in real time. However this method has limitations for use with intracellular organisms, since the dye binds to bacterial surface proteins and this may change host pathogen interactions. In addition, the FITC signal may be lost due to photobleaching (Shaner et al., 2005), can be quenched in acidic conditions and will be diluted out during cell replication.

Green Fluorescent protein (GFP) has been successfully used to visualise intracellular organisms in many bacterial species (Campoy et al., 2011, Gille et al., 2006, Clemens et al., 2009, Voelz et al., 2010). Originally cloned from the jellyfish Aequorea victoria, GFP fluoresces green at an excitation wavelength of 488nm, emitting green light at an optimal wavelength of 507nm and requiring no co-factors or specific substrates. The gene can be introduced into bacterial hosts (with expression driven by a bacterial promoter) either on a plasmid or introduced into the bacterial cell chromosome.
Recent publications have used GBS strains carrying a GFP expressing plasmid as a reporter to monitor endogenous promoter activity (Gleich-Theurer et al., 2009) and to quantify phagocytosis of the organism by monocytes using flow cytometry (Gille et al., 2009). Plasmid expressed GFP in the related organism *Streptococcus gordonii* has been used to visualise biofilm formation (Aspiras et al., 2000, Hansen et al., 2001)

To track the fate of phagocytosed GBS it would be desirable to visualise by microscopy live intracellular organisms for the duration of the survival assay. Time lapse imaging could be used to follow the infection in real time, identifying if the organism is residing in phagosomes for the duration of the assay, if any replication of the organism is occurring and if the organism is able to kill or escape the host cell. Having a live fluorescent marker on the organism in conjunction with other fluorescent cellular markers would also enable determination of the subcellular location of the internalised GBS.

In order to be able to use a GFP strain for these purposes the GFP expressing strain needs to be reliable and show a comparable intracellular survival phenotype to a wild type strain. Fluorescence must be consistently expressed independently of replication, growth phase and environment and be able to be detected. The following work has looked at three GFP expressing plasmids for use in GBS; pMA2 from *Streptococcus gordonii*, pBSU98 from a GFP expressing strain of streptococci and pBSU101, a vector developed for use in Group B *Streptococcus*. 
RESULTS

6.1 The plasmid pMA2 displays variable GFP expression

The Streptococcus -E.coli shuttle vector pMA2, developed for expression of GFP in Streptococcus gordonii, has the GFP gene under the control of the promoter for the hppA gene (Aspiras et al., 2000). The hppA promoter is an active endogenous Streptococcus gordonii promoter driving expression of the hppA gene which encodes an oligopeptide binding lipoprotein.

When this plasmid is transformed into GBS the resultant colonies appear green on solid media (Figure 28a) and using time lapse microscopy intracellular organisms can be seen and followed inside J774 macrophages for over 40min (Figure 28b). However in a population carrying the plasmid only around 10% of the organisms appeared to display fluorescence. Thus the mean fluorescence level for a population of bacteria, as detected for instance by plate reader analysis, is too low to be useful (Figure 29). In addition, the heterogeneity in fluorescence expression, combined with the moderate uptake of the organism into macrophages, meant that the vector pMA2 was not a viable option for detecting intracellular organisms.

To examine this in more detail, the hppA promoter region on this plasmid was sequenced using the primer Pr6F and aligned with the promoter region that had been amplified from the S.gordonii chromosome during initial plasmid construction (Aspiras et al., 2000). This sequence revealed mutations throughout the promoter sequence.
(Figure 30b) which may potentially be one cause of the heterogeneous efficiency of GFP transcription.

Therefore, in conclusion plasmid pMA2 GFP expression is weak and heterogeneous and, although carrying this plasmid does not affect the growth of the organism, fluorescence levels are too poor to be useful for visualising intracellular organisms.
Figure 28: Colonies of NEM316:pMA2 express GFP

Colonies of NEM316 carrying pMA2 express GFP. a) Individual colonies of NEM316:pMA2 (i) grown on solid media and viewed with an epi-fluorescence stereo microscope can be seen as green when using the blue filter (ii). b) J774 cells infected with NEM:pMA2 were visualised using a time lapse imaging capture system. NEM316:pMA2 (arrowed) can be seen by detection of GFP expression. The organism is seen residing inside the J774 cell as it moves around the field of view. Selected stills are presented from a series of images taken every two minutes.
Figure 29: Plasmid pMA2 does not impose a fitness cost

Plasmid pMA2 does not infer a fitness cost to NEM316:pMA2, however GFP expression is weak. Figure 29a shows growth of WT NEM316 and NEM316:pMA2 measured by OD600 readings every 20 minutes for 11 hours. Figure 29b Shows fluorescence (excitation 485-12, emission 520) every 20 minutes for 11 hours, no fluorescence can be detected above the level of the baseline. The images presented are representative curves of three repeats.
Figure 30: Sequence of the hppA promoter region

The sequence of the hppA promoter region on plasmid pMA2 is different from the published sequence. The promoter sequence of pMA2 was sequenced from the primer Pr6F, which reads through PhppA and into the sequence of the GFP gene (Figure 30a). The sequence was aligned using clustalW (Figure 30b) against the published sequence for the region that had been amplified using primers HJ25 (not shown, due to it being upstream of PrF) and HJ26 during plasmid construction. Consensus sequence is marked as stars. The promoter sequence from the pMA2 plasmid used to express GFP in GBS shows multiple nucleotide changes from the published sequence.
6.2 Strains BSU98 and BSU99 have an intracellular survival defect, which may be due to a fitness cost associated with the GFP expressing plasmid.

Strains BSU98 and BSU99 were developed to measure intracellular survival in human monocytes using flow cytometry (Gille et al., 2009). Strain BSU98 has been constructed from the parental strain 090R carrying a pAT28 plasmid with EGFP expressed under the control of the *cfb* (CAMP factor) promoter. Strain BSU99 is carrying a control plasmid, which is unable to express GFP (Gille et al., 2009). The parental strain, 090R, is a poorly encapsulated variant of the serotype Ia strain 090 (Wessels et al., 1989). This strain was generated by serial passage in anti-serotype Ia antisera by Rebecca Lancefield in the 1920’s (Craig Rubens, personal communication) and is therefore not a well characterised strain.

When tested for intracellular survival under our assay conditions strains BSU98 and BSU99 show decreased intracellular survival in macrophages compared to strain NEM316 (Figure 31). They do however show strong GFP expression which can be clearly visualised within macrophages (Figure 32).

The poor intracellular survival of strains BSU98 and BSU99 (which may be due to the strain background not being representative of wildtype GBS strains) led us to conclude that these strains would not be useful for monitoring intracellular dynamics of streptococcal infection. This poor intracellular survival is in line with the published sensitivity of these strains to killing over 180 minutes by cord blood monocytes and peripheral blood monocytes (Gille et al., 2009).
CHAPTER 6: Development and Evaluation of GFP expressing-streptococci

Figure 31: Intracellular survival of strains BSU98 and BSU99

The capacity for intracellular survival in J774 macrophages is reduced in strains BSU98 and BSU99. Strains BSU98 and BSU99 were used to infect J774 macrophages. At three, six and nine hours post infection the number of viable intracellular organisms were enumerated by CFU count. Wild type NEM316 and \textit{L. lactis} were included as controls. Survival of BSU98 and BSU99 is markedly reduced compared to NEM316.

Figure 32: Intracellular expression of GFP by strain BSU98

GFP expressing strain BSU98 can be clearly visualised inside macrophages using fluorescence microscopy. J774 macrophages were infected with BSU98. GFP expression was visualised using microscopy. Figure 32 panel (i) shows a false coloured image of GFP expressing BSU98 within a J774 macrophage. A phase image of the same cell can be seen in (ii); chains of streptococci are marked with an arrow.
6.3 Evaluation of NEM316:pBSU98

Since GFP expression was high from strain BSU98, the vector (pBSU98) was removed from BSU98 and transformed into the NEM316 strain to construct the tagged strain NEM316:pBSU98. NEM316:pBSU98 showed high levels of GFP expression both on solid medium (Figure 33) and in RPMI (Figure 34). Growth of NEM316:pBSU98 was however significantly retarded compared to wild type NEM316 (Figure 34). The decrease in fitness of these strains could be due to toxicity associated with the GFP protein or the cost to the host associated with high level protein expression.

Thus, plasmid pBSU98 confers high levels of GFP fluorescence in the host strain, but this is detrimental to the growth rate of the organism, limiting its usefulness for studies of intracellular survival.

6.4 Fluorescence emitted by the GFP protein is stable and appears to vary with growth phase.

In addition to the strain-specific growth issues described above, we noted a number of other limitations with this experimental approach. Firstly, it is well documented that the GFP protein is generally very stable (Cubitt et al., 1995).

In line with this, we observed that fluorescence can be detected from intracellular organisms that are non culturable having been killed prior to infection (Figure 35).
Persistence of fluorescence in killed organisms may make this method of visualising intracellular organism inappropriate as dead organisms will potentially still be visible.

Secondly, colonies of NEM316:pBSU98 showed a high level GFP expression, which appeared to be growth phase or density dependent, since colonies on the outside of a semi confluent lawn of organism had higher detectable levels of fluorescence than those inside (Figure 33). In addition, although levels of GFP fluorescence increased with cell density during exponential growth (Figure 34), a slight delay of approximately one hour was observed before detectable fluorescence started to increase. Fluorescence intensity continued to increase after NEM316:pBSU98 had reached stationary phase.

Accordingly, the dynamics of GFP protein expression and degradation and how the promoter driving expression is regulated must be considered in constructing a reporter for use in monitoring intracellular organisms.
Figure 33: GFP expression on solid media

Strain NEM316 carrying plasmid pBSU98 shows good GFP expression on solid media, which is growth phase or density dependant. Streaks of a lawn of strain NEM316:pBSU98 grown on THY\textsubscript{spec} plates were visualised using an epi fluorescence stereo microscope. The two images presented are from the same plate with the same starting inoculum streaked in a dilution series, such that image (ii) is ten times more concentrated than image (i). Colony density can be seen in the light image in panel a) with the corresponding GFP image below in panel b). Good GFP expression can be seen in this strain. Colonies that are on the edge of the lawn exhibit higher levels of detectable GFP compared to those in the higher density region at the centre of the lawn.
CHAPTER 6: Development and Evaluation of GFP expressing-streptococci

Figure 34: Growth rate of strain NEM316:pBSU98

Levels of GFP fluorescence increase with increasing optical density in NEM316:pBSU98 however this strain shows a decreased growth rate. NEM316:pBSU98 was cultured in RPMI medium. OD$_{600}$ (a) and (b) fluorescence (excitation 485-12 emission 520) measurements were taken every 20 minutes for 11 hours. Compared to NEM316, NEM316:pBSU98 shows a decreased growth rate and takes longer to reach maximum OD. GFP fluorescence levels seen in b) increase in accordance with optical density increase. The approximate phase of exponential growth for NEM316:pBSU98 is indicated with arrows on both the OD and fluorescence measurements. Maximum OD is reached at six hours in NEM316:pBSU98. The level of fluorescence detected continues to increase past this point reaching maximum at nine hours.
### Figure 35: Stability of GFP

The GFP protein is stable in killed microorganisms. GFP-expressing streptococci were killed by heat, antibiotics or UV. After infection into J774 macrophages GFP expression was visualised using microscopy. GFP expression can be seen in non-culturable organisms.

<table>
<thead>
<tr>
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<th>Growth in THY</th>
<th>Intracellular fluorescence</th>
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<tr>
<td><strong>Live</strong></td>
<td>+ve</td>
<td>+ve</td>
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<tr>
<td><strong>Heat Killed</strong></td>
<td>-ve</td>
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<td><strong>Gentamycin</strong></td>
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6.5 Plasmids encoding the GFP gene confer a fitness cost to the host cell

Having identified the detrimental effect of the presence of the plasmid pBSU98 to the host cell both on intracellular survival and during growth in defined media, NEM316 was transformed with a pair of plasmids, pBSU101 (NEM316:pBSU101) carrying the EGFP gene under the control of the cfb promoter and pBSU100 (NEM316:pBSU100) the same construct but without the promoter. The presence of the control plasmid pBSU100 allowed us to separate the cost of carrying the plasmid and the cost of expressing the GFP protein.

The intracellular survival of three individual transformants of the two strains can be seen in Figure 35. The survival assay was performed both with and without spectinomycin (to select for the plasmid). There is no difference in survival in the presence or absence of spectinomycin for either of the strains (excluding 100 clone B which may have lost the plasmid). Multiple clones were selected to try to select the most stable and healthiest clone for use in visualising intracellular organisms.

Strains harbouring both plasmids showed a decreased ability to cope with macrophage killing (Figure 35). Strains that are carrying the plasmids show visible phenotype changes; colonies look small, dry and hyperpigmented compared to the wild type. Recovery of the strains from frozen stocks is inconsistent and the resultant colonies are slow and show variable growth. This has made assays with these strains difficult to perform.
In conclusion, although the pBSU98 and pBSU101 plasmids confer high level GFP fluorescence the host cells do not show wild type phenotypes, thus preventing proper analysis of the strains in order to determine their viability in being used for live imaging.
Strains of NEM316 carrying the plasmid pBSU100 or pBSU101 have decreased intracellular survival at six hours post infection. Three individual colonies from a transformation were selected and used to infect J774 macrophages. The assay was carried out in the presence and absence of spectinomycin. rCFU is presented at six hours post infection. No difference can be seen between the viable count in the presence or absence of spectinomycin with the exception of 100B which may have lost the plasmid. Intracellular survival is decreased in all clones carrying either plasmid compared to the wild type. This was statistically significant in strains 100A and 100C but not when tested in the others, this may reflect the low number of repeats. Repeating the assay was challenging due to the poor and inconsistent growth of strains. Statistical significance (Mann-Whitney U p<0.05) in survival compared to wild type is marked by a star n=3.
DISCUSSION

To facilitate intracellular visualisation of group B *Streptococcus* it would be desirable to be able to express Green Fluorescent protein (GFP) in the organism. We have tested three plasmids, pMA2 previously used in *Streptococcus gordonii* (Aspiras *et al.*, 2000) and pBSU98 and pBSU101 used in *Streptococcus agalactiae* (Gille *et al.*, 2009, Aymanns *et al.*, 2011). Plasmid pMA2 did not affect growth of the bacterial cell but, as seen in *Streptococcus gordonii*, had weak and heterologous GFP expression (Aspiras *et al.*, 2000). Plasmids pBSU98 and pBSU101 expressed strong fluorescence but to the detriment of growth of the host bacterium. pBSU98 and pBSU100/101 are related plasmids carrying the gene for Enhanced Green Fluorescent Protein (EGFP) which shows increased solubility and fluorescence intensity compared to wild type GFP and is now in widespread use, hence it will be referred to as GFP in the remainder of the text.

Optimal folding of GFP is seen in the presence of oxygen and at a pH of 7 (Cubitt *et al.*, 1995, Hansen *et al.*, 2001). The microaerophilic conditions and reducing environment of the streptococcal cytoplasm have previously been suggested as the cause of heterogenous GFP fluorescence detection in *Streptococcus gordonii* carrying plasmid pMA2 (Aspiras *et al.*, 2000). However high level GFP fluorescence can be detected in GBS carrying pBSU98 and 101, suggesting that correct fluorophore formation is able to occur within the Group B streptococcal cytoplasm. The poor GFP fluorescence detected in NEM316:pMA2 could be due to poor gene
expression. Transcription through the $hppA$ promoter may be sub-optimal due to species specific gene regulation. In addition the observed changes in the $hppA$ promoter sequence could lead to inefficient recognition and binding of transcription factors and RNA polymerase.

The requirement of carefully controlled conditions for optimal GFP folding may explain the increase in GFP fluorescence seen at the edge of the colony of NEM316:pBSU98. The edge effect may be due to limited availability of oxygen in the dense region of growth on the plate. However, (Hansen et al., 2001) report folding of the protein in conditions of near anaerobiosis and instead suggest that the phenomenon of increased GFP expression seen at the edge of a colony could be reflective of the acidification of the medium by the growing organism (Hansen et al., 2001). In areas of dense growth, metabolic end products may lower the environmental pH, whereas in less dense regions the media and colony pH may stay nearer the optimal pH for fluorophore maturation. The same group observe that during the prolonged stationary phase of $S.gordonii$ fluorescence is not able to be detected, hence it is possible that colonies in the centre of the streak are older and thus in a prolonged stationary phase. Finally, it has also been suggested that the thick cell wall of streptococci prevents detection of fluorescence (Aspiras et al., 2000, Hansen et al., 2001).

GFP has a long half-life within the cytoplasm, of up to 24 hours (Qazi et al., 2001, Cubitt et al., 1995). Similarly, we have observed continued GFP fluorescence in organisms that have been subjected to killing and are not able to be cultured.
Fluorescence in dead organisms will reduce the potential of this protein as a reporter for intracellular organisms, since fluorescence may still be seen in non-viable organisms providing they are intact and the protein is not subjected to denaturing conditions. In line with this, the decrease in mean GFP fluorescence as detected by flow cytometry by (Gille et al., 2006) is not as rapid as the decrease in the number of viable intracellular organisms (Gille et al., 2009). During growth of NEM316:pBSU98 fluorescence could be detected increasing with cell density. However, there is a prolonged lag phase before the detectable fluorescence increases, this may be due to delay in gene expression through the promoter but could also be attributed to the time required to generate a mature fluorophore. (Qazi et al., 2001) observed that GFP fluorescence took longer to be detected than luciferase generated bioluminescence when both genes were under the control of the same promoter.

Interestingly fluorescence can be seen continuing to increase after the OD$_{600}$ has plateaued, indicating that the population has reached stationary phase and there is no more cell division. This continued increase in fluorescence could be due to the $cfb$ promoter still being active in stationary phase. However this again supports the notion that GFP is not a good real time reporter of promoter activity due to the time required for post translational modification of the fluorophore (Qazi et al., 2001). The fluorescence detected does reach a maximum level, it is not possible to tell if this is due to a decrease in promoter activity at late stationary phase, a decrease in the protein folding due to environmental conditions, or the amount of GFP protein able to be tolerated by each cell.
Plasmids pBSU98 and pBSU101 express GFP under the control of the cfb promoter. The cfb promoter is a strong promoter (Gleich-Theurer et al., 2009) which makes it ideal for high level expression of GFP. The cfb gene encodes the CAMP factor, (Podbielski et al., 1994), a pore forming toxin which is not directly implicated in the pathogenesis of GBS (Hensler et al., 2008, Lang and Palmer, 2003). The cfb gene is regulated, since expression is decreased at pH7.0 (Santi et al., 2009a) and up regulated in a CsrR (CovR) deletion mutant suggesting regulation can occur through this two component system (Jiang et al., 2005). Regulation of the cfb promoter may give inconsistent gene expression and subsequent fluorescent levels during intracellular monitoring, making it non-ideal for our purposes.

Initially the poor survival of NEM316:pBSU98 was thought to be due to the high level of GFP protein being produced by the organism, with the potential for misfolding in the less than optimal conditions in the GBS cytoplasm. Misfolded proteins may form into weakly fluorescent inclusion bodies. When GBS was transformed with pBSU100 containing a promoter-less copy of the GFP gene, the fitness cost was still observed. The backbone for these constructs is the multi-copy number plasmid pAT28. Multi copy number plasmids may divert host cell resources into plasmid replication and maintenance. pAT28 (Trieu-Cuot et al., 1990) encodes a spectinomycin adenyltransferase which may act as an energy drain on the host cell and in addition by-products generated by this enzyme may affect the health of the cell. The observed hyperpigmented phenotype of NEM:pBSU100 and NEM:pBSU101
suggests that the *CylE* gene is upregulated. This gene is also up regulated in strains deficient in CovS/CovR two component system which as shown in chapter three exhibit a decreased capacity to survive within murine cells.

In summary, the three plasmids evaluated are not currently suitable for live intracellular imaging, due either to low fluorescence levels or their tendency to induce atypical phenotypes (primarily poor growth) in host cells.
CHAPTER 6: Development and Evaluation of GFP expressing Streptococci

FUTURE WORK: Development of a fluorescent strain of GBS

To date there are only a few publications using GFP in streptococci (Aymanns et al., 2011, Gille et al., 2009, Gille et al., 2006, Gleich-Theurer et al., 2009, Hertzén et al., 2012, Qazi et al., 2001, Qazi et al., 2004). Discussion with colleagues working on Gram positive organisms around the world has highlighted that GFP expression is a challenge that is not limited to GBS. In the future, inserting the GFP gene into the GBS chromosome would be one strategy to remove the possible cost of maintaining a plasmid with antibiotic selection. Expression may be reduced in this situation, due to only having one copy of the gene, in spite of this under a strong promoter such as the cfb promoter protein expression may still remain high enough to be detected. However, this chromosomal insertion of the GFP gene into the GBS chromosome has been tried by researchers with little success (Brandon Kim, personal communication).

Secondly, the GFP protein has been cloned and codon optimized for use in a mammalian host. Modification of the GFP sequence to reflect the low CG content genome, the codon preference and tRNA prevalence of GBS may improve expression of the gene. In support of this, the expression of Yellow fluorescent protein and Cyan fluorescent protein has been improved in Bacillus by modifying the gene sequence to include codons favoured by this organism (Sastalla et al., 2009). The same study demonstrated an improvement in GFP intensity with an optimised sequence variant, however the GFPmut1 described in this study has a low GC content (39%) and as such did not require substantial modification for optimal
expression in *Bacillus*. Similarly the EGFP gene used in plasmid pBSU101 has a GFP content of 40% this is not much higher than the GC content of the GBS genome which is 35%. Condon optimisation and reduction of CG content in the EGFP gene in pBSU101 may reduce the fitness cost of carrying this plasmid to GBS and improve GFP expression.

To address the issue of prolonged GFP stability following host cell death, changing the C terminal end of the protein may reduce the half-life of the protein as it would facilitate recognition by host cell proteases. Tools including optimal promoter and terminator sequences have been optimised by (Ruiz-Cruz *et al.*, 2010) and such systems could be helpful in developing the optimal GFP expressing plasmid for GBS. Ideally a promoter for GFP expression would be constitutively expressed and not regulated, promoters for housekeeping genes (Voelz *et al.*, 2010) or a synthetic promoter such as the synthetic constitutive lactococcal promoter CP25 could be considered (Lun and Willson, 2004).

An approach that was tried during this study to improve fluorescent detection of GBS was to replace the GFP gene in the pMA2 vector with mCherry. This was not completed due to time constraints and technical difficulties. mCherry is a fluorescent protein which fluoresces red upon excitation at 587nm, emission 610nm. The protein has been cloned originally from a coral, *Discosoma sp.* (Shaner *et al.*, 2005). These characteristics may give mCherry an advantage over GFP in terms of folding and maturation in the cytoplasm, although there are no published reports of the use of mCherry in streptococci.
In conclusion the three plasmids evaluated in this work are not currently suitable for live intracellular imaging but future work to develop a vector optimised for fluorescent protein expression in Gram positive organisms would aid the investigation of host pathogen interactions.
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