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Review Article

An opportunistic pathogen under stress: how Group B *Streptococcus* responds to cytotoxic reactive species and conditions of metal ion imbalance to survive

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Abstract

Group B *Streptococcus* (GBS; also known as *Streptococcus agalactiae*) is an opportunistic bacterial pathogen that causes sepsis, meningitis, pneumonia, and skin and soft tissue infections in neonates and healthy or immunocompromised adults. GBS is well-adapted to survive in humans due to a plethora of virulence mechanisms that afford responses to support bacterial survival in dynamic host environments. These mechanisms and responses include counteraction of cell death from exposure to excess metal ions that can cause mismetallation and cytotoxicity, and strategies to combat molecules such as reactive oxygen and nitrogen species that are generated as part of innate host defence. Cytotoxicity from reactive molecules can stem from damage to proteins, DNA, and membrane lipids, potentially leading to bacterial cell death inside phagocytic cells or within extracellular spaces within the host. Deciphering the ways in which GBS responds to the stress of cytotoxic reactive molecules within the host will benefit the development of novel therapeutic and preventative strategies to manage the burden of GBS disease. This review summarizes knowledge of GBS carriage in humans and the mechanisms used by the bacteria to circumvent killing by these important elements of host immune defence: oxidative stress, nitrosative stress, and stress from metal ion intoxication/mismetallation.

Keywords: bacteria; pathogenesis; host-pathogen interactions; metallobiology; metal stress; copper; zinc; metal homeostasis; reactive oxygen species; reactive nitrogen species

Group B *Streptococcus* in the human body

Group B *Streptococcus* (GBS) is a Gram-positive commensal bacterium of the human microbiota in approximately half of adults (Brochet et al. 2006, Easmon 1986, Schuchat 1999). The primary and secondary sites of natural colonization are the lower gastrointestinal and female urogenital tracts, respectively (Anthony et al. 1983, Badri et al. 1977, Barnham 1983, Dillon et al. 1982). GBS in the gastrointestinal tract is a likely source of colonization of the female reproductive tract (FRT), specifically, the vaginal epithelium (Meyn et al. 2009). GBS was first reported as a human pathogen in 1938 (Fry 1938). Asymptomatic carriage in the urogenital tract has serious implications for pregnant women and their neonates due to the potential for vertical transmission (Easmon 1986, Finch et al. 1976). A rise in human disease due to GBS in the 1970s related to tetracycline use (Da Cunha et al. 2014, Nelson and Levy 2011) heralded the era of GBS being the leading cause of newborn infection, universal screening and intrapartum antibiotic prophylaxis (IAP) (Schrag and Verani 2013) and emergence of antibiotic resistant and hypervirulent subtypes such as Clonal Complex (CC)17. More recent years have brought renewed efforts to understand the pathogenesis of GBS disease and to develop vaccines targeted at maternal immunization to reduce the disease risk in neonates and young infants (Carreras-Abad et al. 2020).

There is substantial interest in the ways in which GBS transitions from a commensal to a pathogen in the body because of the global burden of GBS infection and disease. One area that has emerged in recent years is how GBS responds to and avoids killing by immune defence systems designed to destroy virulent bacteria, namely phagocyte responses that mediate reactive oxygen species (ROS), reactive nitrogen species (RNS), and metal ion mobilization. Here, we review the ways in which GBS survives in the human body with a particular focus on the responses used by the bacteria to circumvent killing by these three key elements of host immune defence.

Distinct GBS lineages interact with humans differently.

Not all GBS strains are equally pathogenic and some strains exhibit a higher propensity to cause disease through penetration of host barriers, making them more invasive than other colonizing strains (Tazi et al. 2012, Teatero et al. 2016). In 2003, a seven-gene multilocus sequence typing scheme (MLST) was introduced for GBS classification (Jones et al. 2003). MLST of capsular polysaccharide (CPS) serotype III strains identified a hypervirulent lineage termed sequence type (ST)-17, which is more likely to cause

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meningitis than any other serotype III strain (Manning et al. 2009). ST-17 strains display a conserved combination of secreted and surface-exposed proteins (Brochet et al. 2006, Tazi et al. 2010). Highly similar STs are grouped into CCs; most human GBS isolates are grouped into six CCs, namely CC1, CC10, CC17, CC19, CC23, and CC26 (Da Cunha et al. 2014, Sorensen et al. 2010). CCs 1 and 23 have been linked to asymptomatic colonization; CCs 17 and 19 comprise predominantly neonatal isolates (Bisharat et al. 2004, Jones et al. 2003, Manning et al. 2009). The ways in which different GBS affect humans in colonization and disease relate to differences in (i) virulence factor repertoire, (ii) resistance to external stress, and (iii) ability to avoid antibacterial immune responses.

Human disease caused by GBS

GBS in neonates and children, and preventative vaccines

GBS is a significant cause of potentially fatal neonatal disease globally (Brochet et al. 2006, Goncalves et al. 2022) and causes life-threatening disease in pregnant women, the elderly, and immunocompromised adults (Dermer et al. 2004, Edwards et al. 2011). Vertical transmission of GBS from mother to infant as can occur during childbirth or by placental penetration *in utero*. Early onset neonatal disease (EOD) describes a manifestation of pathology within the first 7 days after birth (a majority occurring within the first 2 days after birth) and is often associated with sepsis, meningitis, pneumonia, respiratory, and pulmonary distress (Anthony and Okada 1977, Furuta et al. 2022, Wu et al. 2004). Subsequent manifestation of disease, from 1 week up to 90 days, is classified as late onset disease (LOD) that is more commonly associated with bacteraemia, and has a significantly higher incidence of meningitis than EOD (Anthony and Okada 1977, Edwards et al. 2011, Wu et al. 2004) and a higher prevalence of neonatal disease overall (Edwards et al. 2011). GBS disease in children beyond early infancy is more likely to be associated with an underlying medical condition. For example, urinary tract infections (UTIs) and tonsillitis occur more frequently in older children whereas sepsis and meningitis are more common in infants with EOD or LOD (Wu et al. 2004).

A vaccine to prevent GBS disease in neonates remains in development, as reviewed elsewhere (Bianchi-Jassir et al. 2020, Kobayashi et al. 2016). In 2008, the European Commission Seventh Framework instigated the Design of a Vaccine Against Neonatal Infection (DEVANI) program for the purpose of developing strategies to combat GBS neonatal disease (Afshar et al. 2011). Multiple studies over several decades have targeted the sialic acid (Sia)-rich CPS as a vaccine candidate (Carreras-Abad et al. 2020, Chiarot et al. 2018, Paoletti and Kasper 2003). Other targets studied as GBS vaccine antigens include pili (Margarit et al. 2009), GAPDH (Madureira et al. 2011), and a combination of Alpha C and Rib proteins (Brokaw et al. 2022). A study on CPS–tetanus toxoid conjugates developed against all nine GBS serotypes showed that the CPS–tetanus toxoid vaccine enhanced the production of CPS-specific IgG and was active against GBS of the homologous serotype (Paoletti and Kasper 2003). Clinical trials of these conjugate vaccines demonstrated safety and immunogenicity in adults (Paoletti and Kasper 2003).

A hexavalent conjugate vaccine, GBS6, comprised of six different CPS serotypes (Ia, Ib, II, III, IV, and V) was shown to induce transplacental transfer of antibodies from mothers to foetus to provide passive immunity to the newborn (Buurman et al. 2019). In a safety and immunogenicity phase 1/2 clinical trial, GBS6 was well-tolerated in healthy adults and elicited robust immune responses that persisted 6 months after vaccination, supporting further evaluation of its use in pregnant women (Absalon et al. 2021). Such vaccines have been proposed to provide coverage for all at-risk populations (Bianchi-Jassir et al. 2020, Founou et al. 2023). None of the current GBS maternal vaccines in clinical trials, including the conjugated CPS and surface subunit protein vaccines have caused serious adverse events of special interest linked to vaccination, according to recent data analysed by systematic review (Bjerkhaug et al. 2023). Nonetheless, challenges for CPS-based GBS vaccines remain, including the occurrence of CPS switching that could lead to vaccine-escape (Martins et al. 2010), geographical dissimilarities in the prevalence of serotypes in different regions (Seale et al. 2017) and a rise in noncapsulated GBS strains (Li et al. 2018, Martins et al. 2010).

GBS disease in pregnant women

There are few data available on the global burden of maternal invasive GBS disease (iGBS) and related maternal mortality, as recently reviewed (Goncalves et al. 2022). However, estimates of the prevalence of colonization among pregnant women range between 10% and 30%, equating to a global burden of 20 million pregnant women with GBS carriage that may be intermittent, transitory, or persistent (Mei and Silverman 2023, Paul et al. 2023); which equates to a global burden of >40 000 maternal iGBS episodes annually, though this is considered an underestimation (Goncalves et al. 2022). GBS does not colonize the FRT in isolation but rather, as a frequent member of the vaginal microbiota, variations of which may contribute to variable and fluctuating rates of GBS carriage (Brokaw et al. 2021). Aside from representing the main risk factor for neonatal GBS EOD, outcomes of maternal vaginal–rectal colonization with GBS during pregnancy include various forms of skin and soft tissue disease, UTI, intraamniotic infection, bacteremia, and sepsis (Hall et al. 2017). GBS accounts for up to a quarter of cases of bacteremia in hospitalized pregnant women (Drew et al. 2015). Up to 7% of pregnancies may be complicated by GBS maternal UTI, and GBS accounts for ∼10% of all cases of pyelonephritis during pregnancy (Muller et al. 2006, Persson et al. 1986). These kinds of maternal iGBS infections can predispose to various complications during pregnancy, including chorioamnionitis, preterm labour, and an increased risk of vertical transmission of GBS to neonates. Pregnant women who have a positive GBS test result at any point during pregnancy are recommended to be treated with IAP to minimize the risk of vertical transmission of GBS and control the burden of disease in newborns (Mei and Silverman 2023, Paul et al. 2023, Schrag and Verani 2013).

GBS disease in nonpregnant adults

The past two decades have brought an increase in the incidence of GBS disease in nonpregnant adults (Edwards and Baker 2005, Farley et al. 1993, Skoff et al. 2009). In adults GBS disease can involve skin and soft tissues, the respiratory system, circulatory system, bone, abdomen, and central nervous system (Farley et al. 1993, Munoz et al. 1992, Skoff et al. 2009). GBS is an important cause of systemic infection in adults, including bacteraemia (Skoff et al. 2009) and severe streptococcal toxic shock syndrome (Ikebe et al. 2023). A recent 10-year retrospective study reported that three-quarters of cases of GBS bacteraemia were nonpregnancy related and nonneonatal, reflecting a deviation of GBS systemic infection from being primarily a neonatal disease to one that is more frequently unrelated to pregnancy (Alizzi et al. 2020). GBS also causes 1%–2% of all single organism UTIs (Edwards and Baker 2005, Foxman 2010, Ipe et al. 2013). Farley et al. (1993) estimated that 17% of iGBS disease in an adult cohort was nosocomial.

GBS disease in adults is associated with comorbidity and several factors are linked with increased susceptibility to GBS. For example, advanced age and underlying conditions such as diabetes, cardiovascular disease, cancer, and coinfections are primary risk factors (Collin et al. 2020, Farley et al. 1993, Munoz et al. 1992). Ethnicity is also a factor in increased susceptibility to GBS disease with Farley et. al. (1993) reporting the prevalence of invasive disease more than twice as high in black adults than in white adults. The increasing burden of GBS disease in adults (McLaughlin et al. 2021) is the basis for the prominent ranking of GBS amongst the world's foremost human pathogens (Collaborators 2022), and increasing interest in vaccines to reduce the number of adults with acute GBS disease (Ikebe et al. 2023).

GBS disease pathogenesis and virulence factors

GBS disease reflects colonization of host surfaces and tissues, bacterial penetration of physical barriers (e.g. placental, epithelial, and blood–brain barriers), and resistance to immune defences (Maisey et al. 2008a). Additionally, interactions between GBS and other resident microbes at mucosal surfaces, including in the FRT may be synergistic or antagonistic, though their influence on pathogenesis is unknown (Mejia et al. 2023). In the FRT, GBS must overcome local immune responses including the recruitment and activation of immune cells to succeed in persistent colonization (Brokaw et al. 2021). So, while GBS naturally colonizes many adults asymptomatically, as an opportunistic pathogen GBS can become invasive, evade immune responses, and cause disease (Shabayek and Spellerberg 2018). Pathogenesis of GBS disease reflects a multitude of virulence factors; the most prominent of which is the CPS, described over 35 years ago (Rubens et al. 1987). In addition to the CPS, GBS uses various surface-associated and secreted virulence factors, such as adhesins, pili, toxins, and two-component regulators to facilitate colonization and survive in the host. The most well-characterized GBS virulence factors are discussed below to provide a framework for examining the bacterial responses to host-derived ROS, RNS, and metal ion antimicrobial responses.

Colonization, binding, and invasion

Epithelial cells lining the FRT express abundant structural keratin proteins that act primarily as cytoplasmic scaffolds that support cell structure (Coulombe and Omary 2002). Bacteria, including GBS, can utilize keratin for binding, colonization, and invasion (Tamura and Nittayajarn 2000). This has implications for infection of highly keratinized epithelial sites such as the respiratory and vaginal epithelia (Tamura and Nittayajarn 2000). Using a lung-derived epithelial cell line (A549), Tamura and Nittayajarn (2000) reported GBS bind to two forms of cytokeratin 8 (CK8) via cell surface proteins. GBS surface adhesins support binding to vaginal epithelial cells, extracellular matrix proteins, and interactions with other members of the vaginal microbiota (Patras and Nizet 2018); for example, GBS binds to *Candida albicans* in an interaction facilitated by the surface anchored BspA protein, which also assists in epithelial cell adherence (Rego et al. 2016). The most prominent GBS surface adhesins, namely pili and afimbrial adhesins are discussed below.

Pili

Pili were recognized in GBS in 2005 during screening of genomes for surface-exposed proteins as possible vaccine antigens (Lauer et al. 2005). Pili support adhesion and attachment of bacteria to host cells by promoting initial association with host cells (Telford et al. 2006). Specifically in GBS, pili mediate adhesion and invasion of host pulmonary cells, and epithelial cells including lung, cervical, and brain microvascular epithelial cells, and also play a role in the formation of bacterial aggregates and biofilms (Konto-Ghiorghi et al. 2009, Krishnan et al. 2007, Maisey et al. 2007, Pezzicoli et al. 2008, Sharma et al. 2013). GBS pili are multimeric structures consisting of three pilin proteins, (i) a PilA subunit at the tip of each pilus, (ii) PilB subunits that make up most of the pilus backbone, and (iii) a PilC subunit at the base (Dramsi et al. 2006, Maisey et al. 2008a, Rosini et al. 2006).

Three types of pili have been identified in GBS, namely pilus type 1, 2a, and 2b, with each GBS strain carrying one or two types (Rinaudo et al. 2010). The genes involved in the synthesis and assembly of these three GBS pili are clustered in characteristic genomic loci (termed PI-1, PI-2a, and PI-2b). PI-1 is coded in a pathogenicity island, while the PI-2 locus is part of the stable bacterial genome (Rosini et al. 2006). In general, pili expression by GBS is accepted to augment virulence based on observations of adhesion capability and evasion of host defence. For example, GBS mutants lacking PilA and PilC are attenuated for adherence to epithelial cells (Dramsi et al. 2006). Banerjee et al. (2011) reported further roles for PilA in inducing polymorphonuclear leukocyte (PMNL, neutrophils) recruitment and increasing permeability of the blood–brain barrier creating an opportunity for GBS invasion and leading to higher GBS loads in the brain. GBS lacking PilB are attenuated, more susceptible to phagocytes, and more readily killed by antimicrobial peptides (Maisey et al. 2007, b). PI-2b augments GBS adhesion to and invasion of brain endothelial cells (Lazzarin et al. 2017) but was also found to increase phagocytosis by macrophages (Perichon et al. 2019). Fine-tuning of PI-2b expression by GBS *in vivo*, perhaps divergently by hypervirulent lineages (Perichon et al. 2017), might confer a selective advantage by optimizing adherence to host tissues or mitigating immune responses (Perichon et al. 2019).

Afimbrial adhesins

Several GBS afimbrial adhesins mediate bacterial–host receptor interactions (Lindahl et al. 2005) and are involved in attachment to host cells and/or the extracellular matrix (Cheng et al. 2002). Genome sequencing of GBS strain 515, a clinical isolate from an infected neonate, identified a cell wall-anchored protein encoded by *sal0825* (Tettelin et al. 2005), which is one of seven surface proteins conserved across GBS strains (Rosinski-Chupin et al. 2013). The protein contains a typical N-terminal signal peptide and C-terminal LPXTG sorting signal and is involved in mediating attachment to human cells or extracellular matrix components (Jiang and Wessels 2014). This protein was renamed BsaB (Bacterial surface adhesion of GBS), with functional analysis revealing it mediates biofilm formation, and binding to human fibronectin and laminin, and human vaginal (VK2) and cervical epithelial cells (ME-180) (Jiang and Wessels 2014). ScpB is another GBS surface protein that mediates binding to fibronectin (Beckmann et al. 2002, Cheng et al. 2002). FbsA (a cell wall-anchored protein) and FbsB (a secreted protein) are two structurally unrelated proteins that can bind to fibrinogen *in vitro*; expression of both proteins was associated with GBS binding interactions (Al Safadi et al. 2011, Schubert et al. 2002). FbsA was associated with adhesion to epithelial cells, for which FbsB is required (Gutekunst et al. 2004).

Srr-1 and Srr-2 are LPXTG glycoproteins that have also been reported to contribute to fibrinogen binding (Seo et al. 2013). Srr-1 mediates invasion of brain vascular endothelial cells and

translocation through the blood–brain barrier (Seo et al. 2012, van Sorge et al. 2009). Samen et al. (2007) characterized a 157-amino acid binding region of Srr-1 that targets a 255-amino acid region of human keratin 4 (K4). Samen et al. (2007) further reported the participation of GBS Srr-1 in keratin binding to larynx epithelial (HEp-2) cells. More recently, a fibrinogen-binding region was also identified in Srr proteins (Seo et al. 2012, 2013). Further investigation across multiple GBS serotypes found that corresponding Srr-1 mutant strains exhibited a significant decrease in their capacity to bind to fibrinogen as well as a 50% reduction in adhesion to cervical and vaginal epithelial cells compared to the parental wild-type (WT) strains (Wang et al. 2014); i.e. implicating Srr-1 in vaginal colonization. Srr-1 has been further implicated in both attachment to and invasion of brain endothelial tissue; thereby facilitating meningitis (Seo et al. 2012, van Sorge et al. 2009). Both Srr-1 and Srr-2 proteins have a demonstrated binding affinity to fibrinogen, however, Srr-2 binds to fibrinogen with significantly greater affinity (Seo et al. 2013). It is, therefore likely that the expression of Srr-2 contributes to the increased virulence of ST-17 strains of GBS; resulting from elevated levels of fibrinogen binding observed when compared against the fibrinogen-binding affinity of their non-ST-17, Srr-1 expressing, counterparts (Dramsi et al. 2012, Seo et al. 2013). A more extensive review of GBS afimbrial adhesins that mediate binding to host extracellular matrix and cell surfaces, invasion of and survival within host cells, and that neutralize phagocytosis and/or modulate the immune response is provided elsewhere (Pietrocola et al. 2018).

β-haemolysin and pigment

The pore-forming β-hemolysin/cytolysin (β-h/c) toxin (also termed 'hemolytic pigment toxin') produced by most human GBS strains is a key virulence factor (Rosa-Fraile et al. 2014) and supports pathogenicity in several *in vitro* and *in vivo* models (Doran et al. 2003, Ring et al. 2002, Whidbey et al. 2013). GBS β-h/c promotes invasion of human epithelial and endothelial cells of the lung and the blood–brain barrier, and the release of proinflammatory cytokines (Doran et al. 2002, 2003, Gibson et al. 1999). *In vivo* studies have shown that β-h/c contributes to the development of meningitis, pneumonia, arthritis, and sepsis (Doran et al. 2003, Nizet et al. 1996, Puliti et al. 2000, Ring et al. 2002). In the urinary tract, GBS β-h/c confers virulence by mediating cytotoxicity, cytokine synthesis and inflammation (Leclercq et al. 2016). In the FRT, GBS lacking β-h/c are attenuated for vaginal colonization in mice (Carey et al. 2014). Finally, hyperhemolytic isolates of GBS recovered from a patient with invasive disseminated infection were associated with hyperinflammation and disruption of the coagulation cascade (Siemens et al. 2020).

The inflammatory nature of GBS β-h/c in support of virulence in various models is best considered alongside divergent findings that the toxin is immune modulatory and even suppressive of host responses in some circumstances. For example, in a murine model of ascending infection, a transcriptomic profile of immune cells suggests that β-h/c-producing GBS modulates placental immune cell phenotypes through suppression of the host inflammatory signalling pathway (Kuperwaser et al. 2023). Similarly, in a murine model, survival of GBS was linked to phagocytic cytolysis and apoptosis induced by β-h/c, disrupting host defence (Liu et al. 2004). Hensler et. al. (2008) showed that GBS β-h/c can impair cardiomyocyte viability, which might promote cardiac dysfunction in neonatal GBS disease. Together, these observations on the nature of GBS β-h/c show that it has both cytotoxic and immunomodulatory properties that contribute to virulence and favour survival of GBS (Rosa-Fraile et al. 2014).

GBS produces an ornithine rhamno-polyene called granadaene, which contains a chain of 12 conjugated carbon–carbon double bonds (Spellerberg et al. 1999) that gives rise to a characteristic red pigment produced by GBS under certain conditions. The genes present in the *cyl* operon are responsible for β-h/c expression in GBS (Spellerberg et al. 1999) as well as pigment production (Spellerberg et al. 2000). Granadaene biosynthesis closely resembles the fatty acid biosynthesis pathway, and the *cyl* operon contains many genes that are homologous to enzymes involved in fatty acid biosynthesis (Rosa-Fraile et al. 2014). Pritzlaff et al. (2001) showed that deletion of the *cylE* gene resulted in loss of haemolytic activity that was restored through complementation (Pritzlaff et al. 2001). However, Whidbey et al. (2013) showed that although *cylE* is necessary for haemolytic activity it is not sufficient for the haemolytic phenotype of GBS (Whidbey et al. 2013). The same study also assessed haemolytic activity of the purified GBS pigment, granadaene, observing RBC lysis that was directly attributed to granadaene alone. Proteolytic treatment with proteinase K had no effect on haemolytic activity of the pigment, suggesting that haemolysis was not due to a protein toxin. Together, this work provides strong evidence to show that the haemolytic molecule in GBS is the granadaene pigment, rather than a protein that is encoded by the *cyl* operon. GBS also produces membrane vesicles (MVs) that carry both β-h/c and functionally active granadaene (Armistead et al. 2021). MVs from hyperhaemolytic GBS cause more cell lysis than MVs from nonhaemolytic GBS and were shown to contribute to pathogenesis in mice (Armistead et al. 2021). Another study showed that GBS MVs can incite inflammation in the murine FRT leading to chorioamnionitis and preterm births (Surve et al. 2016). Strain-dependent variation in MV production among GBS suggests that MVs may have lineage-specific functions relating to virulence (Rosa-Fraile et al. 2014).

Sialic acid (Sia)-rich CPS

GBS express CPS with varying repeat unit structures, which acts as a major virulence factor to evade host immune defences (Le Doare and Heath 2013). GBS is grouped serologically into 10 distinct serotypes Ia, Ib, and II–IX according to the composition of CPS (Lindahl et al. 2005, Slotved et al. 2007). Each GBS CPS serotype is antigenically and structurally unique (Le Doare and Heath 2013). GBS CPS consists of high-molecular weight polymers with repeating units composed of glucose, galactose, *N*-acetylglucosamine, and Sia (Paoletti and Kasper 2019). Sialic acid exists in vertebrates as a family of nine-carbon, monosaccharide molecules linked to the outer glycan chains of all cells (Varki 2008). In GBS, Sia on the glycosyl portion of the CPS effects cell–cell interactions, pathogenicity, and immune response (Wessels et al. 1989). For GBS CPS, no role in defence against the antimicrobial effects of ROS, RNS, or metal ions has been defined in contrast to *Streptococcus pneumonia* for which CPS confers resistance to killing by oxidative stress (Brissac et al. 2021). ROS/RNS attack can cause fragmentation of polysaccharide that could presumably alter the function of bacterial CPS (Duan and Kasper 2011).

Epidemiologically, GBS disease is predominated by certain CPS serotypes. Prior to characterization of serotypes IV and V in the mid-1980s, acute disease was represented mostly by serotypes Ia, Ib, II, and III (Jelinkova and Motlova 1985); serotypes Ia and III in particular were consistently overrepresented (Edwards et al. 2011). By 1990, a growing proportion of other serotypes were identified as contributing to the overall disease burden in the US

(Wenger et al. 1990). Serotype V was reported as the most common associated with infection in nonpregnant adults (Navarro-Torne et al. 2021), and serotypes Ia, Ib, II, III, and V were reported as prevalent in the FRT and perianal region of pregnant women, and causal of most invasive human diseases (Weisner et al. 2004). Subsequently, serotype III was reported as most overrepresented among cases of disease in neonates (Weisner et al. 2004) and acute UTI in adults (Ulett et al. 2009). Consistent with a growing recognition of serotype IV in GBS disease, Diedrick et al. (2010) reported an increasing incidence of colonizing serotype IV. Six serotypes (I–V) now account for 98% of GBS isolates colonizing pregnant women and 99% of neonatal cases, as reviewed elsewhere (Furuta et al. 2022).

GBS evasion of host defences

Virulence regulators, host cell mimicry, and evading host antimicrobials

GBS subverts host immune responses using a multitude of mechanisms. Many of these are governed by two-component signal transduction systems that regulate numerous factors contributing to pathogenesis (Thomas and Cook 2020). The best studied two-component system (TCS) of GBS is the CovR/CovS (alternate designation: CsrR/CsrS) transcriptional TCS that regulates ∼7% of the bacterial genome, especially during adaptation to a host (Jiang et al. 2008, Lamy et al. 2004). CovR is a co-ordinator of GBS–host interactions and the CovR-regulon is subjected to host selective pressure (Mazzuoli et al. 2021). Plasticity in CovR-signalling interactions drives intraspecies diversity in the population, ultimately leading to emergence of host-adapted strains with specific pathologies in different niches (Mazzuoli et al. 2021). GBS strains with a *covR* deletion can trigger increased proinflammatory cytokine responses compared to WT strains (Cumley et al. 2012) suggesting that within *covR*/*covS* regulons, there is controlled gene expression to fine-tune host-modulating factors (Lembo et al. 2010). Supporting this is the observation that *covR* deficiency enhances the early production of many cytokines in response to GBS in human uroepithelial cells (Sullivan et al. 2017). Additional to CovR/CovS are more than twenty distinct GBS TCSs that facilitate responses to stressful host environments in ways that are essential to supporting bacterial pathogenicity, as reviewed elsewhere (Thomas and Cook 2020).

Another mechanism GBS use to escape immune responses is via host cell mimicry. The Sia component of the GBS CPS is also abundant in mammalian respiratory, intestinal, and vaginal epithelial cells. Sia-binding Ig-like lectins (Siglecs) are regulatory receptors displayed on host immune cells that recognize GBS Sia as 'self', hence limiting immune activation. GBS Sia binds to host inhibitory Siglecs, resulting in multiple immune modulations. One example is GBS Sia-mediated resistance to platelet responses by inhibiting platelet activation and platelet-mediated antimicrobial activity (Uchiyama et al. 2019). Additionally, interplay between GBS Sia and Siglec-E results in immune evasion by suppressing the release of proinflammatory cytokines and PMNL infiltration, reducing phagocytosis *in vivo* (Chang et al. 2014). Similarly, the interaction of GBS Sia and Siglec-9 reduced PMNL-mediated oxidative burst activity, and decreased the formation of PMNL extracellular DNA traps that contribute to killing of the bacteria (Carlin et al. 2009).

Several other factors also contribute to GBS evasion of host immune responses. For example, biofilm regulatory protein A (BrpA) is a virulence regulator that governs how the bacteria respond to external environmental stress, and plays a role in the ability of GBS to escape PMNL-mediated killing (Patras et al. 2018). Similarly, the GBS immunogenic bacterial adhesin BibA, a highly conserved surface-localized protein that is regulated by CovR/CovS, confers resistance to opsonophagocytic killing (Santi et al. 2009, Dobrut and Brzychczy-Wloch 2021, Santi et al. 2007). A study of WT GBS and *bibA*-deficient GBS showed that BibA is critical for attachment to cervical and lung epithelial cells (Santi et al. 2007). GBS also produces the ectonucleotidase CdnP, which degrades extracellular cyclic-di-AMP and prevents bacterial clearance via the cyclic GMP-AMP synthase (cGAS) and stimulator of interferon genes (STING) pathway (Andrade et al. 2016).

GBS possess several mechanisms to manipulate and overcome immune sensing pathways, including the complement cascade. The complement protein network aids in the clearance of bacteria via activating phagocytic cells or direct killing by forming membrane attack complex (MAC) pores in the bacterial cell wall (Heesterbeek et al. 2018). Recently, a polypeptide complement-interfering protein was described in GBS that binds to the C4b component of the complement cascade, blocking C3b deposition, ultimately resulting in disruption of MAC formation (Giussani et al. 2019, Pietrocola et al. 2016). Another strategy used by GBS to evade complement is manipulating host complement regulators, including factor H (Moore et al. 2021). Sia and a surface β-protein (also called Bac) of GBS can interact with factor H to degrade C3b, disrupting the alternative complement pathway (Maruvada et al. 2009, Xu et al. 2022).

Antimicrobial peptides (AMPs) are vital in innate immune responses to infection and exhibit broad spectrum antibacterial activity. AMPs include cathelicidins, indolicidins, defensins, lactoferrins, and lysozyme (*N*-acetylmuramide glycanhydrolase) (Assoni et al. 2020). Some bacteria can rapidly develop resistance to AMPs (Andersson et al. 2016) and several GBS factors, including TCSs can confer and regulate resistance to AMPs. One recognized mechanism is via DltR/S that modifies GBS cell wall integrity via the incorporation of cationic D-alanyl residues to anionic teichoic acid components that promote resistance to cationic AMPs (Saar-Dover et al. 2012). Similarly, the TCS LiaR/S downregulates the expression of genes involved in cell wall synthesis and cell membrane modification (Klinzing et al. 2013). A mutation in the TCS CiaR/H also reduces susceptibility to cathelicidins (Quach et al. 2009). Finally, PilB of GBS pili prevent the interaction of cathelicidins with membrane targets (Maisey et al. 2008b), and the surface-associated penicillin binding protein a (PBP1a), required for cell viability, is likely to confer resistance to cathelicidins and defensins (Hamilton et al. 2006, Zhu et al. 2021).

There are several virulence factors of GBS that disrupt the signalling cascades that underpin host immune responses that drive inflammatory activation aimed at limiting infection. Hyaluronidase (HylB) for example, mediates host immune evasion in uterine tissue (Vornhagen et al. 2016) and dampens inflammatory cascades by degrading proinflammatory hyaluronan fragments that are released during tissue injury. The degraded fragments bind to host toll-like receptor 2 (TLR2) and TLR4 to initiate a weak inflammation cascade and block GBS recognition by receptors to disrupt host immune responses (Kolar et al. 2015, Kurian and Modi 2022). Expression of HylB by GBS also limits ROS production and confers resistance to PMNL-mediated killing (Coleman et al. 2021). Finally, GBS can thwart the host plasminogen system (also known as the fibrinolytic system) to promote dissemination. GBS NEM316 binds to human plasminogen and enhances host-derived proteolytic activity, which degrades surface adhesive

proteins and the extracellular matrix, promoting GBS dissemination (Magalhaes et al. 2013).

GBS subversion of phagocytes and responses to cellular stress

Phagocytes are the primary host responders to invasion by pathogens and therefore major determinants of killing of GBS by immune defences. Among phagocytic cells PMNLs and macrophages are considered imperative for defence against GBS (Gres et al. 2019). PMNLs provide rapid responses to sites of inflammation (de Oliveira et al. 2016, Kolaczkowska and Kubes 2013) while macrophages contribute to recognition of GBS (Henneke and Berner 2006) and initiation, amplification, and termination of immune responses (Ravi et al. 2023). Phagocytic recognition, engulfment, and killing of bacteria encompasses varied molecules, including extracellular DNA, cytotoxic granules enriched with antimicrobials (e.g. cationic peptides, proteases, and lactoferrin), ROS and RNS (Bogdan et al. 2000, Kumar and Sharma 2010, Mantovani et al. 2011, Sadik et al. 2011) as well as metal ions. Phagocytes kill bacteria by releasing ROS in the respiratory burst, but they also secrete RNS, including nitric oxide ($^{\bullet}$NO) generated from inducible nitric oxide synthase (iNOS). Bacteria can be exposed to ROS and/or $^{\bullet}$NO inside the phagosome and killed through the generation of oxidative and/or nitroxidative∗ activity (Nakatsuka et al. 2023) (∗term per; Lancaster 2006). $^{\bullet}$NO also has the potential of being converted to other antimicrobial RNS, such as nitrate or nitrite. Three enzymatic antimicrobial systems in phagocytes, namely nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, myeloperoxidase (MPO), and iNOS generate diverse reactive species, such as hypochlorous acid (HOCl), superoxide ($O_2^{-\bullet}$), H_2O_2, and $^{\bullet}$NO; the latter can react with $O_2^{-\bullet}$ to generate another phagocytic RNS, peroxynitrite ($ONOO^-$), which is a potent antimicrobial agent (Al-Shehri 2021). Below, we present a summary of what is known for GBS in relation to some of these phagocytic interactions.

PMNLs release fibres of nucleic acids into the extracellular environment in the form of neutrophil extracellular traps (NETs) that adhere to and immobilize pathogens for phagocytosis (Brinkmann et al. 2004). Some bacteria, including GBS, express nucleases that degrade nucleic acids by cleaving nucleotide chains at their phosphodiester bonds as a counter-measure to this host defence. Ferrieri et al. (1980) first reported these enzymes in GBS in 1980. Derre-Bobillot et al. (2013) demonstrated the use of a GBS nuclease (Nuclease A or NucA) in the degradation and evasion of NETs; and a role of NucA in persistent GBS infection. Bonsor et al. (2008) proposed that nucleases entering cells to degrade intracellular nucleotides such as RNA and DNA may also be a virulence strategy. Carey et al. (2014) reports PMNLs as primary responders to GBS β-h/c in a murine model, suggesting NETs induced by β-h/c cause destruction of PMNLs. Supporting this hypothesis, comparing WT and *cylE*-deficient GBS in macrophage and PMNL killing assays, Liu et al. (2004) found that strains lacking β-h/c were more readily cleared by the immune cells, than WT strains expressing β-h/c. Additionally, Boldenow et. al. (2016) showed GBS β-h/c induces PMNL death and subverts killing of GBS by NETs. In contrast, another study found that deletion of the *cylE* gene in a different GBS background did not significantly affect uptake by macrophages compared to the WT strain (Cumley et al. 2012); however, deletion of *covR/S* drastically reduced the ability of the mutant to survive within macrophages. As the CovR/S TCS controls many factors that modulate host factors (as highlighted in the section *'Virulence regulators, host cell mimicry, and evading host antimicrobials'*), the authors concluded that the reduced survival could be due to the macrophages being more activated in the absence of CovR/S-dependent responses (Cumley et al. 2012).

Sialic acid in GBS CPS enables the bacteria to mimic host cells, as previously mentioned, camouflaging the bacteria from host defences (Carlin et al. 2009). For example, CPS residues can bind to PMNL Siglec-9, dampening antibacterial activity (Carlin et al. 2009). Sialic acids also hinder the attachment of Complement 3 Proteins to the bacterial surface, impairing the function of phagocytes as well as the downstream, cytolytic activity of the complement cascade (Marques et al. 1992). Complement Protein 5a (C5a) recruits PMNLs to sites of infection, inducing opsonophagocytic destruction of pathogens (Manthey et al. 2009). GBS expresses the cell envelope protease ScpB (C5a peptidase), which cleaves C5a, thereby dampening PMNL recruitment to sites of infection (Bohnsack et al. 1997, McKenna et al. 2022, Takahashi et al. 1995).

Immune activity in response to infectious stimuli (e.g. recruitment of PMNLs, monocytes to site of infection) involves cytokine responses with Interleukin 6 (IL-6) a predominant proinflammatory mediator (Scheller et al. 2011). IL-6 is made by monocytes infected with Gram-positive bacteria, including GBS (Vallejo et al. 1996) with overproduction of IL-6 potentially causing GBS-induced lung injury (Raykova et al. 2003). Another downstream effect of certain cytokine responses induced by GBS is production of triggers of cell death. For example, GBS induces production of tumor necrosis factor-related apoptosis-inducing ligand in monocytes leading to apoptosis that undermines monocyte-mediated bacterial clearance (Halaas et al. 2004).

The ability to survive the intracellular environment of phagocytes adds to the survival capacity of GBS in the host. Valenti-Weigand et al. (1996) showed that GBS survive in macrophages for 24 h; Cornacchione et al. (1998). GBS survival in murine macrophages for 48 h; attributing this survival to the ability of GBS to impair protein kinase C (PKC), a component in the activation pathway of interferon-γ (IFN-γ). Whilst β-h/c is necessary to induce cytoskeleton alterations that disrupt macrophage activity (Fettucciari et al. 2011), Sagar et al. (2013) found that nonhaemolytic, mutant strains of GBS survived in human THP-1 macrophage-like cells more readily than their haemolytic WT strains through reduced stimulation of TNF-α, indicating the need for GBS to regulate β-h/c expression to promote intracellular survival. Regulation of acidification of the phagosome by GBS CovR/S TCS appears to support survival in phagocytes (Cumley et al. 2012).

Growing biofilms to withstand the effects of stressors in the host

Biofilm formation supports bacterial survival in stress conditions such as from nutrient depravation, pH changes, or ROS (Jefferson 2004), as encountered in host antimicrobial immune responses. GBS can form biofilms of multilayered cell aggregates in a thick adhesive matrix, which can protect from killing by immune cells and promote colonization. The environmental factors that regulate development of biofilms by GBS are not well-defined but include an effect of pH and nutrient-limitation, with acidic conditions and nutrient-limitation generally favouring biofilm formation (D'Urzo et al. 2014, Desai et al. 2021, Ho et al. 2013). Others have shown higher biofilm formation at more neutral rather than acidic conditions (Borges et al. 2012, Yang et al. 2012). GBS Biofilm Regulatory Protein A (BrpA) (Patras et al. 2018) supports biofilm formation along with pilus expression with the type of pilus seemingly important (PI-2a more associated with biofilms than a

combination of PI-2a and PI-1) (Alvim et al. 2019). Finally, the clinical and genetic background of GBS isolates has been linked to distinct biofilm forming phenotypes noting discrepancy between studies. In one study, GBS strains from invasive disease cases and/or belonging to the ST-17 and ST-19 lineages were more likely to form weak biofilms, compared to strains producing strong biofilms that were recovered more frequently from individuals with asymptomatic colonization (Parker et al. 2016). In contrast, an earlier study reported that ST-17 GBS strains produce more biofilm than non-ST-17 strains (D'Urzo et al. 2014). The contribution of biofilm formation to supporting GBS survival in stress conditions from ROS, RNS and metal ions are untested.

GBS subterfuge of killing by ROS, RNS, and metal ion imbalance

Phagocytosed bacteria encounter a range of reactive species, including ROS, RNS, and metal ion mobilization responses that are generated by the phagocytic cell as part of host defence. As host-derived reactive species, ROS and RNS can directly damage essential bacterial proteins and other cell components and consequently impair bacterial metabolism and cell growth/survival (Ezraty et al. 2017, Flannagan et al. 2009). Rather than direct damage to the bacterial cell, host metal ion mobilization responses can cause imbalance in bacterial metal homeostasis that can lead to mismetalatation (and thereby dysfunction) of proteins and in the case of Fe, trigger Fenton chemistry (where electron transfer from ferrous iron to hydrogen peroxide (H_2O_2), generates a hydroxyl radical as an oxidizing product) (Imlay 2008). GBS has evolved multiple mechanisms to subvert killing by these reactive species. In this way, GBS is endowed with systems that not only enable manipulation of host immune responses (described above) but also allow the bacteria to resist antibacterial host defences mediated by ROS, RNS, and metal ions.

ROS

ROS are activated derivatives of molecular oxygen, and include singlet oxygen ($^1O_2$), superoxide anion radical (O_2^-), H_2O_2, hydroxyl radical ($^\bullet OH$), hypohalous acids, and peroxynitrite (Imlay 2008). Among nonradical ($^1O_2$, H_2O_2) and radical (O_2^-, $^\bullet OH$) species, superoxide and hydroxyl radicals are the major ROS in living organisms with the latter being regarded as the most biologically active free radical (Lipinski 2011). ROS-driven cytotoxicity in bacteria stems from metabolic insufficiency due to inactivation of enzymes and crippling DNA damage (Imlay 2008). In bacteria, specialized enzymes offer central defence against oxidative stress; catalases detoxify (reduce) H_2O_2 by catalysing its decomposition to O_2 and H_2O (Yuan et al. 2021), whereas superoxide dismutases (SODs) convert superoxide anions into H_2O_2 and oxygen (Nobrega and Pauleta 2019). Bacteria use distinct mechanisms to detect and respond to discrete forms of ROS, which makes sense because stress from different ROS (such as O_2^- and H_2O_2) does not always occur simultaneously, and these ROS differ in their biochemical actions (Imlay 2008). Other specialized enzymes also detoxify the bacterial cell from ROS, including peroxidases (Barreiro et al. 2023). Nonenzymatic strategies based on chemical scavengers to detoxify H_2O_2 also exist in some bacteria (Capek and Vecerek 2023, Kim et al. 2016).

GBS does not express catalase; nonetheless it can tolerate high levels of oxidative stress, survive exposure to high levels of H_2O_2, and inhibit the ROS burst in macrophages (Cumley et al. 2012, Korir et al. 2017, 2018, Poyart et al. 2001). This illustrates the centrality of strategies independent of catalase in GBS to detoxify the cells from ROS. One factor that confers GBS resistance to oxidative stress is SOD, encoded by *sodA* (Poyart et al. 2001). Poyart et al. (2001) observed that a GBS *sodA* mutant was less able to survive and grow compared to WT when incubated with either 20 mM H_2O_2 or paraquat (generates ROS); the enzymatic activity of SodA that is protective in these assays is likely to be indirect from the SOD acting on superoxide generated from the paraquat. Increased susceptibility of *sodA*-deficient GBS to killing by macrophages as compared to the WT suggests that *sodA* enables GBS to survive in the phagosome despite production of ROS or oxidative burst activity triggered by phagocytosis.

Manganese (Mn) is an essential cofactor for SodA, and a connection between Mn and resistance to oxidative stress exists in many bacteria (Horsburgh et al. 2002, Jakubovics et al. 2002, Martin et al. 1984). This is also the case for GBS; Shabayek et al. (2016) describes the involvement of the iron (Fe)/Mn transporter MntH in the response of GBS to ROS (Shabayek et al. 2016). They found that a GBS *mntH* mutant was attenuated for adherence, survival in acidic environments and in macrophages, and was more sensitive to killing by H_2O_2. Supplementation with Mn restored survival of the *mntH* mutant to WT levels during initial stages of exposure to H_2O_2. Similar observations have been reported for MntH homologues in Group A *Streptococcus* (Janulczyk et al. 2003) and *Escherichia coli* (Kehres et al. 2000), where mutation of these genes causes increased susceptibility to killing by H_2O_2. In Gram-positive bacteria that have a higher requirement for Mn (Capek and Vecerek 2023), Mn can also mitigate oxidative stress by chemically scavenging superoxide and/or H_2O_2 (Culotta and Daly 2013, Horsburgh et al. 2002). *In S. pneumoniae*, for example, Mn provides extra protection against oxidative stress, supplemental to the primary source of protection provided by SOD (Eijkelkamp et al. 2014). Additionally, Mn can directly protect *Staphylococcus aureus* from ROS in the absence of SOD (Karavolos et al. 2003) and high Mn concentrations are thought to protect lactobacilli from ROS (Bosma et al. 2021). It is likely that in 'Mn-philic' organisms, high levels of cytoplasmic Mn (up to 35 mM in lactobacilli) provide protection against ROS (Bosma et al. 2021, Capek and Vecerek 2023). In distantly related bacteria, such as *E. coli*, the beneficial effects of Mn correlate with its ability to cofactor enzymes where Mn does not protect peroxide-stressed cells by scavenging peroxide (Anjem et al. 2009). A simplified view of the role Mn and its transporters in resisting oxidative stress is illustrated in Fig. 1.

Another enzyme that contributes to GBS resistance to H_2O_2 and inhibition of the ROS burst in macrophages is the putative NADH peroxidase Npx. Korir et al. (2018) found that expression of *npx* is highly upregulated during intracellular survival within a macrophage and plays an important role in reducing ROS production in the phagocytic cells; moreover, a *npx* mutant was more susceptible to killing by H_2O_2 as compared to WT (Korir et al. 2018). Nox-2, another GBS NADH oxidase, also confers resistance to oxidative stress. Yamamoto et al. (2006) found that a *nox2*-deficient mutant was significantly more sensitive to ROS generated by paraquat as compared to WT GBS and suggested this was due a defect in fatty acid synthesis and altered cell membrane (Yamamoto et al. 2006). Additionally, the GBS genome encodes two other putative peroxidases: a thiol peroxidase (*tpx*) and an alkyl hydroperoxide reductase (*ahpC*), both which are uncharacterized but could contribute to ROS detoxification (Glaser et al. 2002). Interestingly, Korir et al. (2018) found that a *npx*-deficient mutant expressed higher levels of *tpx* relative to WT, suggesting that *tpx* may be upregulated to compensate for the loss of Npx function following exposure to H_2O_2 (Korir et al. 2018).

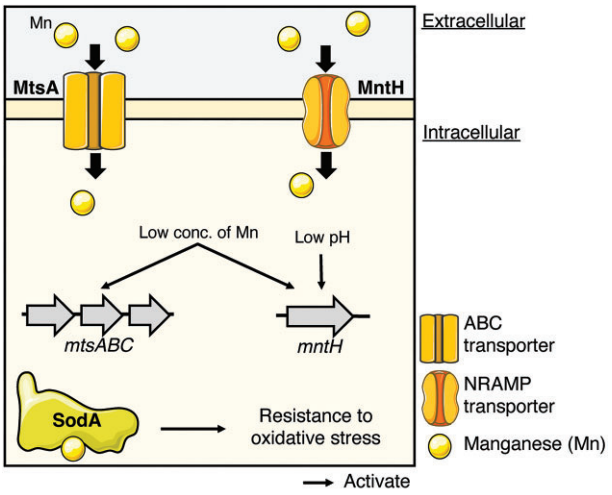

Figure 1. Examples of oxidative stress resistance mechanisms in GBS. Expression of *mtsABC* (ABC transporter) and *mntH* (NRAMP-family transporter) are upregulated during Mn limitation; additionally, *mntH* is also upregulated at low pH. Both transporters import extracellular Mn into the cell, where it acts as a cofactor to SOD (SodA), the primary pathway for GBS to detoxify ROS (Bray et al. 2009, Burcham et al. 2022, Shabayek et al. 2016).

Besides enzymatic processes, GBS also employ other factors to resist killing by ROS. Glutahione (GSH) is an antioxidant that is analogous in all kingdoms of life and plays a role in protection against ROS (Fahey 2013). GBS produce three times more GSH when grown under oxidative conditions compared to when the bacterium is grown under reducing conditions, synthesizing it using a bifunctional enzyme encoded by *gshAB* (Janowiak and Griffith 2005). In order to determine the impact of GSH synthesis on the sensitivity to ROS, Walker et al. (2019) generated *gshAB* mutants in three different GBS strains and found all the mutants were more sensitive to killing by ROS as compared to WT. Additionally, they showed that the three GBS strains used produced varying amounts of GSH, with their data suggesting that this variation was not due to differences in the *gshAB* promoter region or amino acid sequence, but rather due to the involvement of other regulatory elements. Interestingly, they observed an inverse relationship between pigment and GSH production, with a highly pigmented strain producing the least amount of GSH, and vice versa. As the pigment is also a potent antioxidant that facilitates neutralization of ROS, Walker et al. (2019) hypothesized that highly pigmented strains may not require large quantities of other antioxidants like GSH to resist ROS (Walker et al. 2019). Subsequently, they showed that the GBS strains lacking *gshAB* were significantly less virulent in a mouse model of sepsis compared to the WT.

As highlighted in the section '*β-haemolysin and pigment*' the GBS orange carotenoid pigment granadaene, produced from *cylE* that also encodes for the pore-forming β-h/c, protects GBS from killing by ROS (Spellerberg et al. 2000). Liu et al. (2004) found that a GBS *cylE* mutant was more sensitive to killing by H_2O_2, hypochlorite, superoxide, and singlet oxygen, compared to the WT; addition of filtered pigment extract from the WT strain also rescued singlet oxygen-mediated killing of the *cylE* mutant. The conjugated double bonds in the polyene backbone of carotenoids influence their ability to act as antioxidants (Stahl and Sies 2003). The similarity of conjugated double bonds of granadaene to other traditional carotenoids provides it with the ability to detoxify ROS (Jusuf et al. 2021). Jusuf et al. (2021) observed that photobleaching the granadaene pigment breaks up the linear chain of conjugated carbon–carbon bonds in the pigment (Jusuf et al. 2021). They also showed that photobleaching reduced haemolytic activity by 40.5%, confirming that the granadaene pigment plays a role in GBS haemolytic activity. Photobleaching WT GBS reduced antioxidant activity and the ability of GBS to resist oxidative stress, which was not seen when a *cylE* mutant strain was photobleached.

GBS MVs that carry β-h/c and functionally active granadaene can also inhibit the production of ROS by PMNLs and macrophages, enabling escape of oxidative killing (Armistead et al. 2021). The protective effect afforded by MV-derived β-h/c and pigment towards oxidative killing of GBS is depicted in Fig. 2. Collectively, therefore, GBS is equipped to neutralize distinct ROS using an array of defence mechanisms that involve SodA, Mn, Npx, Nox-2, β-h/c, GSH, and probably additional yet-to-be-identified factors (Landwehr-Kenzel and Henneke 2014).

RNS

Nitric oxide ($^{\bullet}$NO) and other RNS derived from arginine have been a focus of bacterial pathogenesis studies, since the gene encoding for the synthesis of $^{\bullet}$NO in response to environmental stimuli, *iNOS*, first appeared in host response to infection studies between 1985 and 1990, as reviewed elsewhere (Ulett and Potter 2011). Despite this, no GBS genes characterized to date are known to contribute to resistance towards RNS or $^{\bullet}$NO stress. Here, we will highlight studies that have explored GBS-mediated induction of RNS and $^{\bullet}$NO.

In mammals, the inducible isoform of $^{\bullet}$NO synthase (*iNOS*) is expressed in a wide range of cells and produces the largest amount of $^{\bullet}$NO during sepsis and inflammation, as reviewed elsewhere (Wong and Billiar 1995). Goodrum and Poulson-Dunlap found that respiratory epithelial cells would only induce *iNOS* expression and secrete $^{\bullet}$NO when cocultured with GBS-infected Human peripheral blood mononuclear cells (PMBCs) or treated with the supernatant of separately cultured GBS-infected PMBCs and attributed this to cytokines released by the infected cells (Goodrum and Poulson-Dunlap 20024). Additionally, GBS uptake by macrophages is dependent on complement receptor type 3 (CR3, CD11b/CD18) with GBS inducing $^{\bullet}$NO production in murine macrophages in a CR3-dependent manner (Goodrum et al. 1994). Leib et al. (1998) infected mice with GBS intranasally and found a dramatic increase in *iNOS* expression in alveolar macrophages (Leib et al. 1998). Similarly, Glibetic et al. (2001) examined *iNOS* expression in response to GBS by treating primary cultures of newborn piglet cerebral microvascular endothelial cells (CMVL) with heat-killed GBS. The authors found that *iNOS* mRNA was significantly induced 24 h after the addition of GBS, and iNOS protein was expressed in a time-dependent manner.

A study performed by Ulett and Adderson found a rapid increase in $^{\bullet}$NO production in GBS-infected murine macrophages postinfection (Ulett and Adderson 2005). Killing of intracellular GBS was unaffected when $^{\bullet}$NO was neutralized by the addition of NG-monomethyl-L-arginine (L-NMMA), an inhibitor of nitric oxide synthase (Semba et al. 1995). Using *S*-nitroso-*N*-acetylpenicillamine (SNAP), a nitrosothiol derivative that releases $^{\bullet}$NO under physiological conditions, they also found SNAP did not cause any significant impairment towards growth of GBS in culture, in contrast to group A streptococcus and *S. aureus* (Ulett and Adderson 2005). Ring et al. (2000) hypothesized that GBS β-h/c may activate *iNOS* in macrophages. Using isogenic nonhaemolytic and hyperhaemolytic mutants of GBS, they found a correlation

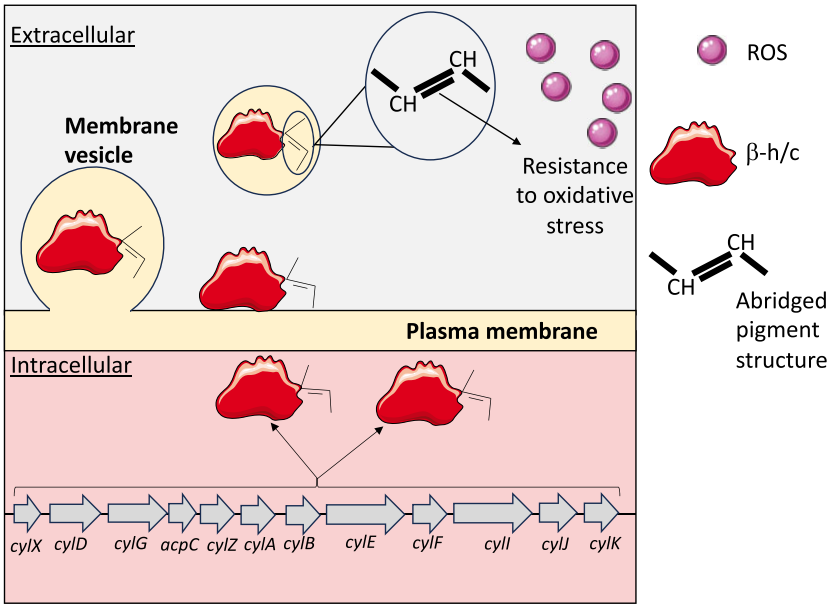

Figure 2. The protective effect afforded by MV-derived β-h/c and pigment towards oxidative killing of GBS. The GBS *cyl* operon consists of 12 genes *cylX-K* (shown at bottom), which are involved in the biosynthesis of β-h/c (red cargo) and granadaene (Pritzlaff et al. 2001, Spellerberg et al. 1999, Whidbey et al. 2013). CylD, CylG, ApcC, CylZ, and CylI are homologous with enzymes that are involved in fatty acid biosynthesis (Pritzlaff et al. 2001, Spellerberg et al. 2000). The granadaene pigment structure has a linear chain of 12 conjugated C = C bonds (shown abridged off β-h/c) providing it with antioxidant abilities to quench and detoxify ROS (Jusuf et al. 2021, Stahl and Sies 2003). The pigment is either bound to the surface of the cell or released in MVs and requires contact with host cells to cause lysis (Armistead et al. 2021, Marchlewicz and Duncan 1980, Platt 1995).

between expression of β-h/c and increased nitrite in macrophages, likely indicative of the detoxification of $^{\bullet}$NO (Ring et al. 2000). They found that treating macrophages with partially purified GBS β-h/c also led to accumulation of nitrite and *iNOS* induction, suggesting an essential role for β-h/c in inducing *iNOS* expression (Ring et al. 2000). Finally, while human PMNLs and macrophages produce RNS in some conditions, their production is relatively limited compared to murine phagocytes, as discussed elsewhere (Al-Shehri 2021).

Maintaining metal ion homeostasis

Transition metals play a vital role in numerous key biological processes and are required by eukaryotic and prokaryotic cells to function primarily as cofactors in the catalytic sites of enzymes (Andreini et al. 2008, Osman and Cavet 2008, Watly et al. 2016). Maintaining metal ion homeostasis is a complex process and is reliant on the tight regulation of metal transport into and out of the cell to prevent adverse effects that result from either metal ion excess or starvation. In conditions of excess metals, bacteria typically express efflux systems to respond to the build-up of intracellular metals and evade mismetallation and toxicity. On the other hand, when faced with metal limitation/starvation, bacteria upregulate expression of import mechanisms, downregulate some metal-dependent processes, mobilize stored metals, and/or activate other cellular pathways that avoid use of the limiting metals (Merchant and Helmann 2012). Adaptation to metal excess and limitation in streptococci is complex and involves several defined systems that have been the subject of recent reviews (Akbari et al. 2022). This section will focus on the mechanisms of maintenance of metal ion homeostasis, specifically for Copper (Cu) and Zinc (Zn), during conditions of metal starvation and the responses of GBS to metal excess that afford resistance to intoxication by these metals.

Maintaining sufficient intracellular metal ion levels via import is crucial for GBS pathogenicity. For example, a functional Zn import system is required for iGBS disease in a mouse model (Burcham et al. 2020); Mn import supports GBS survival in the FRT, and GBS lacking systems that maintain homeostasis of metals such as Zn, Cu, Nickel (Ni), magnesium, and Mn are attenuated for colonization of the FRT (Burcham et al. 2022). Functional intracellular metal homeostasis is also required for GBS persistence in a murine model of nondiabetic wound infection, with Mn and Zn uptake systems playing an important role in GBS survival within that niche (Akbari et al. 2023). GBS also require Mn uptake to survive in human whole blood and plasma, and at low pH (Shabayek et al. 2016, Zhu et al. 2020).

However, metals can also be toxic to bacteria when present at high levels due to their reactivity and/or ability to mismetallate proteins by replacing the cognate metal ion of metal sensing/binding proteins (Eom and Song 2019, Imlay 2014, Waldron and Robinson 2009). This is particularly true for Cu and Zn, which are highly reactive metals as described in the Irving–Willams series (Irving and Williams 1953). Excess Cu in bacteria can lead to the inactivation of enzymes, increased redox stress, and disruption of metabolism (Djoko et al. 2015a). Similarly, high levels of Zn can disrupt carbon metabolism and interfere with the uptake of Mn, another essential metal, which in turn can compromise oxidative stress management in the bacteria (Eijkelkamp et al. 2014, McDevitt et al. 2011, Ong et al. 2015). As such, compounds that alter intracellular metal homeostasis are being explored as an alternative antimicrobial therapeutic option to aid in the treatment of multidrug resistant bacterial infections (Bohlmann et al. 2018, De Oliveira et al. 2020, Harbison-Price et al. 2020, Jen et al. 2021).

The mammalian host exploits this double-edged sword of metal ion essentiality and toxicity via elaborate mechanisms that bring about metal limitation or intoxication in pathogenic bacteria, in a process termed nutritional immunity (Hood and Skaar

2012). First, the host can restrict access to these essential metals by employing host-expressed metal binding proteins to sequester excess metals to prevent uptake by the bacteria, which in turn can restrict bacterial growth and ability to cause disease. One such host protein is the PMNL-associated protein calprotectin, which has a picomolar binding affinity to Zn (Brophy et al. 2012, Burcham et al. 2020, Corbin et al. 2008, Kehl-Fie et al. 2011, Nakashige et al. 2016). Conversely, host immune cells such as PMNLs and macrophages can also expose internalized bacteria to high concentrations of intracellular pools of Cu or Zn to exert antimicrobial effects (Achard et al. 2012, Djoko et al. 2015b, Kapetanovic et al. 2016, Stocks et al. 2019). This review will focus specifically on the mechanisms of GBS resistance to Cu and Zn intoxication, and how these systems are managed.

Transport-dependent mechanisms of GBS metal ion resistance

Cu transport systems

Discrete genetic systems were first identified in studies of other streptococci and enterococci species that are used to maintain a strict intracellular equilibrium of Cu (Mitrakul et al. 2004, Vats and Lee 2001, Young et al. 2015). In streptococci and enterococci, the canonical *cop* operon is utilized for Cu efflux—this operon comprises *copA* (encoding a P-type ATPase efflux pump that expels Cu from the cell), *copZ* (encoding a Cu chaperone that shuttles intracellular Cu), and *copY* (encoding a Cu-responsive transcriptional repressor) (Cobine et al. 1999, Shafeeq et al. 2011, O'Brien et al. 2020).

In silico analyses of the GBS *cop* operon shows that it is moderately conserved compared to other Streptococci, including *S. mutans*, *S. pneumoniae*, *S. pyogenes*, and *S. thermophilus*, as well as *Enterococcus* spp. (Sullivan et al. 2021a). Sullivan et al. (2021a) and colleagues used RNA sequencing of GBS strain 874391 (serotype III, sequence type 17) to characterize the Cu-responsive transcriptome, and found that the GBS *cop* operon was upregulated 3.6–4.0-fold in the presence of a subinhibitory concentration of Cu. Subsequent analyses found that this effect was titratable, with GBS upregulating *copA* expression 3.7–14.2-fold when exposed to increasing concentrations of Cu up to 1.5 mM. Deletion of *copA* resulted in severe attenuation of growth in nutrient-rich media at Cu concentrations ≥1 mM; this effect was amplified under nutrient-limited conditions, where the concentration of Cu required to inhibit growth of Δ*copA* GBS was considerably lower (0.5 mM). Moreover, cellular Cu measurements using inductively coupled plasma optical emission spectroscopy (ICP-OES) showed that Δ*copA* GBS accumulated significantly more Cu than WT when exposed to Cu stress.

In an *in vitro* model of infection, the Cu efflux pump CopA played no role in the intracellular survival of GBS in human macrophage-like cells. Conversely, in a murine model of disseminated infection, deletion of *copA* resulted in fewer GBS being recovered from the liver, spleen, and blood of mice, suggesting that Cu efflux contributes to the ability of GBS to colonize different niches within the host (Sullivan et al. 2021a). Mutants in *copZ* were identified by Burcham et al. (2022) to be underrepresented in an *in vivo* transposon mutagenesis screen in a murine vaginal colonization model (Burcham et al. 2022) further supporting the view that GBS maintenance of Cu homeostasis through Cu efflux contributes to virulence.

Besides *copA*, *cadD* (encoding a putative metal transport protein) was also found to mediate Cu efflux in GBS—deletion of *cadD* led to accumulation of intracellular Cu and an increased sensitivity to killing by Cu stress (Korir et al. 2022). Interestingly, the product of *cadD* also mediated efflux of excess intracellular Ni, cobalt, and Zn, and supported GBS survival in macrophages and virulence in a mouse pregnancy infection model. In *S. pneumoniae*, *cadD* also amplifies Zn and Mn efflux during conditions of Cd accumulation subverting the primary functional roles of CzcD and MntE and further dysregulating transition metal ion homeostasis (Begg et al. 2015).

Zn transport systems

Zn uptake systems in Streptococci include an ABC transporter AdcBC, along with one or more cognate substrate-binding proteins (SBP) such as AdcA, AdcAII, Lmb, or Lsp, with *Streptococcus* spp. encoding different combinations of SBPs (Burcham et al. 2020, Moulin et al. 2016, Shafeeq et al. 2013, Tedde et al. 2016). Additionally, many Streptococci express polyhistidine triad proteins which can bind Zn and facilitate uptake (Plumptre et al. 2012); GBS possesses two such proteins—ShtI and ShtII (Moulin et al. 2016, 2019), the former of which contributes to intracellular survival in macrophages and disseminated infection in mice (Sullivan et al. 2023). GBS can overcome calprotectin-imposed Zn limitation using uptake systems such as AdcA, AdcAII, and Lmb to promote infection (Burcham et al. 2020).

In *S. pneumoniae* and *S. pyogenes*, Zn efflux is meditated by two proteins—CzcD and SczA. CzcD is a member of the cation diffusion facilitator family of proteins that functions as a Zn efflux pump and its expression is controlled by Zn-responsive transcriptional activator encoded by *sczA* (Kloosterman et al. 2007, Martin et al. 2017, McDevitt et al. 2011, Ong et al. 2014). These systems have recently been studied in GBS to identify their roles in resisting Zn intoxication.

Sullivan et al. (2021b) analysed expression of the GBS Zn efflux pump *czcD*, which increased from 3- to 18.9-fold when exposed to Zn concentrations ranging from 0.25 to 1.5 mM. Deletion of *czcD* attenuated GBS growth in rich media supplemented with Zn; an effect enhanced in nutrient-limited media, with 0.1 mM Zn sufficient to perturb growth of Δ*czcD* GBS. ICP-OES analyses showed intracellular levels of Zn are significantly increased in Δ*czcD* GBS, compared to the WT, when exposed to Zn stress, supporting a role for CzcD in maintaining cellular Zn levels during intoxication. Deletion of *czcD* did not affect intracellular survival of GBS in either murine macrophages or human macrophage-like cells but resulted in fewer GBS being recovered from the liver and bladder of mice compared to the WT, suggesting that similar to Cu efflux, a functional Zn efflux system also contributes to the ability of GBS to colonize different niches within the host (Sullivan et al. 2021b).

A recent transcriptomic analysis found that expression of the *cadD* locus in GBS is Zn-responsive, with *cadD* transcripts increasing in abundance more than 2-fold in the presence of Zn as compared to media alone (Korir et al. 2022). Moreover, the product of *cadD* mediates efflux of excess intracellular Zn (as well as several other divalent metal cations) to protect GBS from Zn stress, which may promote infection of the FRT (Korir et al. 2022).

Regulation of copA and czcD

In GBS, expression of *copA* is regulated by the Cu-binding transcriptional regulator CopY. Deletion of *copY* led to an ∼207-fold increase in *copA* transcription, even in the absence of Cu, consistent with a repressor function for CopY. Additionally, in Δ*copY* GBS, levels of cellular Cu were significantly lower than the WT, consistent with the observation that expression of the CopA Cu efflux pump is significantly elevated in this mutant (Sullivan et al. 2021b). On the other hand, expression of *czcD* is activated by the

Zn-sensing transcriptional regulator *sczA*. GBS *czcD* transcripts were upregulated following exposure to Zn, but this transcription was not activated when *sczA* was deleted, with *czcD* remaining at basal levels in the Δ*sczA* background (Sullivan et al. 2021b1). Interestingly, CopY also mediates resistance to Zn intoxication—mutation of *copY* resulted in the attenuated growth of GBS in both rich and nutrient-limited media supplemented with Zn, with transcriptomic analyses revealing unique links between the systems used to resist Cu and Zn (Sullivan et al. 2022). On the other hand, mutation of *sczA* had no effect of GBS growth under Cu stress, indicating that this cross-over effect is limited to CopY only. Strikingly, previous studies with *S. pneumoniae* showed that Zn can also bind strongly to CopY, leading to the enhancement of DNA binding (Glauninger et al. 2018, Neubert et al. 2017); this suggests that Zn-bound CopY could result in altered regulatory outcomes which may be favourable under Zn stress. A complex interplay between global response regulator CovR/CovS and other Zn-responsive genes was also uncovered, with CovR playing a key role in regulating the global transcriptional responses of GBS to Zn (Sullivan et al. 2022). A summary of factors in GBS that mediate transport-dependent resistance to metal intoxication is depicted in Fig. 3.

Transport-independant mechanisms of GBS metal ion resistance

Bacterial factors that subvert metal stress through mechanisms that are independent of metal efflux systems are summarized recently elsewhere (Sullivan et al. 2024). For resistance to Cu and Zn stress in GBS these mechanisms extend beyond the *copA–copY* and *czcD–sczA* efflux mechanisms. RNA-seq analyses, coupled with Transposon Directed Insertion Site Sequencing (TraDIS) experiments performed on GBS strain 874391 uncovered a suite of genes that are differentially expressed and/or required for survival under either Cu of Zn stress (Goh et al. 2022, Sullivan et al. 2021a,b, 2022). These genes encode for diverse processes related to import/export, metabolism, cell structure and signalling, and nucleotide/riboflavin biosynthesis, none of which have been linked to metal ion resistance in other bacteria.

In response to either Cu or Zn stress, GBS differentially expresses numerous metal transporters including *nikABCD*, *mtsABC* (both upregulated), and *fetAB* (downregulated), predicted to transport Ni, Mn, and Fe, respectively (Sullivan et al. 2021a,b). Additionally, *cadD*, another transporter that facilitates the transport of multiple metal ions, is also upregulated in GBS under Zn stress (Korir et al. 2022). Interestingly, supplemental Mn reverses the Zn sensitivity of a GBS Zn efflux mutant (Sullivan et al. 2021a). Similarly, in *S. pneumoniae*, Cu intoxication is partially rescued with excess Mn (Johnson et al. 2015). This suggests the importance of other metal transporters in overcoming metal ion stress, possibly by preventing mismetallation of essential proteins. Additionally, GBS under Cu or Zn stress downregulate the Fe transporter *fetAB*, resulting in elevated levels of Fe. Taken together, this suggests that a broader dysregulation of metal management (encompassing Mn and Fe) in GBS occurs during Cu or Zn intoxication, a notion which is consistent with previous observations in *S. pneumoniae* (Eijkelkamp et al. 2014).

The arginine deiminase system comprises a three-enzyme pathway to convert arginine to ornithine. The arginine deiminase enzyme, encoded by *arcA*, is upregulated >20-fold in GBS in response to high levels of Zn. Deletion of *arcA* renders GBS more susceptible to Zn intoxication, and deletion of *arcR*, a putative repressor of the *arcABCD* operon, increases resistance to Zn stress (Sullivan et al. 2021b). Supplementation with ornithine, but not arginine, rescues the effect of Zn intoxication of a WT strain. In a separate study utilizing a forward genetic screen in high Zn concentrations, an enrichment of transposons was observed in *arcR* and another putative repressor of the arginine deiminase pathway—*argR* (Sullivan et al. 2022). Taken together, this suggests the arginine deiminase system in GBS supports survival during Zn stress, perhaps via the production of ornithine.

A putative histidine ABC-type transporter system was also identified in a transposon screen to uncover GBS genes required for survival in either high Cu or Zn concentrations (Goh et al. 2022, Sullivan et al. 2022). This system comprises *hisM* (permease), *hisJ* (ATP-binding), and *hisP* (substrate-binding), and likely facilitates the import of histidine into the cell; deletion of *hisMJP* in GBS increases sensitivity to Cu stress. Interestingly, a study with *Caenorhabditis elegans* showed that elevated histidine levels facilitate resistance to Zn toxicity (Murphy et al. 2011). Histidine is an amino acid that contains an imidazole group and can bind metals (including Cu and Zn) in solution or at catalytic sites of proteins (Sigel and McCormick 1971, Zhou et al. 2013)—the exact mechanism of how elevated histidine levels protect bacteria from Cu or Zn intoxication is an area that warrants future investigation.

Intriguingly, transposon-screening experiments also identified an overlapping subset of four genes (*plyB*, *yceG*, *rfaB*, and *stp1*), predicted to be involved in cell wall synthesis, that are required for resistance to both Cu and Zn stress (Goh et al. 2022, Sullivan et al. 2022), indicating that there may be additional interactions between cellular responses to Cu and Zn that go beyond the control of CopY or CovR. Transport-independent factors that are differentially regulated and/or mediate GBS metal resistance are summarized in Fig. 4.

Zn resistance phenotypes amongst GBS strains

Although molecular systems for metal ion detoxification are present in GBS, the degree of resistance can vary between strains. For example, Francis et al. (2022) reported that invasive strains of GBS were significantly more resistant to Zn intoxication than colonizing strains isolated from rectovaginal swabs of women (Francis et al. 2022). The authors found that CPS serotype III and ST-17 or 19 strains were also able to grow at higher concentrations of Zn as compared to CPS serotype Ib and ST-1 or 12 strains.

A study by Varghese et al. (2023) and colleagues also found that although the Zn resistance phenotype did indeed vary amongst different GBS strains, they could not attribute any clear role for CPS serotype or ST in mediating higher resistance to Zn (Varghese et al. 2023). However, it is important to note both studies used different media in their growth assays, and that the latter study used approximately half the number of isolates as compared to the former. Whilst this could explain the contradictory observations between the two studies, both provide evidence that the range of GBS Zn resistance phenotypes vary considerably. Notably, Varghese et al. (2023) also identified the presence of insertion element *IS1381* within the coding region of *czcD* of GBS strain 834, which was highly resistant to Zn. The authors hypothesized that the insertion element caused the dysregulation of *czcD*, which in turn conferred an enhanced resistance of 834 toward Zn stress. However, deletion of *czcD* or *in trans* expression of *czcD* did not affect growth of 834 under Zn stress, providing further evidence that mechanisms separate from Zn efflux mediated by *czcD–sczA* (such as *copY*, *covRS*, and other genes mentioned above) support Zn resistance in GBS.

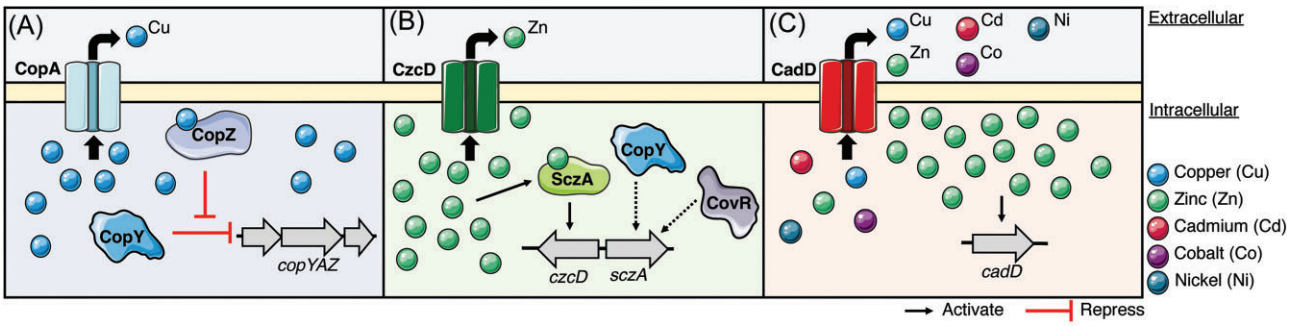

Figure 3. Transport-dependent factors that mediate GBS metal resistance. (A) Copper efflux in GBS is mediated by the transmembrane transporter CopA, which is transcriptionally repressed by CopY in the absence of Cu. Chaperone protein CopZ binds to Cu when intracellular levels of the metal ion is high, which in turn inhibits CopY activity (Neubert et al. 2017, O'Brien et al. 2020, Sullivan et al. 2021a). (B) The Zn-sensing SczA protein activates expression of the CzcD efflux pump when high levels of Zn are present in the cell. CopY and virulence regulator CovR are required for *sczA* expression and in turn, resistance to Zn-mediated stress (Sullivan et al. 2021a, 2022). (C) CadD is a metal efflux pump that plays an important role in metal detoxification. Transcription of *cadD* is upregulated in response to high Zn concentrations, with the CadD protein playing a role in the efflux multiple metal ions, including Cu, Zn, Cadmium (Cd), cobalt, and Ni (Korir et al. 2022). Dotted lines represent activation mechanisms that may be direct or indirect and remain to be characterized.

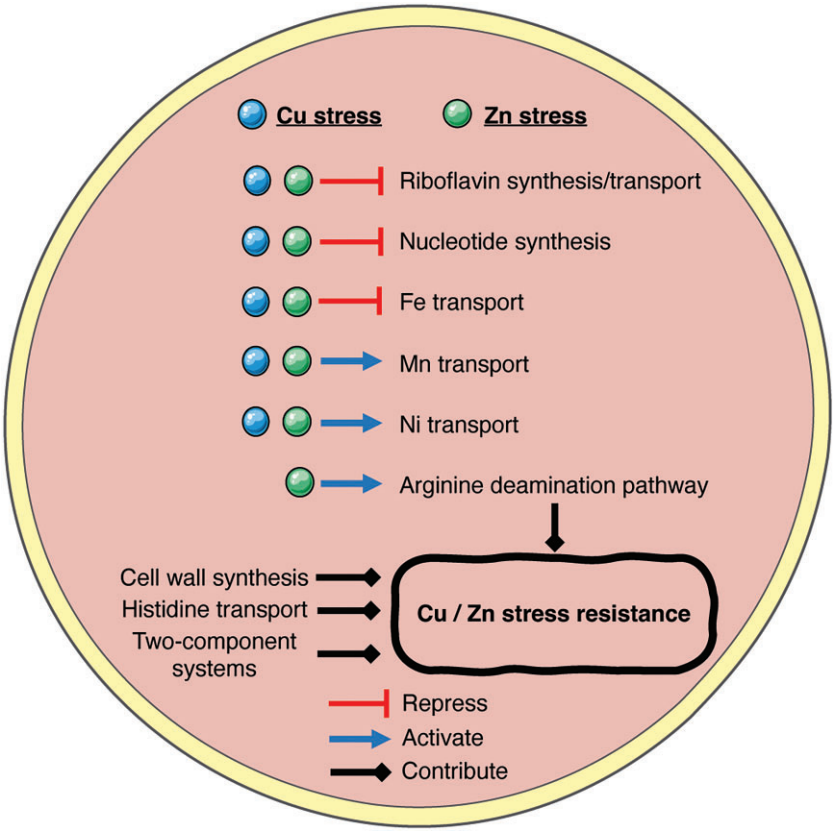

Figure 4. Transport-independent factors that are differentially regulated and/or mediate GBS resistance to Cu or Zn intoxication. Under either Cu or Zn stress, GBS downregulates genes involved in riboflavin and nucleotide synthesis, and Fe transport, but upregulates genes required for Mn and Ni transport. Excess Zn also upregulates genes that play a role in arginine deamination and transport, which are required for Zn resistance. Other genes that are involved in cell wall synthesis (i.e. *celB*, *oafA*, *plyB*, *rfaB*, and *yceG*), histidine transport (*hisMJP*) and TCSs (*covRS* and *stp1/k1*) are also required for GBS to resist high levels of Cu or Zn (Goh et al. 2022, Sullivan et al. 2021a,b).

Conclusion

The increasing prevalence of GBS infections worldwide highlights a need to better understand the mechanisms and complexities that underpin GBS disease. GBS surface-associated and secreted virulence factors have central roles in helping GBS evade host defences, and mechanisms that support GBS survival in conditions of oxidative, nitrosative, and metal ion stress are now well-established. The recent discoveries of how GBS mediates resistance to Zn and Cu intoxication gives new insight into stress responses in this pathogen and provides better understanding of the fundamental biology of GBS resistance to specific toxic insults. These discoveries deliver the potential to develop new mechanisms to target GBS therapeutically and pave the way for testing new avenues for disease prevention.

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