The contribution of YfdX and Ail/OmpX-like proteins to *Salmonella* Typhimurium stress resistance and virulence

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Abstract

Salmonella is a Gram-negative, food-borne pathogen and a major cause of worldwide morbidity and mortality. *Salmonella* encounters a wide variety of stressors in its environments, including changes in pH and heat, antimicrobial compounds, and reactive oxygen and nitrogen species. These conditions can result in serious damage to the bacterium, in particular the cell wall. To combat this, envelope stress response (ESR) pathways are in place to sense and respond to protein damage induced by such conditions and restore envelope homeostasis. There is a degree of overlap in terms of environmental cues and regulon members across the ESR pathway, many of which are important for *Salmonella* infection.

We have been investigating genes that are co-regulated by more than one ESR, as we hypothesise that they will be more important for maintaining envelope homeostasis and might be potential therapeutic targets. We discovered that the hypothetical protein STM3030 and the outer membrane protein STM3031, which is an Ail/OmpX-like protein, were both transcriptionally regulated by the CpxR and BaeR ESR regulators in *Salmonella*. Both proteins have been associated with antibiotic resistance, with STM3030 having chaperone activity, and a Typhi homologue of STM3031 a role in adhesion to host cells.

This study aims to characterise the roles of STM3030 and STM3031 in the *S.* Typhimurium ESR and infection. Using single and double deletion mutants, we have identified a role for these proteins in adhesion of *Salmonella* Typhimurium to Caco-2 epithelial cells, as well as determined their role in a range of envelope damaging conditions.

The results of this study show that STM3030 and STM3031 cooperate during host cell adhesion, after which only STM3031 is needed for invasion of host cells, contradicting past studies, and revealing novel virulence functions of STM3030 and STM3031. This study highlights the need for further investigation into *Salmonella* molecular mechanisms of disease.

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1. Introduction

1.1. Phylogeny

1.1.1. Origins

Salmonella is a Gram-negative intracellular pathogen of the Enterobacteriaceae family, consisting of two genetically distinct species *Salmonella bongori* and *Salmonella enterica*. The former, *S. bongori*, is limited to cold-blooded animals and the latter, *S. enterica*, is associated mammals and birds. The distinction of the two species is based on differences in the 16S rRNA sequence analysis (Eng *et al*., 2015). *S. enterica* is classified into six subspecies, of which one, *S. enterica* subspecies *enterica*, is comprised of more than 2,600 serovars. A classification scheme by Kauffmann and White was developed to classify these serovars based on somatic (O), capsular (K), and flagellar (H) antigenic determinants (Kim *et al.*, 2006). The somatic O antigen is a heat-stable antigen and is the oligosaccharide component of the lipopolysaccharide found in the outer membrane. The surface K antigens, the least common antigen, are heat-sensitive polysaccharides found in the capsular surface. The virulence (Vi) antigen is a subtype of K antigen and is found in *S.* Typhi. The flagellar H antigen is heat-labile, meaning it is destroyed or altered by heat, are found in the flagella, and are heavily involved in host immune reaction. Specific H antigens are expressed by various serotypes, which defines the immunological identity of the serovar (Kim *et al.*, 2006).

Four *S. enterica* serovars are of greatest human clinical relevance. These four serovars are Typhi and Paratyphi A, which are known as typhoidal serovars, and Typhimurium and Enteritidis, known as non-typhoidal serovars. Typhoidal serovars are human restricted without animal reservoirs and are the cause of enteric fever, while non-typhoidal serovars have wider range of hosts, including mammals other than humans (Saleh *et al*., 2019). *Salmonella* was identified by Theobald Smith, who isolated strains from the intestines of pigs, in 1855, and named the bacterium after American pathologist, Dr Daniel Elmer Salmon, who took credit for Smith's work (Eng *et al*., 2015). It was later discovered that *Salmonella* had not been the pathogen of interest in these pigs, but a virus instead; *Salmonella* is now known to rarely cause enteric symptoms in pigs (Fàbrega & Vila, 2013).

1.2. Infection

1.2.1. *Salmonella* infection

Salmonella is responsible for causing enteric fever, also known as typhoid and paratyphoid fever; gastroenteritis, also known as food poisoning or salmonellosis, and bacteraemia. Gastroenteritis is the most common *Salmonella* infection worldwide, after which come bacteraemia and enteric fever (Eng *et al*., 2015). The severity of infections by *Salmonella* varies depending on the specific serovar and overall health of the host. Infection by *Salmonella* usually occurs through contact with contaminated food, usually poultry, eggs, and dairy. However, fresh fruits and vegetables, especially ones without a removeable peel, contribute greatly to foodborne *Salmonella* transmission due to possible washing with contaminated water. Slaughter of animals in abattoirs is heavily implicated in the contamination of carcasses by *Salmonella* (Eng *et al*., 2015).

To survive, *Salmonella* has had to adapt to the challenging conditions within its animal and non-animal hosts, and being an intracellular pathogen allows for better ways to create and maintain infection. Many such pathogens have evolved mechanisms to pass through host barriers, namely intestinal barriers, to be able to infect and survive within their host (Ribet and Cossart, 2015). An intracellular lifestyle offers many advantages, using their position within cells avoid immunological surveillance and have access to a wider range of nutrients found within cells (Ribet and Cossart, 2015). Intracellular pathogens spread from cell through extracellular fluids, however, extracellular fluids are protected by humoral immunity, whereby antibodies secreted by plasma cells prevent spread of infection by eliminating extracellular microbes (Takaya, *et al.*, 2020). Both *S.* Typhi and *S.* Typhimurium rely on being internalised by a variety of phagocytic cells, most of which have short-lived, and non-phagocytic cells (Malik-Kale *et al*., 2011). *Salmonella* strains lacking the ability to persist intracellularly within the host cell are found to be non-virulent, highlighting the importance of the intracellular lifestyle in *Salmonella* pathogenesis (Eng, *et al.*, 2015). This internalisation happens either through phagocytosis or *Salmonella*-induced internalisation; in the latter instance, *Salmonella* forcibly penetrates a non-phagocytic cell via a “trigger” mechanism, which is a part of the *Salmonella* pathogenicity island-1 (SPI-1) type III secretion system (T3SS), that injects bacterial effectors into the vacuole, triggering cellular responses and a ruffling of the non-phagocytic cell membrane (Birhanu *et al*., 2018; Ribet and Cossart, 2015). The modified

vacuole then prevents fusion of lysosomes, which contain digestive enzymes for destroying invading pathogens, allowing for *Salmonella* to survive and replicate intracellularly *(Eng, et al*., 2015). Secretion of lysosomes and other digestive enzymes that fuse to infected host cells and destroy intracellular bacteria is an integral response to the detection of foreign bodies (Eng, *et al*., 2015). The relatively short lifespan of the phagocytic cells that internalise *Salmonella* means that, for survival purposes, *Salmonella* must escape into new host cells every 1 to 7 days, passing through the heavily surveyed extracellular spaces (Takaya, *et al*., 2020). It is currently unknown exactly how *Salmonella* manages to evade antibodies within the extracellular spaces.

The first line of defence against infection is the host innate immunity, it relies on the complement system to effectively identify and destroy any potential pathogens. Phagocytic cells, such as macrophages, dendritic cells, M cells, neutrophils, and enterocytes, which are also known as innate cells, are critical in the clearing any such potential pathogens. Upon infection, these cells produce cytokines, such as tumour necrosis factor (TNFα), and chemokines, which are released to alert and recruit other immune cells to the site of infection. Numbers of macrophages, neutrophils and dendritic cells increase during early *Salmonella* infection and start producing cytokines Type II interferon (IFNγ) is another cytokine responsible for enhancing the immune response to *Salmonella* and is very important for innate and adaptive immunity (Takaya *et al.*, 2020). Intracellular survival of *Salmonella* within macrophages allows the bacteria to be transported into the mononuclear phagocyte system (MPS), which is linked to a chronic carrier state of *Salmonella* infection (Monack, *et al*., 2004).

Various studies have revealed a *Salmonella* specific multilayer suppression of host humoral immunity, with several stages of the humoral immunity being affected. During *Salmonella* infection, B cell lymphopoiesis, expression of MHC class II molecules, germinal centre (GC) formation, persistence of IgG-secreting plasma cells, and IgG antibody serum concentration are all negatively impacted (Cunningham, *et al*., 2007; Takaya, *et al*., 2020). GC formation is responsible for differentiation of B cells into short-lived plasma cells, memory B cells, or long-lived plasma cells; delays in GC formation, as seen in *Salmonella* infection, stops immune cells from maturing and activating as normal (Cunningham, *et al.*, 2007). Past studies in mice have shown that due to this host humoral immunity suppression, B cells have no function in primary immune response to *Salmonella* and antibodies produced by B cells have no role in clearing *Salmonella* (McSorley and Jenkins, 2000). On the other hand, clearance of *Salmonella* from

host tissues involves $CD4^+$ helper T cells, along with innate cells, and results in long-term specific immunity (Takaya, *et al.*, 2020). A further differentiated subset of $CD4^+$ cells, Th1 cells, demonstrate a principal role while CD8 T cells demonstrate a moderate role in primary immunity to *Salmonella* (Takaya, *et al*., 2020).

Alongside having an immunosuppressive and evasive role, *Salmonella* has also been connected to autoimmune diseases to a certain extent. The combination of the gut microbiome is heavily implicated in immune-mediated conditions and *Salmonella*, being an enteric pathogen that can negatively affect the gut microbiome, has led to links between the two being investigated. A study by Ktsoyan *et al.* report a presence of autoantibodies in 85.7% of patients with a *S.* Typhimurium infection. Another study by Soloski and Metcalf determined nontyphoidal *Salmonella* infections generate autoantibodies that have been implicated in the development of autoimmune diseases such as reactive arthritis (ReA), formerly known as Reiter's syndrome. Other enteric pathogens besides *Salmonella* are known causative agents of ReA. Although the specific mechanisms by which *Salmonella* triggers ReA remain undetermined, the presence of Human leukocyte antigen (HLA) B27 (HLA-B27) in the host is strongly associated with development of ReA and other similar inflammatory diseases (Gaston & Lillicrap, 2003).

While internalisation is an essential aspect of *Salmonella* pathogenesis, the first step towards any sort of host colonisation is adhesion to host surfaces, from that invasion of the host cells can happen (Ribet and Cossart, 2015; Klemm *et al.*, 2010). Adhesion is a crucial element of the infection process at the earlier stages as it allows for the persistence of extracellular bacteria and the internalisation into host cells after adhesion to cell surfaces (Birhanu *et al*., 2018). Adhesins, which are bacterial cell-surface components essential for attachment to host cells and other surfaces, allow for specific recognition and attachment to structures on target surfaces, either biotic or abiotic (Klemm *et al.*, 2010). Once bacteria adhere to host cell surface, they infiltrate the host cell either through phagocytosis or using the "trigger" mechanism. However, adhesion does not only play a role in pathogenesis, but also in survival outside of the host. Adhesins can bind to abiotic surfaces, such as plastic and metal, by forming dormant three-dimensional sessile colonies embedded within an extracellular matrix that provide a barrier to the outside environment (Klemm *et al.*, 2010; Ramachandran *et al.*, 2016). These sessile colonies aid in survival when exposed to nutrient scarcity, heat and pH changes, and antimicrobial compounds (Fàbrega & Vila, 2013). *Salmonella* biofilms have been implicated in a variety of conditions, from, but not limited to, chronic *Salmonella* infections to irreversible

adherence to medical supplies or sewage pipes, implanted devices, and to antibiotic resistance (Crawford *et al*., 2010; Tursi, *et al*., 2020). In chronic *Salmonella* infections, most notably in *S.* Typhi, biofilms form on cholesterol gallstones within the gallbladder, causing the bacteria to persist within the host (Crawford *et al*., 2010). This persistent carrier state is often asymptomatic and increases the person-to-person transmission of typhoid fever, with antibiotic treatment often proving ineffective, and has been popularised in the media through the story of "Typhoid Mary" (Crawford *et al*., 2010). Mary Mallon was a cook in the United States in the early 1900s and is believed to be the first identified carrier of typhoid fever; 51 cases and 3 deaths have been attributed to her, although a further 3,000 cases are thought to have been linked to her (The New York Times, 1938; Marineli, *et al*., 2013). However, *S.* Typhi is not the sole *Salmonella* serovar to produce chronic carriers, *S.* Typhimurium does too, with up to 2.2% of patients with a non-Typhoidal *Salmonella* infection being identified as chronic carriers, lasting from 30 days to as long as 8.3 years (Takaya, et al., 2020). However, these chronic infections have been explored to a lesser extent. As adhesion is paramount to pathogenesis and survival outside of the host, its prevention is a promising method of interfering with early-stage *Salmonella* infection. Fimbriae are the predominant adhesins involved in abiotic and biotic adhesion and their biosynthesis is a potential antibiotic or immunisation target. A fimbriae-specific immunisation would protect the host as it would inhibit fimbriae-receptor interaction and stop infection at the key point of contact. This has been attempted various times to no avail as the variation of genetic sequences in these proteins was too great (Klemm *et al.*, 2010).

There are overarching common infection mechanisms employed by both *S.* Typhi and *S.* Typhimurium, however due to their distinctive genomes, the type of disease caused by either serovar differs greatly. Mode of entry for both serovars is through the oral route via contaminated food or water, and into the small intestine, but mechanisms of infection of the serovars diverge from their point entry into intestinal epithelial cells.

1.2.2. Typhi versus Typhimurium

Typhoidal serovars are host restricted, therefore *S.* Typhi is only able to create infection in humans, and are the cause of enteric fever, while non-typhoidal serovars are generalists with a wide range of hosts where they may be commensals or cause infection (Saleh *et al*., 2019). *Salmonella* and *Escherichia* diverged from a common ancestor around 100-150 million years

ago, which is estimated from the 10% difference in core genomes (Sabbagh *et al*., 2010; Baker and Dougan, 2007). The CT18 *S.* Typhi and LT2 *S.* Typhimurium strains share approximately 89% of their genes, and there are 601 genes unique to *S.* Typhi and 479 genes unique to *S.* Typhimurium (Sabbagh *et al*., 2010). The emergence of the *S.* Typhi serovar is thought to be due to a loss of gene function, highlighted by the presence of pseudogenes in *S.* Typhi that remain functional in *S.* Typhimurium, which are conserved in *S.* Typhi isolates and could explain *S.* Typhi's human host restriction (Sabbagh *et al*., 2010). Several pseudogenes found in *S.* Typhi are also found in *S.* Paratyphi, implying shared evolutionary origin (Baker and Dougan, 2007). Accumulation of specific mutations inactivated approximately 5% of *S.* Typhi's genes (Baker and Dougan, 2007). Reduced genetic variation has been observed in various *S.* Typhi isolates, whereby they have conserved and clonal relation to one another, making *S.* Typhi a monomorphic organism and a product of reductive evolution.

The differences between *S.* Typhi and *S.* Typhimurium have been heavily researched, with comparative genomic strategies being used to identify phenotypic variances, most of which have to do with host adaptation and virulence. The Vi antigen, a polysaccharide capsule important for virulence, specific to *S.* Typhi allows it to elude immune responses and systematically circulate within human hosts, as opposed to *S.* Typhimurium's modus operandi of triggering an immune response and causing inflammation in the intestines (Sabbagh *et al*., 2010). The Vi antigen is thought to have been recently acquired by *S.* Typhi through horizontal transfer (Baker and Dougan, 2007). Genes related to *Salmonella* infection are located in *Salmonella* pathogenicity islands (SPIs) gene clusters, also thought to be acquired through horizontal transfer, of which there are twenty-one known in *Salmonella*; SPIs-19, 20 and 21 are not present in either *S.* Typhi or *S.* Typhimurium. Eleven common SPIs are found in *S.* Typhi and *S.* Typhimurium, SPIs 1 to 6, 9, 11, 12, 13, and 16. Only present in *S.* Typhi are SPIs-8 and 10, and only present in *S.* Typhimurium is SPI-14 (Shah *et al*., 2005; Sabbagh *et al*., 2010). Further comparative analysis identified 469 genes involved anaerobic metabolism of *S.* Typhimurium, a gastrointestinal pathogen, as decaying in *S.* Typhi and *S.* Paratyphi, accounting for the difference in the ability of *S.* Typhimurium to exploit inflammation-derived nutrients and *S.* Typhi to evade the immune system (Nuccio and Bäumler, 2014). Additionally, most genes in *S.* Typhimurium associated with intestinal colonisation are deactivated in *S.* Typhi (Sabbagh *et al*., 2010).

1.2.3. Salmonellosis

During NTS mediated infection, *Salmonella* bacterial cells adhere to intestinal epithelia upon contact using various adhesins, which as previously mentioned, play an integral part in infection. *Salmonella* has been observed to prefer, although not limited to, infecting microfold cells (M cells) of the Peyer's patches (PPs); PPs are lymphoid tissues found in the small intestine while M cells, found within PPs, transport antigens to lymphoid tissue to initiate immune responses (Fàbrega & Vila, 2013; Kobayashi, *et al*., 2019). At this point, phagocytosis of *Salmonella* by dendritic cells also occurs, allowing for the bacteria to be dispersed through the lymphatic and blood systems (Eng, *et al*., 2015). During this infective process, most bacterial cells are eliminated by infected host cells, triggering inflammation and an immune response, which is a survival strategy used by *Salmonella*. Inflammation leads to the production of reactive oxygen species (ROS) by neutrophils. Hydrogen sulphide (H_2S), generated by commensal bacteria, oxidises into thiosulphate, and further oxidised into tetrathionate by RO*S.* Tetrathionate can be used by *S.* Typhimurium, as it is a facultative anaerobe, as a terminal electron acceptor for survival in anaerobic conditions and to out-compete commensal bacteria, as commensal bacteria are unable to utilise tetrathionate (Ribet and Cossart, 2015; Fujimoto, *et al*., 2018). Intestinal cells are damaged during this inflammatory process, leading to necrosis of epithelial cells, allowing infection of other surrounding host cells (Hume *et al.*, 2017).

Though damage done by infection seems extensive, Salmonellosis is a self-limiting infection that rarely requires antibiotic treatment. Symptoms include diarrhoea, vomiting, nausea, abdominal cramps, and muscle pains; these generic symptoms are common to many enteric pathogens and make it difficult to distinguish specific illnesses from one other. However, in approximately 5% of cases, secondary bacteraemias can develop, which can be fatal. Bacteraemia is a condition where *Salmonella* enters the bloodstream following invasion of the intestinal barriers; it is associated with high fever but not with rose spots, which is linked to enteric fever (Eng, *et al.*, 2015). Occurrence of bacteraemia in NTS as opposed to typhoidal *Salmonella* is thought to be because of the *spy* virulence gene in NTS (Eng, *et al*., 2015). Bacteraemias usually happen in young children, people with comorbidities, and immunocompromised individuals (Fàbrega & Vila, 2013). In such cases, antibiotic treatment is recommended, using fluoroquinolones, ampicillin, and extended spectrum cephalosporins.

1.2.4. iNTS

The most concerning clinical manifestation of NTS is an invasive form of non-typhoidal bacteraemia commonly known as invasive NTS (iNTS) caused by the invasion of *Salmonella* into the bloodstream, which if left untreated can be fatal. The clinical presentations include a fever, an enlarged liver and spleen, and respiratory symptoms, with an absence of salmonellosis-associated symptoms (Feasey, *et al.*, 2012). The development of iNTS is prevalent in individuals who have natural or acquired immunodeficiencies, namely sickle-cell disease, malaria, and HIV, though malnutrition and anaemia can contribute (Uche *et al.*, 2017). Additionally, iNTS has been found to be the second most cause of bacterial meningitis in Malawi (Feasey, *et al*., 2012). Most cases of iNTS, although probably underestimated, are found in sub-Saharan Africa, specifically in areas with a higher HIV and malaria prevalence; surveillance programmes in 10 African countries revealed iNTS accounts for 17% of cases of bacteraemia (Haselbeck, *et al*., 2017). Of particular concern, a multidrug resistant (MDR) *S.* Typhimurium ST313 strain linked to bacteraemias more than to salmonellosis with higher transmissibility rates in human than other strains (Haselbeck, *et al*., 2017). ST313 has evolved to be more invasive than its counterparts as it is more efficiently phagocytosed and resistant to destruction by human macrophages (Majowicz, *et al*., 2010). This strain has caused epidemics in several Africa countries (Feasey, *et al.*, 2012). The emergence of NTS MDR strains in countries with high prevalence of HIV and reduced access to suitable medical care is alarming.

1.2.5. Enteric fever

Though it invades epithelial cells in the small intestine similarly to *S.* Typhimurium, *S.* Typhi does not cause cell necrosis to avoid causing inflammation and any ensuing immune responses (Velge *et al.*, 2012). It causes a systemic infection, going beyond the intestine and into surrounding organs. The mechanisms employed to cross mucosal barriers into the bloodstream is correlated with severe clinical symptoms (Ribet and Cossart, 2015). With an incubation of around a week and symptoms consisting of fever, headaches, abdominal pain, and diarrhoea, the systemic infection caused typhoid fever typically requires antibiotic treatment, as untreated typhoid fever can persist for long periods of time. Additional symptoms include muscle pains, bradycardia, an enlarged liver, and spleen, and rose spots. Gastrointestinal complications, such as pancreatitis, hepatitis, and cholecystitis, can also develop in areas of endemicity, with the most severe complication being haemorrhages initiated by perforation of PPs (Eng, *et al*.,

2015). As mentioned previously, infiltration into the MPS results in relapse in 10% of individuals with typhoid fever (Eng, *et al*., 2015). Chronic carrier states are also more prevalent and increase person-to-person transmission. There is inadequate knowledge on how typhoid fever is cleared in the host as *in vivo* comparisons of *Salmonella* are impossible, since *S.* Typhi is host-restricted to humans and is not infectious in mice (Takaya, *et al*., 2020).

Control of typhoid fever in low- and middle-income countries continues to be a struggle due to factors such as access to water safety, suitable sanitation, and hygiene. Unlike for *S.* Typhimurium, vaccines against typhoid fever are available: a live-attenuated Ty21a Vaccine and a subunit Vi Capsular Polysaccharide Vaccine (ViPS). A third conjugate vaccine, Typbar TCV, has since replaced use of the ViPS vaccine in many endemic countries (Syed, *et al*., 2020). Though there are various limitations to the available vaccines, such as an efficacy of only 30% to 70% and no lifelong immunity, which quickly reduces the possibility of consequential disease mitigation, they remain an important factor in prevention of typhoid fever (Milligan, *et al*., 2018). Additional promising vaccines are currently in development.

1.2.6. Incidence

An estimated 93.8 million cases of salmonellosis occur worldwide each year, of which 80.3 million cases are thought to be foodborne, with 155,000 deaths (Majowicz, *et al*., 2010). Not included in the salmonellosis predictions are cases of iNTS, of which there are an estimated 3.4 million annually, of which approximately 63.7% are children, with 681,316 deaths *(*Ao*, et al*., 2015). Around 10.9 million cases of typhoid fever occurred in 2017, with 116,800 deaths (GBD 2017 Typhoid and Paratyphoid Collaborators, 2019).

Overall, the global burden of enteric fever, salmonellosis, and iNTS remains high; for this reason, specific molecular mechanisms of infection must be investigated, which could reveal potential therapeutic or vaccine targets. With antibiotic resistance on the rise in both serovars, alternative therapies must be found as the implications of pan-resistance to antibiotics pose a serious threat to millions worldwide.

1.3. Envelope Stress Response

1.3.1. *Salmonella* Stress Responses

Survival is dependent on an organism's ability to sense and react to their environment, particularly within a host for an enteropathogenic bacterium, like *Salmonella*. The ability to assess and respond accordingly to various stressors is mediated by a variety of stress responses which are categorised based on the stress(es) involved and their cellular targets. As a Gram-negative bacterium, *Salmonella*'s bacterial envelope is comprised, from the inside outwards, of the inner membrane (IM), the periplasmic space (PP) that contains a peptidoglycan (PG) layer, and the outer membrane (OM) (MacRitchie *et al*., 2008) (Figure 2). The OM contains lipopolysaccharides and functions as a permeability barrier, which allows transport through porins. The IM, made up of proteins and phospholipids, acts as a hydrophobic barrier to maintain the necessary intracellular concentrations of ions and molecules, amongst other functions such as energy production and maintenance. The peptidoglycan layer within the periplasmic space is responsible for retaining cell shape MacRitchie *et al*., 2008). This bacterial envelope is the first line of defence against any external conditions. Damage or potential damage to these structures, induce the envelope stress response (ESR) (Hu *et al.*, 2011).

Changes in temperature, pH, antimicrobial compounds, and reactive oxygen and nitrogen species are examples of stressors that can cause damage to the envelope, and particularly to the OM (Runkel *et al*., 2013). Various ESR pathways have evolved to combat these conditions, which work to sense protein damage and subsequently fix them, ultimately restoring homeostasis. These pathways are involved in the biogenesis, and the maintenance and repair of the bacterial envelope, while also regulating various functions such as motility, biofilm formation, and colony formation (Hu *et al.*, 2011). Examples of these ESR pathways are the extracytoplasmic sigma factor σ^E (*rpoE*) pathway, regulator of capsule synthesis (Rcs) phosphorelay system, and the phage shock protein (Psp) system, as well as the CpxAR, BaeSR, and ZraSR two-component signal transduction (2CST) systems. These pathways consist of mostly system specific components, however there is evidence of functional overlap and compensatory expression between some systems (Pando *et al*., 2017). The σ^E pathway is involved in the maintenance of the OM and is activated by the accumulation of misfolded outer membrane proteins (OMPs) or lipopolysaccharide (LPS), triggering a sequence of proteolytic cleavage events (Hews *et al.*, 2019). The regulator of capsule synthesis (Rcs) phosphorelay

system is made of two sensor kinase proteins, RcsC and RcsD, and a response regulator, RcsB. It also includes an accessory coregulator, RcsA, and an outer membrane bound lipoprotein, RcsF. The Rcs regulon has been implicated in the control of virulence genes in *Salmonella* Typhi and Typhimurium. Disruption of the peptidoglycan layer and presence of cationic antimicrobial peptides (CAMPs), such as polymyxin B, are known to activate the Rcs pathway (Farris *et al*., 2010). In *E. coli*, Rcs has a function in the intrinsic resistance of *E. coli* to β-lactam antibiotics (Laubacher and Ades, 2008). The Psp system detects any possible problems that could result in the increase of permeability of the IM and cause the dissipation of the proton motive force (PMF), which halts ATP production (Farha *et al.*, 2013). Interestingly, the Psp does not regulate gene expression in the same way that σ^E and 2CSTs do (Flores-Kim and Darwin, 2016). Functional overlaps exist between different ESRs, specifically between BaeSR and CpxAR as they both regulate *spy*, *acrD* and *mdtA*, but also between σE, for example *htrA*, *skp*, *rpoD*, *rpoE*, *rpoH*, and *rseABC* (Appia-Ayme *et al*., 2011; Wells *et al.*, 2015).

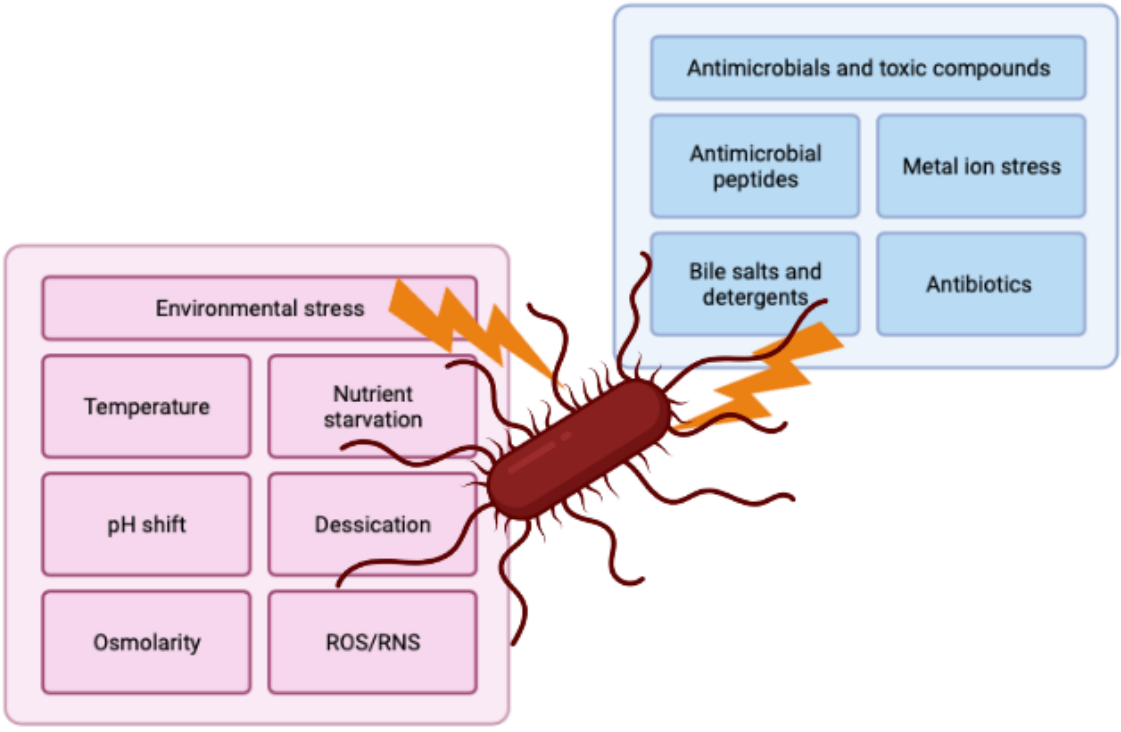

Figure 1. Chemical and physical stresses encountered by *Salmonella* in host and non-host environments. Pink boxes represent the environmental stressors faced by a bacterium, blue boxes represent stressors of an antibiotic or toxic nature faced by a bacterium. Listed stressors can be encountered in or outside of a host.

The 2CST systems are comprised of a transmembrane sensor histidine kinase (HK), located in the inner membrane, and a cytoplasmic response regulator (RR). The HK autophosphorylates upon sensing any perturbations, while the response regulator, when activated through transfer of the phosphate group from the HK to the RR, binds to specific DNA-binding sites to initiate the transcription of genes involved in various processes to restore homeostasis (Vogt and Raivio, 2011). In the absence of an inducing signal, the RR is maintained in an inactive state by the HK, which acts as a phosphatase. The 2CSTs of interest in this study are the Cpx (conjugative pilus expression) and Bae (bacterial adaptive response) responses. Using 2CSTs as therapeutic targets is advantageous as they are common in bacteria, but not in mammals, and since many ESR effectors are enzymes, general or specific sensor histidine kinase or response regulator inhibitors could therefore be used therapeutically (Guerrero *et al*., 2011; Rowley *et al.*, 2006).

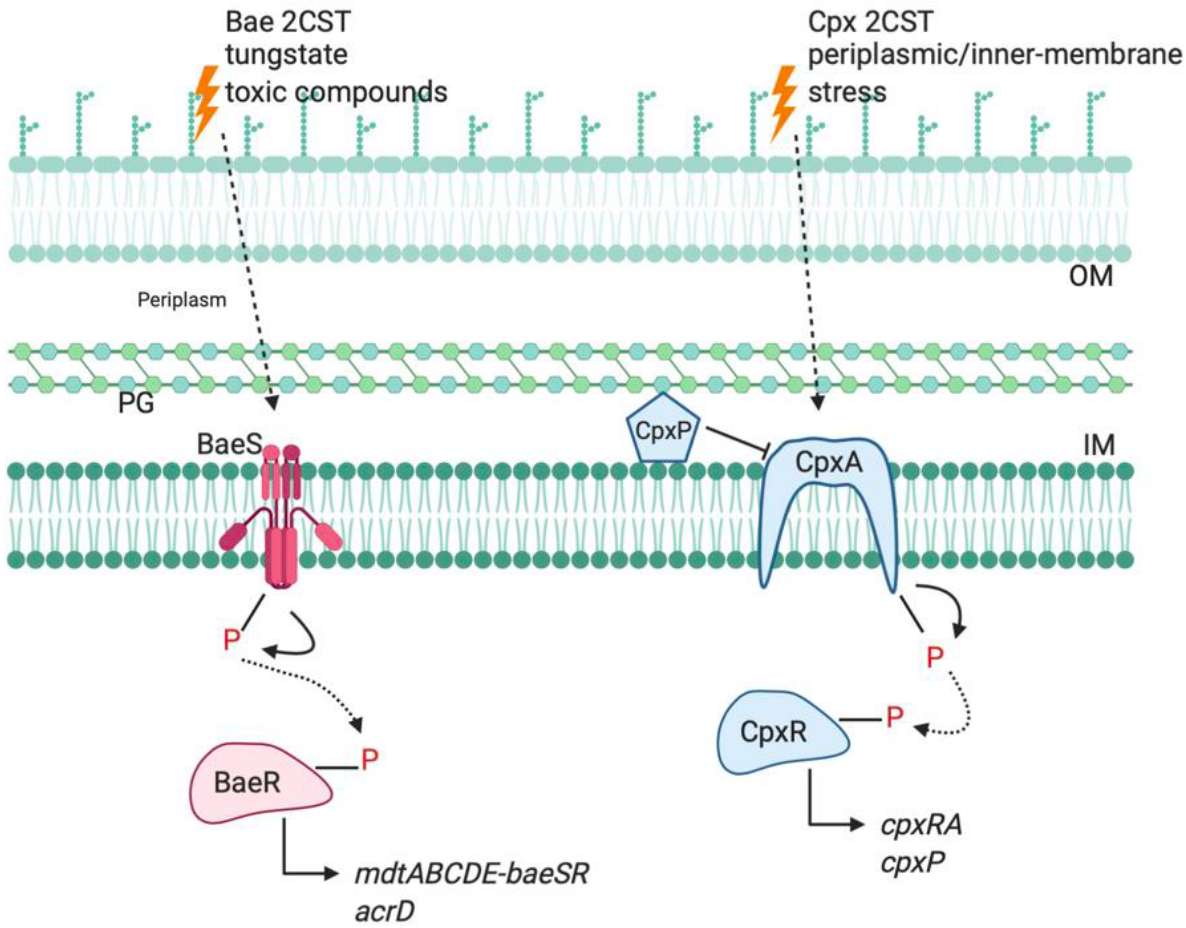

Figure 2. The BaeSR and CpxAR two-component systems envelope stress response pathways. Dashed arrows represent stress signals; dotted arrows represent movement of phosphate (P); solid arrows represent induction of genes (adapted from Runkel *et al*., 2013)

1.3.2. Cpx 2CST

In the Cpx 2CST, CpxR, the response regulator, is in an unphosphorylated state under non-inducing conditions. When induced by a specific envelope stress, such as a misfolded periplasmic protein, CpxA, the sensor kinase autophosphorylates and the phosphate group is transferred to CpxR, in a process called transphosphorylation. The activated form of CpxR, CpxR-P, binds to target regions of DNA, which initiates a response to repair or remove damaged proteins (Hews *et al*., 2019). CpxP is a periplasmic accessory protein functioning as a negative regulator for the Cpx pathway, forming a dimer with CpxA under non-inducing conditions, and dissociating from CpxA, allowing for autophosphorylation and transphosphorylation of CpxR, under inducible conditions. The regulatory cascade for both the Cpx and Bae 2CSTs can be seen in Figure 2. The Cpx 2CST has been linked to *Salmonella* virulence and functions in response to periplasmic and inner-membrane stress, namely misfolded proteins (Humphreys *et al*., 2004; Fujimoto *et al*., 2018). Phosphorylation of CpxR induces expression of *degP*, a periplasmic protease that degrades misfolded protein, as well as other genes involved in protein folding and drug efflux (Fujimoto *et al*., 2018). The Cpx pathway has a significant role in adhesion regulation and consequently in biofilm formation; In *E. coli*, activation of the pathway negatively regulates surface organelles, such as F pili, P pili, and curli, involved in adherence to abiotic surfaces (Gubbins, *et al*., 2002). Mutations in the *cpxA* gene impact the ability to form biofilms as adherence to surfaces was compromised (Dorel, *et al*., 1999). Cpx-mediated envelope stress response has been implicated in *Salmonella* gut infection. In *E. coli*, the Cpx pathway is also known to regulate adhesion and has a role in biofilm formation (Otto and Silhavy, 2002).

1.3.2. BaeSR 2CST

The Bae 2CST pathway is like that of the Cpx 2CST, whereby BaeS, the HK, autophosphorylates upon sensing an envelope stress signal, transferring the phosphate group to the response regulator, BaeR. BaeR then activates the expression of the *mdt* operon and *acrD*, which encode for the MdtABC and AcrD drug efflux systems. Contact with antimicrobials or toxic compounds, such as sodium tungstate, induces the Bae pathway and the drug efflux systems function to remove these compounds from the cell. The *mdtABCD* genes make an operon along with the *baeS* and *baeR* genes, and being immediately upstream of the *baeSR* genes, create a positive feedback loop (Nishino *et al*., 2007; Guerrero *et al*., 2011). The

Bae 2CST has been associated with resistance to antibiotics and toxic compounds, as well as increased metal resistance, all of which is due to its role in inducing drug efflux systems (Hews *et al*., 2019; Guerrero *et al*., 2011; Nishino *et al.*, 2007; Appia-Ayme, *et al*., 2011). Bae was shown to regulate drug efflux through the combined role of MdtA, and either AcrD or AcrB in heavy metal detoxification (Appia-Ayme, *et al*., 2011). In a study by Nishino *et al.* where *baeR* was overexpressed resistance to oxacillin, cloxacillin, and nafcillin, three antibiotics of the penicillin class, increased significantly (Nishino, *et al*., 2007). It has also been noted that *baeR* is more involved in resistance to a broad-spectrum antibiotic, such as ceftriaxone, than in resistance to a narrow-spectrum antibiotic, such as cephalothin (Hu *et al.*, 2005). Unlike Cpx, Bae does not contribute to establishing infection (Guerrero *et al*., 2011).

1.4. STM3030 and STM3031

1.4.1. Structures and functions

The hypothetical Yfdx-family protein, STM3030, and the outer-membrane Ail/OmpX-like protein, STM3031, are two proteins found in *Salmonella* Typhimurium. The ORFs are located 46 base pairs apart on the genome and are part of the same operon. However, results from Lin *et al.* in 2019 suggest that *stm3030* and *stm3031* do not belong to the same operon; mRNA levels of *stm3030* in a *stm3031* knockout strain and mRNA levels of *stm3031* in a *stm3030* knockout strain were similar to the mRNA levels of each gene in the wildtype strain. Results from the same study suggest that expression of the *stm3031* gene is regulated by the BaeR regulator protein of the BaeSR 2CST, while the *stm3030* gene is not as BaeR does not bind to *stm3030*. However, a study by Hu *et al.* suggests that it is the *baeR* regulator gene of the BaeSR 2CST that regulates STM3031 protein production. The study also reports that CpxR regulator protein of the CpxAR 2CST is a likely transcription factor for *stm3031* gene expression, and that CpxR also modulates BaeR. Bioinformatics studies show a down regulation of the *stm3030* and *stm3031* genes in the absence of CpxR, although no CpxR-P binding sites were located within what was predicted to be a *stm3030-stm3031* operon (Wells, 2015).

STM3030 binding stabilises STM3031 (Lin *et al*., 2019). This stabilisation function in addition to the observed chaperone function of the STY3178 *S.* Typhi homologue suggests a likely chaperone function of STM3030, and a GST-pull down assay by Lin *et al.* is in accordance with this hypothesis (Saha *et al.*, 2016).

Decreased levels of the STM3031 protein were noted in Δ*baeR* and Δ*cpxAR* strains and both genes are significantly down regulated in the absence of CpxR (Hu *et al.*, 2005; Wells, 2015). BaeR has been reported to control expression of the outer membrane protein OmpW, as well as STM3031, which have been linked to ceftriaxone resistance (Hu *et al*., 2005). All of this implies an indirect or combined modulation of *stm3031* by Cpx or Bae 2CSTs. There is limited literature on the regulation and function of STM3030 and STM3031 in *S.* Typhimurium, as well as a full characterisation of their physiological roles, however what little is known points to a role in antibiotic resistance and a potential role in the ESR.

It has been proposed that outer membrane proteins could be involved in antibiotic resistance through changes in permeability to β-lactam drugs and cooperation with efflux pumps, however most studies of antibiotic resistance mechanisms in *Salmonella* have focused on the acquisition of resistance plasmids encoding for extended-spectrum and AmpC β-lactamases (Hu *et al.*, 2005; Hu *et al.*, 2011). This is because in many Gram-negative bacteria, such as *Escherichia coli* and *Klebsiella pneumoniae*, resistance to extended-spectrum cephalosporins is linked to the production of these β-lactamases, amongst other mechanisms (Hu *et al.*, 2005). However, the efflux of β-lactam drugs by efflux pumps, such as ArcD, that cooperate with outer membrane proteins, has shown to be a significant mechanism of antibiotic resistance (Webbet & Piddock, 2003). In 2005, Hu *et al.* compared outer membrane protein expression levels in an adapted *S.* Typhimurium strain against a ceftriaxone susceptible *S.* Typhimurium strain and two outer membrane proteins, OmpW and STM3031, were identified as being influenced by *baeR* and associated with ceftriaxone resistance. As STM3031 was a newly identified protein, its overall function remained unknown with only a putative antibiotic function being assigned to it. Subsequent studies by Hu *et al.* in 2009 and 2011 further elaborated on STM3031's function in ceftriaxone antibiotic resistance, implicating it directly through upregulation by the CpxAR and BaeSR 2CSTs. The proposed antibiotic resistance mechanism of STM3031 functions by increasing ArcD efflux pump activity and reducing OmpD porin levels. AcrD is a resistance-nodulation-division (RND) efflux pump that requires an outer membrane protein to function while the OmpD porin functions an outer membrane channel (Yamasaki, *et al.*, 2011). An increase in efflux pump activity would enhance export of antimicrobial drugs out of the bacterial cell, while a decrease in porin activity would reduce the diffusion of antimicrobial drugs into the bacterial cells, reducing permeability. A biofilm formation phenotype of a STM3030 deletion strain and of a STM3030 and STM3031 double deletion strain was observed by Wells *et al*, at 30% and 55% increase of biofilm formation

respectively. STM3031 homologues in *E. coli*, OmpX, and *Yersinia pestis*, Ail, have roles in adhesion and virulence, which suggests STM3031 may also have a role in adhesion (Vogt & Schulz, 1999). Adhesion is an essential aspect of biofilm formation, and it has been hypothesised that STM3031, because of the function of its known homologues and an increase in STM3031 expression during stationary phases of bacterial growth, has a function in the transition between planktonic and biofilm forms (Wells, 2015). As the STM3030 deletion increases biofilm formation, it can be put forward that STM3030 is an adhesion suppressant that promotes the transition from biofilm form back into planktonic form. This is however putative and requires in depth analysis to confirm.

While the antibiotic resistant function of STM3031 has been relatively investigated, there is scarce information on the role and structure of STM3030. Lin *et al.*, postulated that through STM3030's role as chaperone for STM3031, the stabilisation effect on STM3031 could be what confers antibiotic resistance. This is a possibility as the homologue to STM3030 in *S.* Typhi, STY3178, has a reported antibiotic binding function and absence of STY3178 was linked to increased β-lactam antibiotic susceptibility (Saha *et al.*, 2016; Lee *et al.*, 2019). It is in fact homologues of STM3030, and even STM3031, that could shed some light on their functions

1.4.2. Homologues in *Salmonella* and other species

Homologues of STM3030 and STM3031 in *Salmonella* and other bacterial species have been studied in greater detail than in *S.* Typhimurium, allowing us to extrapolate on the known function of homologues and apply them to STM3030 and STM3031.

STM3030 belongs to the YfdX protein family, sharing a 99% sequence identity with STY3178 in the *S.* Typhi CT18 strain. YfdX family proteins are found in various pathogenic bacteria, such as *E. coli*, *Hafnia alvei*, *Shigella dysenteriae* and *Klebsiella pneumoniae*, and are implicated in multidrug resistance and are predicted as periplasmic proteins (Saha *et al.*, 2016; Lee *et al.*, 2019). STY3178 has only recently been biophysically characterised; it is a soluble protein with antibiotic binding and chaperone functions (Mondal *et al.*, 2017). Experimental modelling of STY3178 propose that it has a primarily α-helical nature, with some β-sheet elements (Saha *et al.*, 2016; Mondal *et al.*, 2017). In *S.* Typhi, Saha *et al.* used servers like Cello and LocTree to predict that STY3178 has a subcellular localisation in the periplasm. The

location of STY3178 within the periplasm indicates an ATP independent function, as ATP is absent in the periplasmic, and since periplasmic chaperones can function without ATP, STY3178's putative role as a chaperone is entirely possible. In *S.* Typhi, YfdX was proven to have a role in susceptibility to the β-lactam antibiotics penicillin G and carbenicillin; growth was significantly negatively affected upon contact with the antibiotics and survival was increased by complementation (Lee *et al.*, 2019). This reveals that STY3178, while not being functionally characterised, may not be redundant in *Salmonella* Typhi and Typhimurium.

STM3031 was identified as an Ail/OmpX-like protein, having 99% sequence identity with STY3179 in the *S.* Typhi CT18 strain. Homologues of STM3031 have been found in *Y. pestis*, with which STY3179 has 41% sequence similarity; *Yersinia enterocolitica*; *Yersinia pseudotuberculosis; E. coli*; and *Enterobacter aerogenes* (Mecsas *et al.*, 1995; Vogt and Schulz, 1999; Mouammine *et al.*, 2014; Mondal *et al.*, 2017). OmpX and Ail proteins in the bacteria mentioned previously have known roles in biofilm formation, host cell infection and pathogenesis, and resistance to antibiotics and human innate immunity (Mecsas *et al.*, 1995; Mouammine *et al.*, 2014; Mondal *et al.*, 2017; Singh *et al.*, 2020). The *E. coli ompX* gene was initially identified and sequenced by Mecsas *et al.*, revealing the outer membrane protein it encoded had an 83% sequence homology with OmpX in *Enterobacter cloacae*, prompting them to use the same name. The *S.* Typhi Ty2 homologue of STM3031, T2942, has a β-barrel structure that consists of eight anti-parallel β-sheets and four outer membrane loops (Chowdhury *et al.*, 2015). In 2015, Chowdhury *et al.* carried out infection assays on T2942 from the *S.* Typhi LT2 strain, reporting an adhesion and invasion phenotype, demonstrating the protein was essential for pathogenesis. While T2942 was shown to be important for bacterial pathogenesis, the same study concluded that STM3031 is not required for adhesion or invasion of *S.* Typhimurium LT2. The study concluded that in *S.* Typhimurium, STM3031 must utilise or collaborate with other factors for adhesion and pathogenesis. Knowledge gaps regarding the *S.* Typhimurium STM3030 protein exists as limited studies have been conducted to confirm function.

The interaction between STM3030 and STM3031 is of great interest as it is an important step in understanding bacterial virulence, considering the roles of their homologues across a variety of bacterial species. Mondal *et al.* proposed that a co-occurrence of these proteins did not mean they would necessarily interact, however they could not exclude this possibility. The computational studies they conducted confirmed the interaction of STY3178 and STY3179,

whereby a probable binding interaction between the two was identified. STY3178 indeed stabilises STY3179; however, extra-cellular loop residues of STY3179 became unstable upon binding, which they suggest could have a function in adherence to host cells, indicating a role in host-pathogen interactions (Mondal *et al.*, 2017).

1.5. Thesis overview

1.5.1. Knowledge gaps

Investigation into the molecular mechanisms of infection employed by *Salmonella* is an important aspect in understanding how this pathogen adapts and survives in its environment. It is an important step in the fight against antibiotic resistance, in the development of novel therapies, and identification of putative vaccine targets. Proteins that have potential roles in aspects of *Salmonella* survival like virulence and antibiotic sensitivity, such as STM3030 and STM3031, could prove to be a useful target. Their mediation by several ESR pathways, which have been identified as possible sources of therapeutic targets due to their critical roles in protecting *Salmonella* in challenging environments, strongly backs up their importance in pathogenicity and persistence.

1.5.2. Aims

The aim of this thesis is to explore the roles of STM3030 and STM3031 in *Salmonella* stress response, antibiotic resistance, and infection. The data gathered in this thesis could apply both *S.* Typhi and *S.* Typhimurium, as well as other enteric pathogens such as *E. coli*, *Yersinia* spp., *Shigella* spp., and *K. pneumoniae* due to the homology of the proteins in all these species. The aims of this thesis are as followed:

- Assess the function of STM3030 and STM3031 during the *Salmonella* Typhimurium ESR.
- Characterise the role of STM3030 and STM3031 in the *Salmonella* Typhimurium strain SL1344 in host cell adhesion and invasion
- Determine the contribution of f STM3030 and STM3031 to antibiotic resistance

2. Materials and methods

2.1. Materials

Chemicals and reagents used are laboratory standard grade or above, purchased from Sigma Aldrich (UK) or Thermo Fisher Scientific (UK) unless otherwise stated. All media and solutions were made using dH_2O.

2.2. Bacterial strains

The SL1344 WT (hereafter referred to as WTSL1344) strain was used as a control strain. For ease of description, SL1344 will be referred to as our 'wildtype' (WT) strain throughout this thesis. The SL1344 Δ*stm3030::Cm* (hereafter referred to as ΔSTM3030) and SL1344 Δ*stm3031::Kan* (hereafter referred to as ΔSTM3031) strains were constructed by lambda red mutagenesis with a chloramphenicol and kanamycin, respectively, cassette replacing the genes, while the SL1344 Δ*stm3030/31::Kan* (hereafter referred to as ΔSTM3030/31) strain was constructed by a p22 transduction of the single mutants to make a double mutant with a kanamycin cassette replacing the gene. This was done by a previous PhD candidate in the Rowley lab and donated for this study (Wells, 2015). The SL1344 Δ*baeR*::pCP20 (hereafter referred to as Δ*baeR*) was donated for this study by another previous PhD candidate in the Rowley lab (Appia-Ayme, *et al*., 2011). A comprehensive list of strains used in this study are described in Table 1.

Table 1: Strains used during this study

Strain	Description	Reference
***Salmonella* strains**		
SL1344	*Salmonella enterica* serovar Typhimurium	(Hoiseth and Stocker, 1981)
***Salmonella* mutant strains**		
ΔSTM3030	Δ*stm3030::Cm*	(Wells, 2015)
ΔSTM3031	Δ*stm3031::Kan*	(Wells, 2015)
ΔSTM3030/31	Δ*stm3030/31::Kan*	(Wells, 2015)
Δ*baeR*	SL1344 ΔbaeR::pCP20	(Appia-Ayme, *et al.*, 2011)

2.3. Bacterial culture conditions

2.3.1. Media

Media compositions are seen in Table 2. When needed, media was supplemented with the relevant antibiotic: chloramphenicol for ΔSTM3030 and kanamycin for ΔSTM3031 and ΔSTM3030/31. Antibiotic stocks were aliquoted in 1 mL fractions, stored at –20ºC.

Table 2: Media composition

Media	**Description**
Luria-Bertani (LB) Broth (g L^{-1})	**(Bertani, 1951)**
10 Sodium chloride 10 Tryptone 5 Yeast Extract	Dissolve in 800 mL of dH_2O. Fill up to 1 L with dH_2O and sterilise by autoclaving.
Luria-Bertani (LB) Agar (g L^{-1})	**Modified from (Bertani, 1951)**
10 Sodium chloride 10 Tryptone 5 Yeast Extract 1.5% (w/v) Agar	Prepare LB broth as mentioned. Pour 200 mL of LB and add 3g of agar. Sterilise by autoclaving.
M9 Minimal Medium (g L^{-1})	**Modified from (Maniatis, 1982)**
5X M9 Salts: 30 Disodium phosphate 15 Monopotassium phosphate 2.5 Sodium chloride 5 Ammonium chloride	Dissolve in 600 mL dH_2O, adjust to 1 L and sterilise by autoclaving.
1X M9 Minimal Media: 40 mL dH2O 10 mL M9 Salts 0.1 mL 1M Magnesium sulphate 0.5 mL Glucose 20% (w/v) 0.005 mL 1M Calcium chloride 0.05 mL Histidine	Adjust 1X M9 minimal media to 50 mL aseptically with sterile dH_2O

2.3.2. Overnight cultures

Bacterial strains were aseptically streaked onto LB agar (1.5% w/v) plates (supplemented with antibiotics where applicable (2.3.1) from DMSO stocks of Microbank™ bead stocks (2.3.3). Streaked plates were incubated overnight at 37ºC and stored at 4ºC for maximum two weeks.

Overnight cultures were made by inoculating 10 mL LB broth with a single colony picked from a streak plate using autoclaved wooden toothpicks. Cultures were incubated at 37ºC with 200-250 rpm agitation for a minimum of 12 hours.

2.3.3. Long-term strain stocks

Strains were stored at -80°C either in Microbank™ bead stocks (Pro-Lab Diagnostics), produced following manufacturer's instructions, or DMSO stocks, made by adding 1.8 mL of fresh overnight culture (2.3.2) to 50 µL DMSO (>99%), inverting to mix and freezing at -80°C.

2.3.4. Aerobic batch culture

Batch culture growth curves were carried out using 50 mL with either LB media or M9 minimal media in sterile 250 mL conical flasks inoculated with 1:100 (v/v) from overnight cultures (2.3.2). Cultures were incubated at 37ºC with 200 rpm agitation for 24 hours.

Cell density was measured by collecting 1 mL of culture at regular one-hour intervals and the optical density (OD) was measured at 600nm. If the OD_{600} > 1, 0.1 mL of the culture was diluted 1:10 (v/v) in the appropriate media before measuring the OD. The OD_{600} of the diluted culture was then calculated by correcting for the dilution factor. Growth rate was calculated by plotting log values of OD_{600} against time. The specific growth rate of the cultures was calculated using Equation 1, where *t* is time, ln is the natural log (loge), N2 is the OD_{600} value at *t*2 and N1 is the OD_{600} at *t*1. All growth curves were conducted in biological triplicates to allow for statistical analysis.

Equation 1:

$$\ln(N2/N1) = \mu(t2-t1)$$

2.4. General laboratory techniques

2.4.1. Sensitivity spot plates

Serial dilutions of overnight cultures (2.3.2) of all strains of interest, including SL1344 WT and Δ*baeR* as comparative controls, were made 10-fold in 1 x PBS to 10^{-6} using an aseptic technique. LB agar (1.5% w/v) plates (control) and the same media containing the concentration of interest of sodium tungstate were prepared and 10 µl of appropriate overnight dilutions (usually 10^{-4} to 10^{-6}) were spotted consecutively onto the control and test plates. After drying at room temperature, spot plates were incubated at 37ºC for at least 12 hours until single colonies were visible. The CFU mL-1 was calculated by multiplying colony numbers by the dilution rate. All spot plates were conducted in biological triplicates to allow for statistical analysis.

2.4.2. Kill curves

OD_{600} values of overnight cultures (2.3.2) of all strains of interest, including SL1344 WT as a comparative control, including SL1344 WT as a comparative control, were measured and used to calculate the volume needed to make a starting culture at OD_{600} 0.01. Sodium tungstate stock solutions were prepared after determining the highest concentration to be tested in the assay. In 24-well plates (Thermo Scientific), the volume of bacteria was added to LB to make up 500 µl in each well. Selected concentration of sodium tungstate was then added to each well. The plate was then placed in plate reader to measure the OD_{600} of the cultures over 24 hours with 5 secs of agitation prior to each reading. Log values of OD_{600} were plotted against time.

Data collected was analysed for statistical significance using Prism 9 software. Data was tested for normality using a Shapiro-Wilk test. Normal data was analysed using an ordinary one-way or 2way ANOVA and Tukey's or Šídák multiple comparison's test. P values for statistical significance for all tests were *P<0.0332, **P<0.0021, ***P<0.002, ****P<0.0001.

2.4.3. Preparation of antibiotic stock solutions

A suitable range of antibiotic concentrations to be tested was chosen. Stocks were then prepared by dilution antibiotic powder using dH_2O according to manufacturer's instructions. Final solution was then filter-sterilised and stored at 4ºC.

2.4.4. Minimum Inhibitory Concentration (MIC) assays

OD_{600} values of overnight cultures (2.3.2) of all strains of interest, including SL1344 WT as a comparative control, including SL1344 WT as a comparative control, were measured and used to calculate the volume needed to make a starting culture at OD_{600} 0.08. Antibiotic stock solutions using the antibiotic of interest were prepared after determining the highest concentration to be tested in the assay, stock solutions were made to be 2x this concentration (2.4.2). The antibiotics used for this method were cephalothin and ceftriaxone. In a 96-well plate, 100 µl of bacteria was added to each well. The 96-well plates (Thermo Scientific) were divided into 44 wells containing culture from one strain and 44 containing another, with 8 remaining wells containing only LB as control. In the first column, 100 µl of antibiotic stock solution was added. First column was mixed a few times and 100 µl was taken from the first column into the second column. This was repeated across the plate for the number of dilutions needed (usually 10^{-9}), resulting in a 1:2 serial dilution of antibiotic concentrations across the plate and a starting concentration of 0.04 for cultures in each well. The plate was incubated at 37ºC for at least 12 hours. The plate was then placed in spectrophotometer plate reader to measure the OD_{600} of the cultures. Log values of OD_{600} were plotted against concentration of antibiotic.

Data collected was analysed for statistical significance using Prism 9 software. Data was tested for normality using a Shapiro-Wilk test. Normal data was analysed using an ordinary one-way ANOVA and Tukey's multiple comparison's test. Non-normal data was analysed using a nonparametric Kruskal-Wallis test. P values for statistical significance for all tests were *P<0.0332, **P<0.0021, ***P<0.002, ****P<0.0001.

2.4.5. Cell lines and culture medium

The human colon adenocarcinoma Caco-2 cell line was cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 1X non-essential amino acids (NEAA) and 2 mM L-glutamine (Sigma). Cells were maintained at 37 °C in a 5% (v/v) CO_2 atmosphere.

2.4.6. Cell culture

New Caco-2 cell stocks were prepared by removing stock from liquid nitrogen storage. 1 mL solution of cells was added 5 mL supplemented DMEM for epithelial cell lines and centrifuged for 6 minutes with 1,000 rpm at 25ºC. Supernatant was removed without disturbing the pellet. Pellet was resuspended in 1 mL supplemented DMEM and then all added to 6 mL DMEM in T25 flask. Passage from cell stock was noted, passage increased on next trypsinisation of adherent cell lines. Cells were monitored and split into T25 flasks once at confluency, usually after 7 days.

2.4.7. Adhesion assays

Caco-2 cells were grown in 24-well tissue culture plates (Thermo Scientific) for 7 days to obtain a confluent monolayer. Overnight cultures (2.3.2) of all strains of interest, including SL1344 WT as a comparative control, including SL1344 WT as a comparative control, were made. The next day, supplemented DMEM was removed from Caco-2 cells and replaced with 1 mL plain DMEM (not containing FBS). To each well, 20 µl of overnight culture was added and plate was incubated for 15 minutes. After bacterial incubation of Caco-2 cells, cells were washed 3 times with PBS and 1 mL of 1% Triton was added to each well and incubated at room temperature for 10 minutes. Cell monolayer was scraped off from bottom of well plate and lysate was transferred to Eppendorf tube. Serial dilutions until 10^{-4} of lysate were made and 100 µl was plated onto LB agar plates. After drying at room temperature, spot plates were incubated at 37ºC for at least 12 hours until single colonies were visible. The CFU mL-1 was calculated by multiplying colony numbers by the dilution rate. Average number of bacterial cells were calculated. All spot plates were conducted in biological quadruplicate to allow for statistical analysis.

Separately, using overnight cultures, serial dilutions were made 10-fold in 1 x PBS to 10^{-8} using aseptic technique and 10 µl of several dilutions (usually 10^{-5} to 10^{-8}) were spotted onto LB agar (1.5% w/v) plates. After drying at room temperature, spot plates were incubated at 37ºC for at least 12 hours until single colonies were visible. The CFU mL^{-1} was calculated by multiplying colony numbers by the dilution rate. Percentage attachment of bacterial cells was calculated using CFU mL^{-1} number. All spot plates were conducted in biological quadruplicate to allow for statistical analysis. Relative adhesion was then calculated to compare percentage adhesion of mutant strains compared to wildtype.

Data collected was analysed for statistical significance using Prism 9 software. Data was tested for normality using a Shapiro-Wilk test. Normal data was analysed using an ordinary one-way ANOVA and Tukey's multiple comparison's test. Non-normal data was analysed using a nonparametric Kruskal-Wallis test. Statistical analysis for individual strains was carried out using an unpaired T test when Shapiro-Wilk test indicated normal data and a Mann-Whitney when Shapiro-Wilk test indicated non-normal data. P values for statistical significance for all tests were *P<0.0332, **P<0.0021, ***P<0.002, ****P<0.0001

2.4.8. Gentamicin protection assay

Invasion assays follow similar procedure to adhesion assays (2.4.6), but 100 µl of overnight culture is added to each well and left to incubate for 60 minutes. After bacterial incubation of Caco-2 cells, cells were washed 3 times with PBS and cells were supplemented with 100 µl mL^{-1} of gentamicin and incubated for 60 minutes. After gentamicin incubation of Caco-2 cells, cells were washed 4 times with PBS. For an extended incubation, cells were supplemented with 50 µl mL^{-1} of gentamicin and incubated for 24 hours at 37ºC. 1 mL of 1% Triton was added to each well and incubated at room temperature for 10 minutes. Cell monolayer was scraped off from bottom of well plate and the lysate was transferred to Eppendorf tubes. Serial dilutions to 10^{-4} of lysate were made and 100 µl was plated onto LB agar plates. After drying at room temperature, spot plates were incubated at 37ºC for at least 12 hours until single colonies were visible. The CFU mL^{-1} was calculated by multiplying colony numbers by the dilution rate. Average number of bacterial cells were calculated. All spot plates were conducted in biological quadruplicate to allow for statistical analysis. Same process to make serial dilutions of overnight cultures was used as for adhesion assays (2.4.6).

Data collected was analysed for statistical significance using Prism 9 software. Data was tested for normality using a Shapiro-Wilk test. Normal data was analysed using an ordinary one-way ANOVA and Tukey's multiple comparison's test. Non-normal data was analysed using a nonparametric Kruskal-Wallis test. Statistical analysis for individual strains was carried out using an unpaired T test when Shapiro-Wilk test indicated normal data and a Mann-Whitney when Shapiro-Wilk test indicated non-normal data. P values for statistical significance for all tests were *P<0.0332, **P<0.0021, ***P<0.002, ****P<0.0001.

3. The role of STM3030 and STM3031 during *Salmonella* stress responses and host cell invasion

3.1. Introduction

3.1.1. Impact of environmental stress on bacterial growth

Environmental stresses directly impact bacterial cell growth through changes in optimal growth pH, nutrient starvation, temperature, osmolarity, and other components that impact the ability of the bacteria to reproduce and maintain homeostasis. The physiology of the bacteria can change drastically according to changes in environmental conditions, which explains why in stressful conditions, bacteria grow at either slower rates or do not grow at all (Tan, *et al*., 2014). Through various ESR mechanisms, as described in 1.3.1, *Salmonella* adapts to its environment by activating specific genes, allowing for survival. Expression of virulence genes has been associated with the ability to adjust to these perturbances (Murakami, *et al*., 2004).

3.1.2. Outer membrane proteins (OMPs) and bacterial growth

OMPs in numerous bacterial species have many diverse roles and have been noted as being highly regulated in a variety of conditions, including in response to growth (Mescas, *et al.*, 1995). Culture media has also been noted to affect expression of OMPs. Some OMPs are channels responsible for the internalisation of nutrients due to their location in the OM and have significant roles in bacterial persistence (Rollauer, *et al*., 2015). Although in *E. coli* the homologue for STM3031, OmpX, has no effect on bacterial growth, no studies have been conducted in *S.* Typhimurium to verify whether this is conserved across species (Chaturvedi and Mahalakshmi, 2017; Mescas, *et al*., 1995).

3.1.3. Phenotypic characterisation using growth curves

Using molecular methods like reverse genetics, where, through recombinant DNA technology, the phenotypic effects of an altered target gene are evaluated, allows for the function of a gene of interest to be determined (Aklilu, 2021). Comparing a mutant strain of a bacteria against the wildtype strain provides further insight into the relative fitness of the mutant strain, highlighting any advantages or disadvantages conferred by gain or loss of that protein. The link between phenotype and genotype is an essential aspect of understanding bacterial physiology, including mechanisms of survival and pathogenicity. A mutation that causes a poor-growth phenotype could indicate roles in mechanisms such as survival, stress response, and virulence

as many of the pathways involved in these stress response processes overlap in function (Murray, *et al*., 2001). The CpxAR and BaeSR ESRs are known to regulate some of the same regulon members, such as *spy*, *acrD*, and *mtdA*, and as they are putative regulators for STM3030 and STM3031, for which the phenotypes are unclear, it allows for a large scope of investigation to determine roles in various ESR responses. In this study, we are in search of a function for the *Salmonella* proteins STM3030 and STM3031. Using two single mutants of STM3030 and STM3031 and a double STM3030/STM3031 mutant, 24-hour growth curves were conducted to ascertain whether either protein is involved in *Salmonella* bacterial growth. This phenotypic characterisation is important for future studies involving these proteins as it paints a bigger picture of their function in relation to ESR systems. The first part of this investigation consisted of growing the wildtype and mutant strains and conduct growth curves in LB media, while the other half consisted of growing the wildtype and mutant strains and conduct growth curves in M9 minimal media.

3.1.4. Toxic compound and antibiotic resistance functions

3.1.4.1. Toxic compounds and antibiotics

A wide variety of compounds, either natural or synthetic, can be harnessed to fight against microbes. Some of these, such as antibiotics, have been used in clinical settings against bacterial infections, operating either by killing the bacteria or inhibiting its growth. Toxic compounds, on the other hand, can be found in natural surroundings and cause environmental stress in microbes, disrupting various essential processes (Bonilla, 2020).

Toxic compounds, such as heavy metals, much like antibiotics can have damaging effects on bacterial growth or assemblage if they cannot be cleared from within the bacterial cell (Riemann & Lindgaard-Jørgensen, 1990). In moderate levels, metal ions are essential for normal bacterial metabolism, however, excess levels can be lethal. The catalytic activity of metals is what intensifies their toxicity; therefore, their levels must be carefully regulated through metal homeostatic mechanisms (Hood & Skaar, 2012). Metal homeostasis consists of a fine balance between activation of pathways to ensure import of select metal ions during metal starvation and induction of efflux pumps to export metal ions upon metal intoxication (Chandrangsu, *et al*., 2017). Limitation of essential metals within a vertebrate host poses a substantial threat to bacterial pathogens as the human body is an environment inherently

depleted of metals in terms of availability to bacteria. This is an adaptation known as 'nutritional immunity', which allows the host to mobilise metal ions while impeding bacterial access to them. Recently, it was uncovered that vertebrates can seize metals from invading microbial pathogens and utilise their toxicity against the bacteria (Skaar & Raffatellu, 2015). For this reason, bacterial pathogens have acquired mechanisms to circumvent such defensive strategies, some of which overlap with antibiotic resistance mechanisms.

Two efflux systems, MdtABC and AcrD, in *Salmonella* have recently been revealed as having previously uncharacterised functions in metal resistance; they are induced by the BaeSR 2CST, which is involved in resistance to antibiotics and toxic compounds (Appia-Ayme, *et al*., 2011). Upon receiving a signal from the BaeR RR, MdtABC and AcrD increase their efflux mechanisms and flush out the drugs. These pumps, along with AcrAB, AcrEF, and MdsABC, belong to the resistance-nodulation cell division (RND) family that form a three-part complex with periplasmic proteins and outer membrane protein channels, and have key roles in resistance to a range of compounds in Gram-negative bacteria (Yamasaki, *et al*., 2011). Overproduction of AcrD has been heavily implicated in increased drug resistance and is an efflux pump of great interest.

Prior to the discovery of penicillin, antibiotics had been unknowingly used for millennia. Records from up to 3,500 years ago have found that the Ancient Chinese, Ancient Egyptians, Ancient Romans, Ancient Greeks, Australian aborigines, and Sudanese Nubians all used antibiotic containing moulds for therapeutic benefits (Wainwright, 1989). Towards the end of the 19th century, scientists linked the development of diseases to pathogens, which lead to the amelioration of sanitation systems and increased hygiene (Zaffiri, *et al*., 2012). In 1910, the first modern antibiotic to be used clinically, Salvarsan, a synthetic arsenic-based compound used to treat syphilis, was introduced (Hutchings, *et al.*, 2019). Then with Alexander Fleming's discovery of penicillin in 1928, where he observed a *Staphylococcus aureus* colony being contaminated by the fungus *Penicillium chrysogenum*, and the subsequent availability of the antibiotic to the wider public in 1945, came the 'Golden Age' of natural antibiotic discovery (Nicolaou & Rigol, 2018). The following decades resulted in some of the fastest advances in modern medicine, causing many to say it was the end of infectious diseases as they had all been solved (Piot, 2013). Most antibiotics of clinical relevance are derivatives of natural products, such as secondary metabolites of bacteria or fungi. Antibiotics can be separated into two categories: bactericidal antibiotics, which kill bacteria, and bacteriostatic antibiotics, which

impede normal bacterial growth (Guerrero, *et al*., 2013). Secondary metabolites are produced by an organism to use for survival purposes but are not required for normal growth or reproduction (Demain & Fang, 2000). The aim of these antibiotics is to outcompete rival organisms or pathogens by inhibiting their growth or killing them; however, since the organisms producing the antibiotics would be susceptible to their own secondary metabolites, they must have genetically encoded survival mechanisms in the form of antibiotic resistance (r) genes, giving them intrinsic resistance (Rosenblatt-Farrell , 2009). These antibiotic r genes can be transferred to other bacteria in a population through 'promiscuous' gene transfer systems, where genes are shared either via conjugal plasmids, transposons, or integrons. This aspect is of greatest importance as antibiotic r genes present in natural bacterial populations represent a large portion of the r genes reported in antibiotic resistant bacteria (Davies & Davies, 2010).

During Fleming's Nobel Prize acceptance speech in 1945, he loosely predicted the dawn of the antibiotic resistance era, stating that the misuse of antibiotics could lead to resistant bacteria (Fleming, 1945). He was quickly proven right as penicillin resistant bacteria with the ability to inactivate the drug became prevalent by 1947 (Miller, 1947). Remarkably, bacterial penicillinases (β-lactamases), enzymes responsible for resistance to penicillin, were observed in 1940, prior to the introduction of therapeutic use of penicillin, begging the question: what originated first, the antibiotic or the antibiotic resistance? As antibiotic r genes are a natural component of bacterial populations and they can be easily exchanged between bacteria, it is inevitable for these resistance genes to eventually be found in all different species of bacteria. Misuse of antibiotics does not itself create antibiotic resistance within bacteria, instead, it selects for already resistant bacteria and effectively increasing their proportional relevance (Rosenblatt-Farrell, 2009). Antibiotic resistance has now been declared by the United Nations as a fundamental threat to human health, development, and security and is one of the top 10 global public health threats.

Functional overlaps between resistance to antibiotics and toxic compound in efflux pumps removal exist, making them a promising target for investigations regarding multidrug resistance in *Salmonella* (Munita & Arias, 2016). As MDR is on the rise and there is a decline in discovery of antibiotic agents, investigation into intrinsic resistance to antibiotics has garnered attention.

3.1.4.2. Sodium tungstate and BaeSR

Sodium tungstate (Na_2WO_4) is an inorganic compound and an intermediate of the metal tungsten. Tungsten is a rare metal and is the heaviest element with a biological function, although it is toxic to most organisms (Smart, *et al*., 2009). Tungsten is usually present in the environment as highly soluble tetrahedral oxyanions, namely tungstate (WO_4^2). In bacteria with no metabolic function for tungsten, tungstate affects with the bacterial envelope and may perturb metalloenzyme stability (Appia-Ayme, *et al.*, 2011). Tungsten is a competitive inhibitor of molybdenum due to similarity in their electrochemical properties; tungstate is able to replace molybdenum in the molybdenum cofactor, which used by some bacteria in their respiratory processes, and efficiently inhibit the production of energy during aerobic respiration (Zu, *et al*., 2011)

The BaeSR was first discovered in *E. coli* in 1993 by Nagasawa *et al.* while screening for other 2CSTs and categorised as an envelope stress response by Raffa and Raivio in 2002, however no function of the 2CST had been identified. The BaeSR regulon was also confirmed to contain the *acrD* gene and the *mdtABCD-baeSR* operon, which encode for drug efflux pumps. A functional overlap between the BaeSR and CpxAR 2CSTs was subsequently revealed as they both mediate induction of *acrD* and *mdtABC* by binding to different sites in the promoter regions of *acrD* and *mdtA* (Hirakawa, *et al*., 2005). However, it was noted that BaeR is the primary regulator with CpxR enhancing the effects of BaeR. The BaeSR 2CST was consequently hypothesised to have a principal function of upregulating efflux pumps as a response to damaging compounds.

Although BaeSR had a known purpose of maintaining drug efflux, there were few phenotypes related to loss of the BaeSR RR, BaeR, prompting further investigation into inducing conditions not yet known. Phenotypic microarrays conducted in *E. coli* found that a *baeSR* mutant demonstrated growth sensitivity to presence of sodium tungstate, amongst other metals (Zhou, *et al*., 2003). To confirm results gathered from phenotypic microarrays, minimum inhibitory concentration (MIC) assays were performed to test the ability of sodium tungstate to induce the BaeSR pathway. Assays revealed sodium tungstate as a substrate for the MdtABC efflux pump (Leblanc, *et al*., 2011). The conserved nature of ESR pathways across some bacteria has driven researchers to inspect whether phenotypes in one species are reproducible in others.

In *S.* Typhimurium, the BaeSR 2CST is observed to have similar functions as in *E. coli* and the finding of sodium tungstate as a novel inducer for BaeSR in *E. coli* lead to investigation of the phenotype in *S.* Typhimurium. In 2011, Appia-Ayme *et al.* established that sodium tungstate was in fact an inducer of BaeSR as sodium tungstate inhibits growth of a *S.* Typhimurium *baeR* mutant, and that an overlap in MdtA, AcrD, and AcrB efflux pump function in detoxification of sodium tungstate exists. Similar to results gathered by Leblanc *et al.*, CpxR regulation of the MdtA and AcrD efflux pumps is auxiliary to BaeR regulation. These results are an indication of *S.* Typhimurium preserving mechanisms of tungstate waste disposal throughout generations as it would have encountered it in its natural environment. Additionally, it was reported that BaeR was not necessary for murine Typhoid infection, making BaeR first envelope stress regulator not needed for establishing infection. This implies BaeSR 2CST function is firmly rooted in regulation of drug efflux.

3.1.4.3. Cephalosporins

Cephalosporin antibiotics are a class of β-lactam antibiotics all based on cephalosporin C. Cephalosporin C is a natural antibiotic isolated from the aerobic mould *Cephalosporium acremonium* in 1945 by Giuseppe Brotzu near a sewage outlet in Sardinia, Italy (Greenwood, 2010). Structurally, cephalosporins have a β-lactam ring fused to a six-membered dihydrothiazine ring, instead of a five-membered dihydrothiazine ring as in penicillins. Introduction of new groups into the cephalosporin C nucleus, 7-aminocephalosporanic acid, allowed for the assembly of the new variations of semisynthetic compounds (Aronson, 2016). Based on cephalosporin C, more than 20 semisynthetic cephalosporins have since been generated and marketed clinically, though many have been discontinued. Much like penicillin, cephalosporins promote breakdown of the bacterial cell wall by binding to penicillin-binding proteins, rendering the cell wall defective and osmotically unstable (Scholar, 2007; Rawls, 2014). β-lactam antibiotics all have a ring structure similar to that of the D-alanine-D-alanine dipeptidase; ordinarily, D-alanine-D-alanine is the substrate of transpeptidase or penicillin binding proteins (PBPs), however due to their resemblance, β-lactam antibiotics bind to PBPs instead. PBPs are essential enzymes for peptidoglycan (PG) synthesis and disruption of the PG compromises cell well integrity, leading to cell death (Li, *et al.*, 2018).

The various cephalosporins have been categorised by their similar antimicrobial properties into five 'generations'. First-generation cephalosporins are most effective against Gram-positive bacteria and much less effective against Gram-negative bacteria; they are no longer widely used. Successive generations have greater efficacy against Gram-negative bacteria (Greenwood, 2010). Of interest to this study are cephalothin, a first-generation cephalosporin, and ceftriaxone, a third-generation cephalosporin. Cephalothin is a narrow-spectrum antibiotic and greatly effective against Gram-positive infections and largely ineffective against Gram-negative bacteria, akin to other first-generation cephalosporins (Scholar, 2007). It is a semisynthetic cephalosporin with a chemical formula of $C_{16}H_{16}N_2O_6S_2$. It is predominantly used as prophylactic treatment to prevent infection rather than treat it. Ceftriaxone, on the other hand, is a broad-spectrum antibiotic and has much superior activity against Gram-positive and Gram-negative (Rawls, 2014). It is also semisynthetic cephalosporin with a chemical formula of $C_{18}H_{18}N_8O_7S_3$. Ceftriaxone, along with fluoroquinolone, is currently the recommended antibiotic for treatment of invasive *Salmonella* infections or to treat patients at risk of developing an invasive *Salmonella* infection (Cuypers, *et al*., 2018).

3.1.4.4. Antibiotic resistance and 2CSTs

Antibiotics, as they are perceived by the bacterial cell as an environmental stressor, are responded to accordingly. As mentioned in 3.1.2., in *S.* Typhimurium, the BaeSR 2CST reacts to toxic compounds as well as antibiotics by regulating drug efflux pumps. Contact with antimicrobials or toxic compounds stimulates the BaeSR pathway and drug efflux pumps remove these compounds from the cell. The *mdtABCD* genes make an operon along with the *baeS* and *baeR* genes, and being immediately upstream of the *baeSR* genes, create a positive feedback loop (Nishino *et al*., 2007; Guerrero *et al*., 2011).

Additionally to having a role in drug efflux maintenance, BaeSR has been linked to antibiotic resistance both in *S.* Typhimurium and *E. coli* (Nishino, *et al*., 2007; Wang, *et al*., 2021). Although the mechanism by which BaeSR confers resistance in *E. coli* is unknown, in *S.* Typhimurium it is thought to do so by controlling expression levels of several OMPs, AcrD, and MdtABC (Hu *et al.*, 2005; Hu *et al.*, 2011). Resistance to β-lactam antibiotics, like cephalosporins, can be acquired through varied means; generally, this happens through production of β-lactamases, which destroy β-lactams before their PBP targets are reached. Alternatively, bacteria can modify the target PBPs, which causes the β-lactam antibiotic to lose

affinity for the PBPs. Lastly, through alteration of outer membrane permeability and increase of efflux pump activity, the antibiotic can be prevented from reaching the targeted PBPs (Li, *et al*., 2018). Antibiotic resistance mediated by the BaeSR 2CST is achieved through this latter process.

BaeSR is not the only 2CST involved in antibiotic resistance in *Salmonella*, the CpxAR 2CST has also been associated with it. In the absence of the ArcB efflux pump, the overexpression of the CpxAR RR, CpxR, can lead to β-lactam resistance (Li, *et al.*, 2018). CpxR is also a modulator of BaeR function, though, as mentioned previously, it is secondary to regulation of *acrD* and *mdtABC* by BaeR through mechanisms of crosstalk. Independently, however, in clinical isolates CpxR contributes to β-lactam, along with other antibiotic types, resistance by inducing other multidrug resistance genes (Huang, *et al*., 2016).

The functional overlap between the BaeSR and CpxAR 2CSTs proves to be an important aspect of antibiotic resistance in *S.* Typhimurium, with BaeSR also being essential for toxic compound waste removal. These roles have underscored the possibility of using 2CSTs as antibiotic targets in the search of alternatives, as they are not present in mammals and virtually ubiquitous in prokaryotes (Guerrero, *et al.*, 2013).

3.1.4.5. Roles of STM3030 and STM3031 in antibiotic resistance

Previous studies by Lin *et al.* in 2019, Hu *et al.* in 2005, 2009, and 2011 reported STM3030 and STM3031 as having functions in resistance to cephalosporins and fluoroquinolones. In this study, a first- and second-generation cephalosporin, cephalothin and ceftriaxone; and a second-generation fluoroquinolone, ciprofloxacin, were used.

3.1.5. Adhesion and invasion functions

3.1.5.1. Bacterial adhesion

Bacterial adhesion is the crucial first step in the processes of host colonisation, invasion, and biofilm formation, making it a key mechanism of pathogenesis for practically all bacterial infections (Klemm, *et al*., 2010). Though the primary method by which *Salmonella* initially enters a host is through ingestion by the host of contaminated items, once in the intestinal

lumen, to invade host cells and establish disease *Salmonella* must adhere to host cell surfaces. As an intracellular pathogen, *Salmonella* has a principal goal of being internalised as the intracellular lifestyle confers various advantages, ranging from evasion of host immune defences to access to a wider selection of nutrients (Ribet & Cossart, 2015).

Adhesive molecules, often classified as adhesins, expressed by bacterial cells interact with coordinating receptors or macromolecules, such as proteoglycans, of host tissues or cells, securing the attachment of the bacterial cell to the host cell (Kline, *et al*., 2009). Different *Salmonella* serovars express different adhesion factors, contributing to the different disease types caused by the various serovars (Velge, *et al*., 2012). Specific adhesins have been categorised as virulence factors in bacterial pathogens that infect mucosal tissues such as the gastrointestinal tract (Klemm, *et al*., 2010). Many adhesins are singular proteins found on bacterial surfaces and some are found at the ends of flagella, fimbriae, or pili, which are thread-like organelles that protrude from the bacteria and allow *Salmonella* cells to attach to epithelial cells in various host species (Klemm, *et al*., 2010; MacKenzie, *et al*., 2017). Fimbriae are described as pili that have a main bacterial attachment function with binding specify conferred by adherence factors or adhesins located at the tip of the scaffold (Pizarro-Cerdá & Cossart, 2006). Though flagella and fimbriae are involved in intestinal colonisation, neither are fully indispensable (Gast & Porter, Jr, 2020). Alongside flagellar, fimbrial, and pili adhesins, other components such as outer membrane proteins (OMPs) have been suggested as adhesins and virulence factors, though they have been less studied than the former (Velge, *et al*., 2012). Furthermore, interactions of *Salmonella* OMPs with host cells have shown to trigger both the innate and adaptive responses of a host (Galdiero, *et al*., 2003). Adhesins prepare the bacterial cells for invasion of the target host cell, which is carried out by the T3SS-1 needle complex (MacKenzie, *et al.*, 2017). The T3SS-1 translocon, a protein complex responsible for the translocation of effector proteins into the host cell, binds to the cholesterol on the host cell plasma membrane with high affinity and is integral for *Salmonella* virulence (Hayward, *et al.*, 2005). It has been reported that while *Salmonella* is not particularly adhesive, the invasion mechanisms are highly efficient, illustrating the importance of how bacteria attach to host cells to initiate their internalisation (Pizarro-Cerdá & Cossart, 2006). Pathogens that through evolution have lost their adhesins are known to become avirulent and some even commensal, illustrating their importance in bacterial pathogenesis (Klemm, *et al*., 2007).

3.1.5.2. Bacterial invasion

Entry into both phagocytic and nonphagocytic host cells is the ultimate objective for *Salmonella* as pathogens have adapted to invade bacteria to take advantage of host cells. The ability to invade host cells and replicate within them is an important aspect of infection for intracellular pathogens and their virulence depends on it (Steele-Mortimer, 2008; Tang, *et al*., 1993). Invasion is a two-step process that includes the initial attachment to the host cell and the ensuing internalisation, the latter cannot occur without the former. Cellular internalisation can be achieved in two ways, bacteria can either allow themselves to be passively phagocytosed by phagocytic cells or can induce their invasion into nonphagocytic cells using internalisation mechanisms; however, *Salmonella* can also forcefully invade nonprofessional phagocytes (Ribet & Cossart, 2015).

Professional phagocytes, including macrophages, dendritic cells (DCs), neutrophils, enterocytes, and M cells from Peyer's patches, while being a frontline defence strategy for the host, provide a most appealing niche for *Salmonella* due to their natural phagocytic functions. *S.* Typhimurium exploits M cells to cross the intestinal barrier by destroying them and gaining access to deeper tissues before an immune response can be initiated (Ribet & Cossart, 2015). Another method by which *S.* Typhimurium quickly traverses the host intestinal epithelium is by invading inter-epithelial DCs and reaching the bloodstream, which results in dissemination of *Salmonella* within in the host (Ribet & Cossart, 2015). These strategies allow *Salmonella* to effectively replicate and create disease within the host, and while large quantities of bacteria are killed during bacterial invasion, this triggers an additional inflammatory response that benefit surviving bacteria, maximising their virulence within the host (Ribet & Cossart, 2015).

Invasion of nonphagocytic intestinal epithelial cells is induced by type III secretion system (T3SS), mainly referred to as T3SS-1, effectors, of which there are over 30. This T3SS is encoded within the *Salmonella* pathogenicity island I (SPI-1), a key determinant of *Salmonella* virulence, which contains the genes of various effector proteins responsible for entry of *Salmonella* into host cells (Fàbrega & Vila, 2013). Contact of the bacteria with nonphagocytic host cell surfaces initiates entry into the cell using the "trigger" mechanism (as described in section 1.2.1) of the T3SS-1, which leads to extensive cytoskeletal rearrangements of the host cell membrane and signature ruffling of the cell membrane, translocation of virulence effector proteins, and subsequent entry into the host cell (Fàbrega & Vila, 2013). Principal T3SS-1

effectors include SipA, SipC, SopB, and SopE2; while SipA and SipC bind to host actin to stabilise and cause their rearrangements, respectively, SopB, SopE, and SopE2 regulate actin activity indirectly by activating Rho GTPases (Malik-Kale, *et al*., 2011). Intriguingly, normal cellular architecture is regained once *Salmonella* has successfully invaded a cell, signifying that the triggering of host Rho GTPases, which is responsible for actin reorganisation, and the actin polymerisation of host cells is a temporary occurrence (Pizarro-Cerdá & Cossart, 2006).

It has long been accepted that T3SS-1-dependant invasion was the sole way nonphagocytic host cells were entered by *Salmonella*. Recently however, *in vivo* studies in bovine, chicken, and murine models have produced results that contradict this and imply that infection can be caused independently of the T3SS-1 (Velge, *et al*., 2012). Mutant SPI-1, which encodes the T3SS-1, *Salmonella* strains that were unable to invade polarised epithelial cells could still invade murine M cells, implicating unknown invasion factors in intestinal colonisation (Clark, *et al.*, 1996). Further findings point to some T3SS-1-independent cell entry mechanisms as being involved in cell specificity and improved entry into different cell types (Velge, *et al*., 2012). Other studies have discovered various uncharacterised *Salmonella* invasion factors, pointing to a host of unknown elements of *Salmonella* infection (Velge, *et al.*, 2012). These results highlight a vast number of unknown virulence processes, which are of great scientific interest as bacterial adhesion is a major aspect in understanding interactions between bacteria and their hosts. The reasons why *Salmonella* developed other invasion mechanisms are undetermined, however, a hypothesised function of support to T3SS-1 invasion mechanism has been proposed, suggesting that these invasion adhesion mechanisms, while being T3SS-1-independent, provide host and cell specificity (Malik-Kale, *et al*., 2011). Though studies have shown T3SS-1-independent *Salmonella* invasion, these have mainly been *in vitro* studies using cell culture techniques, which limits our understanding of *in vivo* relevance to pathogenesis (Chowdhury, *et al.*, 2015).

As disruption of proper adhesion functions affects bacterial virulence, adhesion factors may be useful targets for drug and vaccine development (Velge, *et al*., 2012; Chowdhury, *et al*., 2015). Bacterial adhesion to host cells could be inhibited by blocking adhesin receptors through immunisation of a host or through development of a drug that target adhesins specifically, which would be a promising new generation of anti-bacterial drugs in an age of antibiotic resistance (Klemm, *et al.*, 2010).

3.1.5.3. Adhesion in biofilms

While adhesion is imperative for bacterial infection, it is also of great importance to survival outside the host. Adhesins allow bacteria to attach to both biotic and abiotic surfaces, meaning abiotic surfaces such as glass, plastic, and metal provide bacteria with a surface to attach to for improved survival in abiotic environments. Attachment to these abiotic surfaces permits bacteria to initiate a shift from a unicellular planktonic state to form multicellular sessile colonies known as biofilms. Biofilms, a term coined by Bill Costerton in 1978, were initially assumed to be simple conglomerations of cells, however advanced imaging techniques and fluorescent stains have shown that biofilms are complex and dynamic heterogenous three-dimensional systems surrounded in a self-produced extracellular polymeric matrix (Hall-Stoodley, *et al*., 2004; Donlan, 2002). Physiologically, bacterial cells within a biofilm are different to planktonic cells, whereby genetically identical cells express different genes that result in subpopulations of functionally diverse bacterial cells (Vlamakis, *et al*., 2013; Ramachandran, *et al*., 2016). This matrix, which is extracellular material, accounts for about 90% of the entire biofilm mass, whereas the bacterial cells make up the remaining 10%, illustrating the importance of proper matrix formation for biofilm structure (Flemming & Wingender, 2010). What sets biofilms apart in terms of scientific research interest is their ability to resist antimicrobials and survive for long periods of time in harsh environments, without the bacteria losing their ability to immediately regain normal function by going through controlled lysis, going on to cause infection as well as biofilm dispersal (Paytubi, *et al.*, 2017). The mechanisms behind this aggregation of cells are also poorly understood, contributing to the pressing need for exploration into this topic. The public health implications of biofilms are far reaching and alternative strategies to prevent their formation need to be investigated. While most research has focused on single-species biofilm formation, it is important to note that in their natural environments, biofilms are polymicrobial communities (Vlamakis, *et al.*, 2013).

Adhesion of bacteria to a suitable surface is the first step in biofilm formation, followed by the formation of a microcolony and eventually of a mature biofilm, a process which is reversible (Klemm, *et al*., 2010). Curli and cellulose are two essential biofilm production factors in *Salmonella*, although curli has proven to be more important than cellulose (Paytubi, *et al*., 2017). Disruption of these elements result in morphologically distinct biofilm colonies when grown on Congo red agar plates in a laboratory setting (Jonas, *et al*., 2007). Up to 90% of all bacteria found in non-host environments are thought to remain in these biofilm communities,

which are known to help withstand challenging conditions (Klemm, *et al*., 2010). Optimal conditions for inducing biofilm production are in aerobic conditions with low nutrient availability, low osmolarity, and a low temperature of 28°C; other factors such as pH and presence of antimicrobials also affect their production (Fàbrega & Vila, 2013). In different conditions, two distinct biofilm types can form; in low osmolarity rich medium and a low temperature of 25°C, a pellicle biofilm forms, which is found at the air-liquid interface; in minimal medium, the biofilm switches from a pellicle biofilm to a bottom biofilm, which is found at the solid-liquid interface (Paytubi, *et al*., 2017). This reason for this phenotype switching, though it remains unclear, could be that it confers certain adaptational advantages to *Salmonella* communities, allowing them to deftly acclimate to challenging conditions.

The ability to aggregate in biofilms comes with many benefits, ranging from survival in demanding conditions to causing chronic infections. Formation of biofilms in flow environments, for example, protects bacteria from their removal by hydrodynamic shear forces (Klemm, *et al*., 2010). The configuration of the biofilm matrix, which is composed of up to 97% water, efficiently protects cells from desiccation in dry environments (Berlanga & Guerrero, 2016). The ability to withstand removal by strong forces and survive in dry environments, amongst others, is the perfect combination for persistence within industrial and clinical settings. Various *Salmonella* serovars are known to contaminate work surfaces, machinery, and pipes and drains with biofilms, which in various instances has led to repeated outbreaks of *Salmonella* infections in farms, hospitals, and factories (Klemm, *et al.*, 2010). Though adhesion to abiotic surfaces is a major issue, the ability to adhere to biotic surfaces such as plant surfaces, animal epithelial cells, gallstones, and even cow udders to produce biofilms poses an even greater threat (Paytubi, *et al*., 2017; Wood, *et al*., 1991). Consumption of contaminated fresh vegetables, fruits, milk, and, infamously, raw chicken and eggs have resulted in outbreaks of *Salmonella* due to biofilms have been recorded all over the world (MacKenzie, *et al*., 2017). Outbreaks have also been attributed to food products that are either processed or are in extremely dry conditions such as peanut butter, pistachios, and ready meals (MacKenzie, *et al.*, 2017). Data shows up to 40% of human and livestock bacterial diseases are biofilm-related, emphasising the importance of addressing biofilms in a research capacity (MacKenzie, *et al*., 2017). *S.* Typhi biofilms coating human gallstones have been linked to chronic typhi carriers, where in many cases the host remain asymptomatic and can continue to shed bacterial cells, increasing transmission; the production of biofilms in gallstones has been found to be induced by the presence of bile (MacKenzie, *et al*., 2017). *In vitro*, *S.* Typhimurium

has also been found to form biofilms on gallstones, demonstrating the functional protection of biofilms against elevated bile concentrations (Fàbrega & Vila, 2013).

As previously mentioned, biofilms grown on Congo red agar plates in a laboratory setting exhibit distinct colony morphology, particularly upon disruption of components essential for biofilm formation; the Congo red, an azo dye, accumulates in rdar colonies in presence of curli (MacKenzie, *et al.*, 2017). Normal *Salmonella* biofilms will present with a red, dry, and rough (rdar) morphology, whereas biofilms with a defect in cellulose synthesis exhibit a brown (bdar) morphology, those with defect in curli expression exhibit a pink (pdar) morphology, and those with defects with both curli and cellulose production exhibit a smooth and white (saw) morphology (Jonas, *et al*., 2007). The distinctly recognisable rdar morphotype makes visual assay-based investigation into biofilm formation simple to carry out without needing expensive equipment. Though biofilms formed by both *S.* Typhi, and Typhimurium have been associated with chronic persistence in humans, some studies on *Salmonella* grown on Congo red agar plates have uncovered a link between the inability to form biofilms and increased host adaptation. The rdar morphotype was associated with non-typhoidal *Salmonella* serovars, such as *S.* Typhimurium and Enteritidis, associated with gastroenteritis, whereas in serovars that are host restricted, such as *S.* Typhi and other invasive isolates, the rdar morphotype was not observed (MacKenzie, *et al*., 2017; Römling, *et al*., 2003). Furthermore, a study on a novel iNTS causing *S.* Typhimurium isolate, ST313, revealed that much like *S.* Typhi, ST313 was unable to form biofilms (Ramachandran, *et al*., 2016). A study by MacKenzie *et al.* in 2017 reported that invasive *Salmonella* serovars, including *S.* Typhi and iNTS causing *S.* Typhimurium, are associated with a restricted host range, meaning they cause infection in fewer host species compared to other non-invasive *Salmonella* serovars. This difference in ability to form biofilms could be due to the difference in infection strategies employed by typhoidal and non-typhoidal *Salmonella* serovars. Generally, *S.* Typhimurium aims to cause inflammation and activate host immune systems to mediate infection, whereas *S.* Typhi avoid this in order to create a systemic disease in combination, all these results point to the importance of proper biofilm formation for *S.* Typhimurium virulence in human and animal hosts.

A proposed hypothesis to explain why *Salmonella* forms biofilms is that they help with persistence, particularly outside the host, by protecting bacteria against unfavourable conditions through use of various survival and resistance mechanisms (Fàbrega & Vila, 2013). The combination of purported mediation of transmission by biofilms from one host to another

and the expression of virulence genes being linked to biofilm formation firmly establish biofilms as virulence factors for *S.* Typhimurium (Fàbrega & Vila, 2013). In *S.* Typhimurium, the role of biofilms within the host has yet to be confirmed, unlike in *S.* Typhi, however *in vitro* results on biofilm formation on gallstones, as mentioned previously, are promising and *in vivo* studies must be conducted.

3.1.5.4. Adhesion and invasion in bacterial homologues

Adhesion and invasion are an important aspect of infection for many pathogenic bacteria; the common methods in which bacteria can infect hosts means several infection mechanisms are homologous throughout various bacterial species. This commonality allows scientists to apply knowledge from one species to another as a means of characterising previously unknown factors and getting a better understanding of how bacterial infection works across species.

In *E. coli*, at least two non-fimbrial outer membrane proteins, Rck and PagN, have been implicated in T3SS-1-independent invasion that use a "zipper" mechanism to enter cells. Unlike the "trigger" mechanism, the "zipper" mechanism does not inject effectors into the host cell and instead bacteria surface proteins interact with host proteins to induce the rearrangement of the cytoskeleton and membrane, after which the bacteria are internalised (Ribet & Cossart, 2015). This highlights the possibility of an alternative mode of entry facilitated by outer membrane proteins in *Salmonella*, as homologues for these two OMPs have been observed in *S.* Typhimurium. Other OMPs in different species involved in cell attachment and invasion are Rck and PagC in *Salmonella*, Lom in *E. coli*, Ail in *Yersinia enterocolitica*, and OmpX in *Enterobacter cloacae* (Heffernan, *et al*., 1992). However, some known components that are usually adhesins in one species, in others have been shown to be involved in entry into professional and nonprofessional phagocytes, demonstrating variability of functions between species (Velge, *et al*., 2012).

3.1.5.5. Homology in *Salmonella* serovars

All pathogenic *Salmonella* serovars possess numerous adhesin gene clusters, some of which have been associated with pathogenesis, that encode fimbrial and non-fimbrial adhesins (Malik-Kale, *et al*., 2011; Chowdhury, *et al*., 2015). Though *Salmonella* serovars may share similarities in genetic sequences, adhesins and invasins from one serovar may have a different

function in another. For example, adhesion and invasion are two differentially regulated activities, although very interconnected in *S.* Enteritidis (Gast & Porter, Jr, 2020).

In *S.* Typhi, though genomic sequence analysis has identified fimbrial operons, experimental evidence for their involvement in adhesion and invasion is limited. (Townsend, *et al*., 2001). A study by Chowdhury *et al.* in 2015 identified, through protein BLAST searched, T2942, an *S.* Typhi homologue for *Salmonella* Ail-like proteins that could be involved in adhesion or invasion. The *S.* Typhi T2942 protein has a 100% sequence homology with the *S.* Typhimurium STM3031 protein, which is an Ail/OmpX-like protein. Further bioinformatic analysis of T2942, while using the *Y. pestis* Ail protein as a template, predicted a β-barrel structure comprised of eight anti-parallel β-sheets and four outer-membrane loops and an N-terminal signal peptide (see Fig. 1). T2942 was subsequently confirmed as an OMP with an expected function in adhesion and invasion due to its sequence similarity to the *Y. pestis* Ail OMP. Assays to assess involvement of both T2942 in *S.* Typhi and STM3031 in *S.* Typhimurium in *Salmonella* pathogenesis revealed a role for T2942 in bacterial adhesion and invasion independently of T3SS-1, but not for SMT3031 (Chowdhury, *et al.*, 2015). Chowdhury *et al.* also revealed that a 20-amino acid extracellular loop and three extracellular loop regions of T2942 were specifically involved in host cell invasion and adhesion, respectively. In conclusion, in the *S.* Typhi Ty2 strain, T2944 was shown to be a standalone adhesin and invasin, essential for epithelial adhesion and invasion. The apparent lack of involvement in infection by STM3031 was hypothesised to be because *S.* Typhimurium uses other factors to mediate adhesion and invasion, although the specific factors were not mentioned (Chowdhury, *et al.*, 2015).

3.2. Aim

The aims of this study are to investigate the roles of STM3030 and STM3031 in relation to bacterial growth; resistance to the toxic compound sodium tungstate and antibiotics cephalothin and ceftriaxone; and involvement in adhesion and invasion of human cells when knocked out. The first aim is to do growth curves in LB media and in M9 minimal media; the second aim is to test sodium sensitivity using spot plates and kill curves, then to test antibiotic resistance using MICs; the third aim is to carry out infection assays to assess adhesion and invasion of STM3030 and STM3031 deficient bacterium in human Caco-2 cells.

3.3. Results

3.3.1. Loss of STM3030 and STM3031 has no negative impact on the growth of *S.* Typhimurium SL1344 in LB media

In this study, we are in search of a function for the *Salmonella* proteins STM3030 and STM3031. Using two single mutants of STM3030 and STM3031 and a double STM3030/STM3031 mutant, 24-hour growth curves were conducted to ascertain whether either protein is involved in *Salmonella* bacterial growth. This phenotypic characterisation is important for future studies involving these proteins as it paints a bigger picture of their function in relation to ESR systems. The first part of this investigation consisted of growing the wildtype and mutant strains and conduct growth curves in LB media, while the other half consisted of growing the wildtype and mutant strains and conduct growth curves in M9 minimal media. All growth curves were performed in biological and technical triplicates for both LB media and M9 minimal media growth curves to allow for statistical analysis.

Although no reported growth phenotypes have been observed in STM3030 and STM3031 homologues in other bacterial species, it is important to measure this in our own system to ensure that this is not a cause of any potential phenotypes observed in subsequent experiments.

The effects of the loss of STM3030 and STM3031, individually and together, on *S.* Typhimurium growth were investigated by growing them in LB batch culture. WTSL1344, ΔSTM3030, ΔSTM3031, AND ΔSTM3030/31 were grown over a 24-hour period as described in 2.3.4. Readings of the OD_{600} values were taken every hour during a consecutive eight-hour period of growth and a final reading at the 24-hour time point, the natural log (ln) was plotted against time (Figure 3). The specific growth rate (μ) was calculated using Equation 1 (2.3.4). Growth rates for WTSL1344, ΔSTM3030, ΔSTM3031, and ΔSTM3030/31 were $\mu=0.70\ hr^{-1}$, $\mu=0.69\ hr^{-1}$, $\mu=0.70\ hr^{-1}$, $\mu=0.68\ hr^{-1}$, respectively. No significant difference between strain growth rates (μ) were noted during growth in LB broth.

Visually, there seemed to be a slight deceleration in growth for the ΔSTM3031 strain around the fourth and fifth hour, which is recovered around the sixth hour (Figure 4). However, statistical analysis using one-way ANOVA showed no significant difference between the isogenic parent strain SL1344 and any of the mutant strains tested.

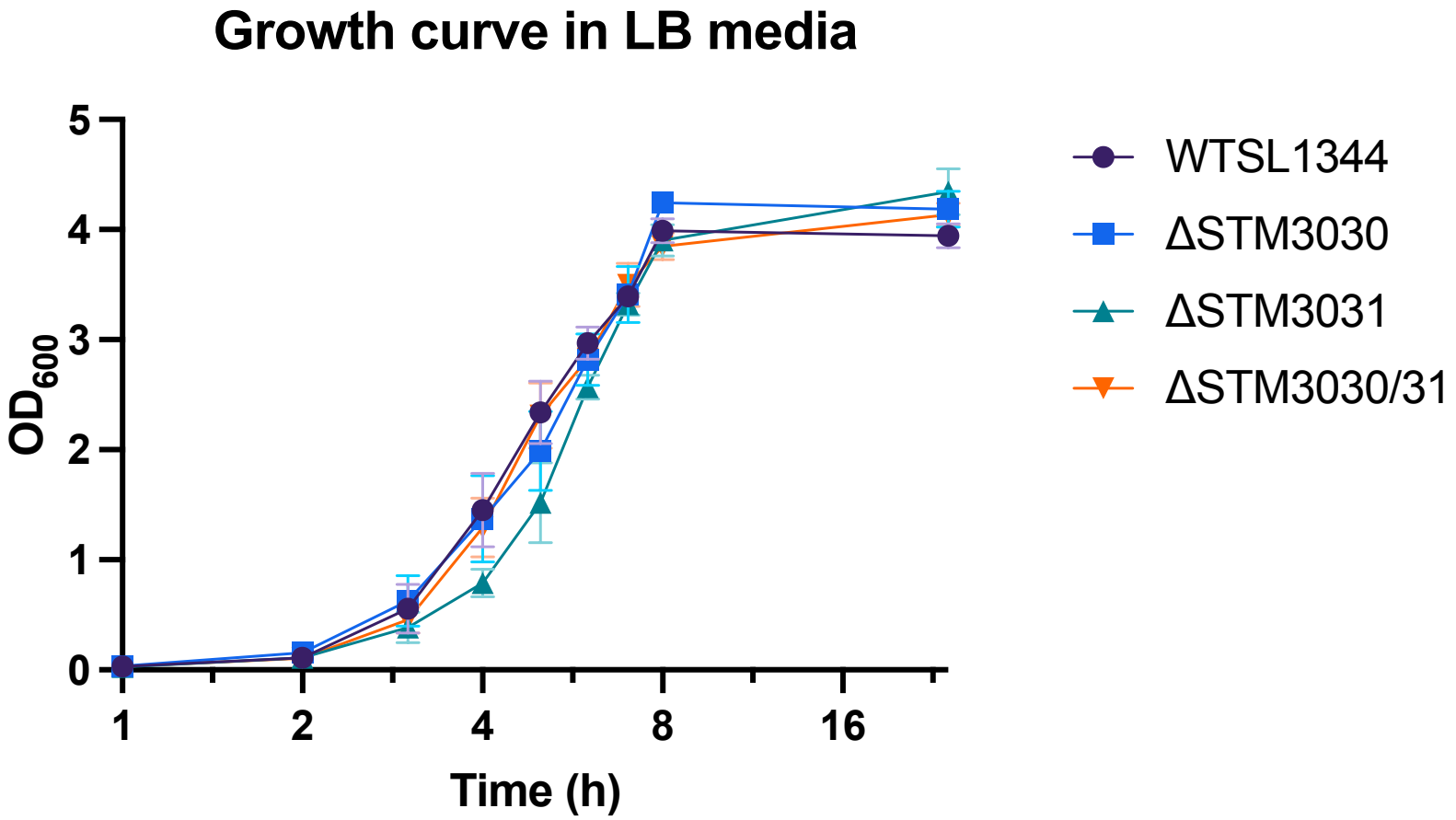

Figure 3. Growth of SL1344 is unaffected by loss of STM3030 and STM3031 in LB media. Growth curves of $\ln(OD_{600})$ values taken hourly over eight consecutive hours and one final reading at 24th hour in LB broth (3.3.2). Growth rates (μ) were 0.70 hr^{-1}, 0.69 hr^{-1}, 0.70 hr^{-1}, and 0.68 hr^{-1} for WTSL1344, ΔSTM3030, ΔSTM3031, and ΔSTM3030/31 respectively. Error bars in all graphs represent SEM from three repeats. *P<0.0332, **P<0.0021, ***P<0.002, ****P<0.0001.

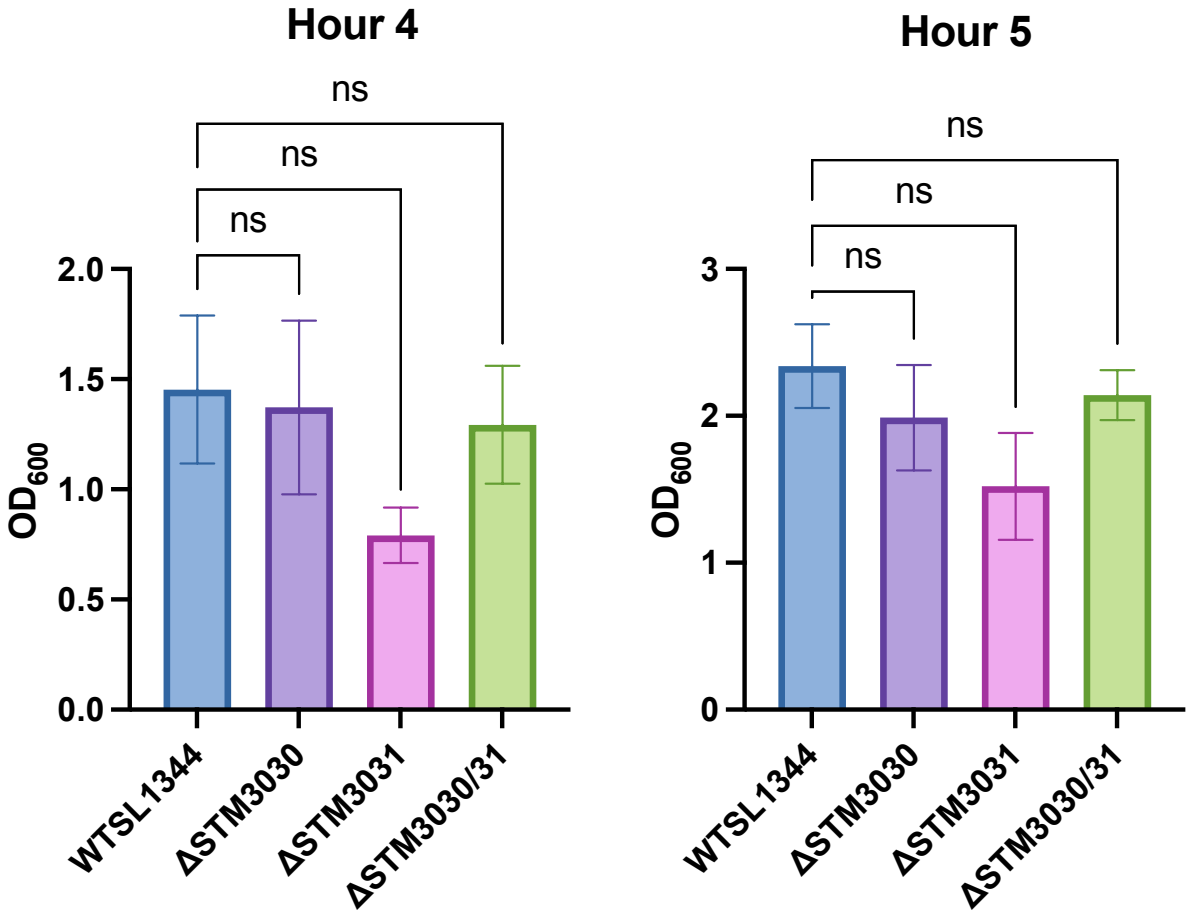

Figure 4. Comparison of OD_{600} readings at fourth and fifth hours during *S.* Typhimurium growth in LB. One-way ANOVA analysis shows no significant difference was found between WTSL1344 and mutant strains. Error bars in all graphs represent SEM from three repeats. *P<0.0332, **P<0.0021, ***P<0.002, ****P<0.0001.

3.3.2. Loss of STM3030 and STM3031 has no negative impact on the growth of *S.* Typhimurium SL1344 in M9 minimal media

To ascertain whether growth in M9 minimal media exposed a growth phenotype for STM3030 and STM3031, the WTSL1344, ΔSTM3030, ΔSTM3031, and ΔSTM3030/31 strains were grown over a 24-hour period as described in 2.3.4 in M9 media batch culture (Figure 5). The same process as for growth curves in LB (3.3.2) was carried out. Growth rates for WTSL1344, Δstm3030, Δstm3031, and Δstm3030/31 were μ=0.53 hr^{-1}, μ=0.52 hr^{-1}, μ=0.50 hr^{-1}, μ=0.50 hr^{-1}, respectively. No significant difference between strain growth rates (μ) were noted during growth in M9 minimal media.

MICs consist of testing strains against increasing antibiotic concentrations to identify the lowest concentration needed for an antibiotic to impact bacterial growth. The lower the MIC value, the higher the efficacity of an antibiotic as it takes less antibiotic to negatively affect bacterial growth, and vice versa.

Towards the eighth hour, growth of both the ΔSTM3031 and ΔSTM3030/31 strains seems to visually decelerate compared the WTSL1344 and ΔSTM3030 strains, which is then recovered by the 24^{th} hour (Figure 6). However, statistical analysis using one-way ANOVA showed no significant difference between the WTSL1344, ΔSTM3031 and ΔSTM3030/31 strains.

Overall, maximum OD_{600} for all strains in M9 minimal media is lower than maximum OD_{600} in LB broth, as M9 minimal media contains the strict minimum required for bacterial growth. Distinction between bacterial growth in LB broth and M9 minimal media is to be expected. Statistical analysis showed a significant difference between growth in LB broth compared to M9 minimal media in both the WTSL1344 and ΔSTM3031 strains, while no significant difference was found between the ΔSTM3030 and ΔSTM3030/31 strains

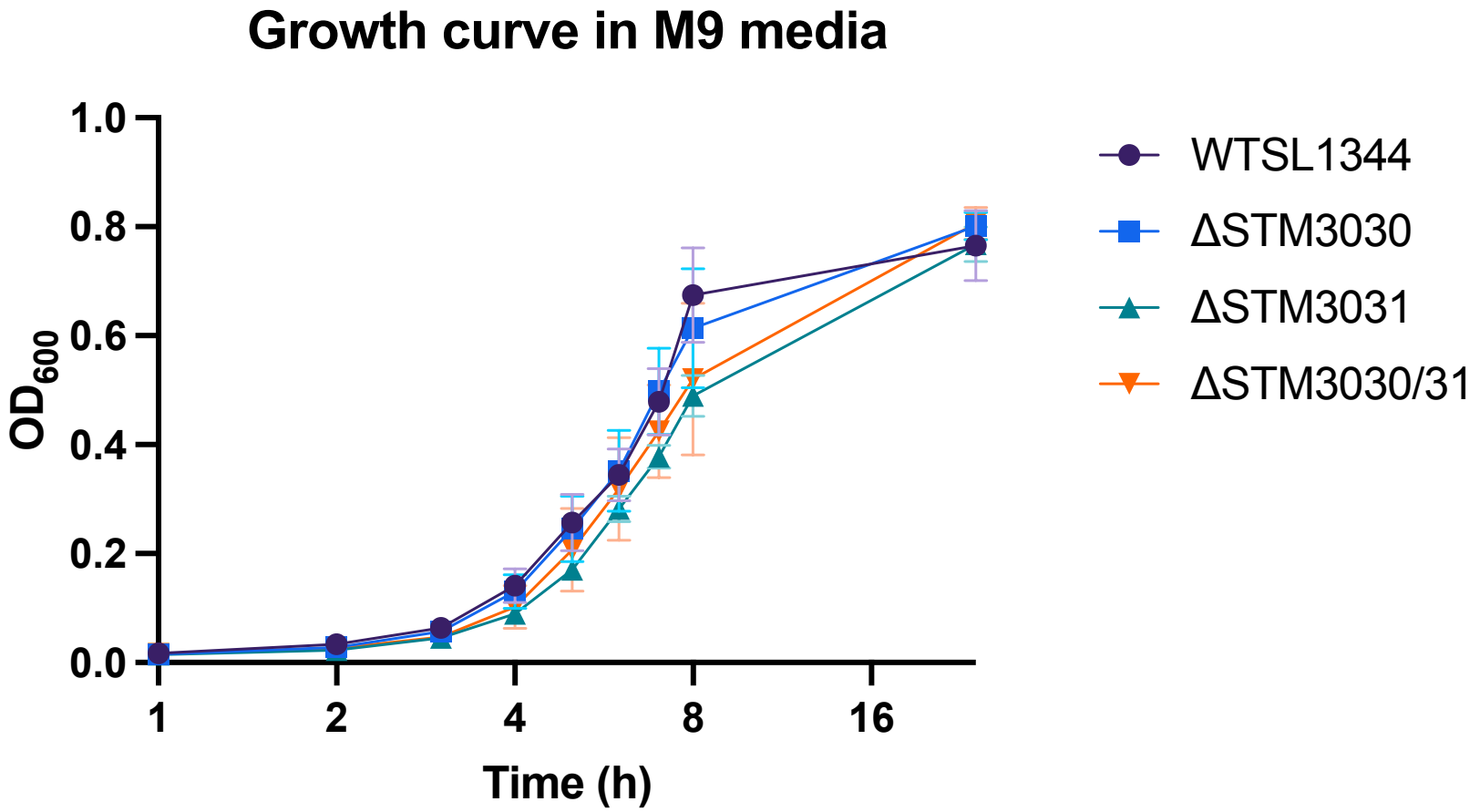

Figure 5. Growth of SL1344 is unaffected by loss of STM3030 and STM3031 in M9 minimal media. Growth curves of ln(OD_{600}) values taken hourly over eight consecutive hours and one final reading at 24th hour in M9 minimal media (3.3.2). Growth rates (μ) were 0.53 hr^{-1}, 0.52 hr^{-1}, 0.50 hr^{-1}, and 0.50 hr^{-1} for WTSL1344, ΔSTM3030, ΔSTM3031, and ΔSTM3030/31 respectively. Error bars in all graphs represent SEM from three repeats. *P<0.0332, **P<0.0021, ***P<0.002, ****P<0.0001.

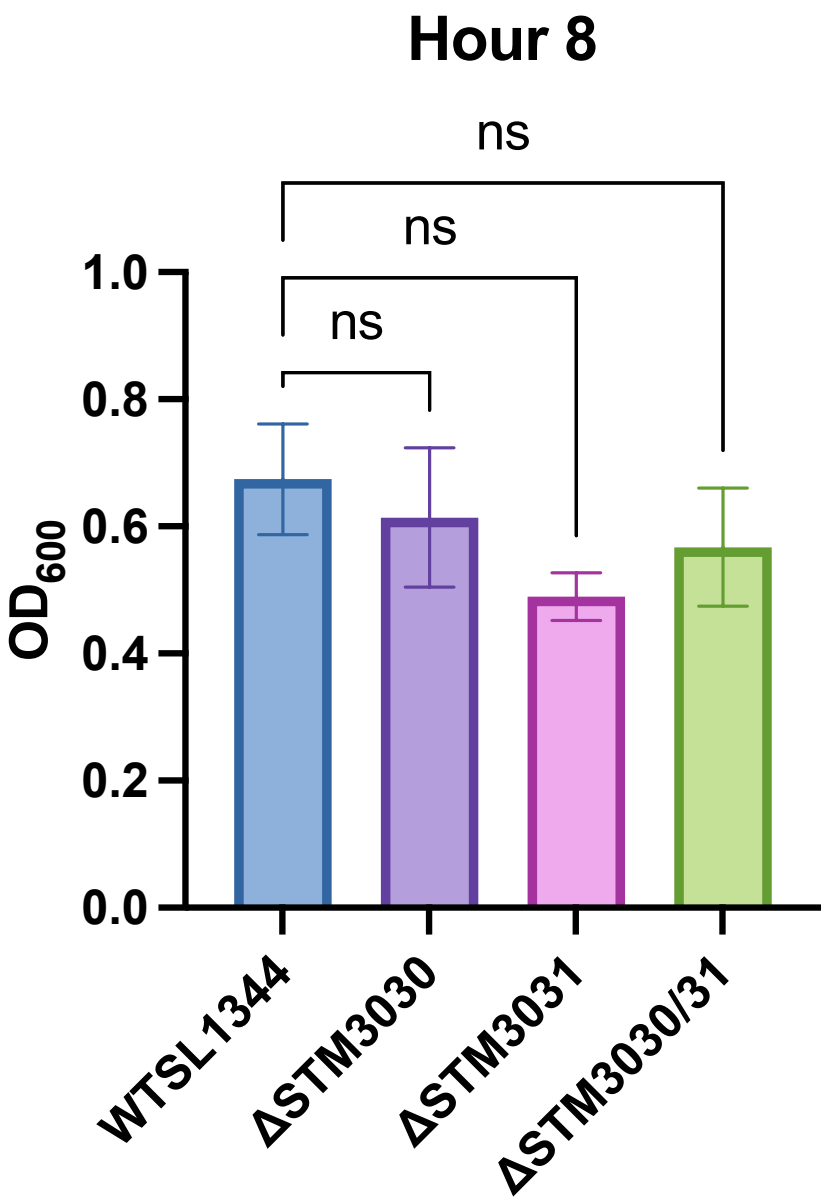

Figure 6. Comparison of OD_{600} readings at the eighth hour during *S.* Typhimurium growth in M9 minimal media. One-way ANOVA analysis shows no significant difference was found between WTSL1344 and mutant strains. Error bars in all graphs represent SEM from three repeats. *P<0.0332, **P<0.0021, ***P<0.002, ****P<0.0001.

3.3.3. STM3030 and STM3031 have no role in sodium tungstate resistance

To further ascertain whether STM3030 and STM3031 are regulated by the BaeSR 2CST, as has previously been reported, the proteins were examined for sodium tungstate sensitivity as sodium tungstate resistance is one of the few known phenotypes associated with BaeSR (Lin, *et al*., 2019; Appia-Ayme, *et al*., 2011). It is possible that if STM3031, but not STM3030, is regulated by BaeSR, there may be a sodium tungstate resistance phenotype, which would also serve to confirm regulation by the 2CST. LB agar plates supplemented with various concentrations of sodium tungstate, from 30 mM to 5 mM, were spotted with serial dilutions of each bacterial strain, which had been cultured overnight, including a BaeR deletion as a positive control and incubated overnight. Results were grouped according to the sodium tungstate concentration and the number of colonies counted after an overnight incubation were plotted for each strain.

Results from the spot plates (Figure 7) demonstrate an expected decreased in tolerance to sodium tungstate by the ΔBaeR strain as the concentration increases, confirming previous results from Appia-Ayme *et al.* At 5 mM (Figure 8A), there is no significant difference between any strain and the wildtype, which can be explained by the relatively low concentration of sodium tungstate potentially not being harmful to the bacterium. However, at 10mM (Figure 8B), there is already a significant decrease in bacterial survival of the ΔBaeR strain, possibly indicating a tolerance threshold is quickly reached. At 20mM (Figure 8C) and 30mM (Figure 8C) of sodium tungstate, there is almost no bacterial growth, which signifies a loss of resistance to sodium tungstate.

There was a minimal difference in growth of the ΔSTM3030, ΔSTM3031, and ΔSTM3030/31 strains compared to the wildtype, which is confirmed by statistical analysis. At all concentrations, the mutant strains of interest maintain a similar level of growth compared to the wildtype and each other.

To test whether any sensitivity could be detected at various time points during a 24-hour period, kill curves using a spectrophotometer plate reader were done. Three concentrations of sodium tungstate were chosen to be investigated: 30 mM, 20 mM, and 10 mM, as there had been no discernible difference between the wildtype and control ΔBaeR strain. For the kill curves, all strains were tested in normal LB and in LB supplemented with a known concentration of

sodium tungstate in 24-well plates with two repeats per strain on each plate, as well as containing a control row of LB. Results for each mutant strain in the sodium tungstate supplemented LB were plotted against the wildtype in the sodium tungstate supplemented LB, to compare tolerance to sodium tungstate.

The kill curve at 30 mM (Figure 9) showed the expected statistically significant loss of tolerance for sodium tungstate by the ΔBaeR strain compared to the wildtype (Figure 9E). The only other statistically significant difference observed was between hours 6.5 and 9 in the ΔSTM3031 strain (Figure 9C), where there was a significant decrease in tolerance compared to the wildtype. Visually, there seemed to be a difference in the ΔSTM3030 strain (Figure 9B) as well, however statistical analysis proves there isn't. A difference was noted between the wildtype in LB and wildtype in sodium tungstate supplemented LB, although this is expected as sodium tungstate is a toxic compound and some sensitivity to it is normal.

The kill curve at 20 mM (Figure 10) unexpectedly only showed a significant loss of tolerance to sodium tungstate by the ΔBaeR strain (Figure 10E) only between hours 3 and 3.5, which contradicts results from the sensitivity spot plates (Figure 8C). There was, however, a significant decrease in tolerance by ΔSTM3030 compared to the wildtype (Figure10B) between hours 6 and 8.5. No other statistically significant decrease in tolerance to sodium tungstate was noted in any other strain.

The kill curve at 10 mM (Figure 11) revealed the most unexpected results as both ΔSTM3031 (Figure 11C) and ΔSTM3030/31 (Figure 11D) showed a significant loss of tolerance to sodium tungstate compared to the wildtype at various hours. This contradicts results from both sensitivity spot plates and kill curves at higher concentrations and merits further investigation. At this concentration, the visual loss of tolerance to sodium tungstate by ΔBaeR is not validated by statistical analysis, which can be explained by the wider standard error of the ΔBaeR strain results.

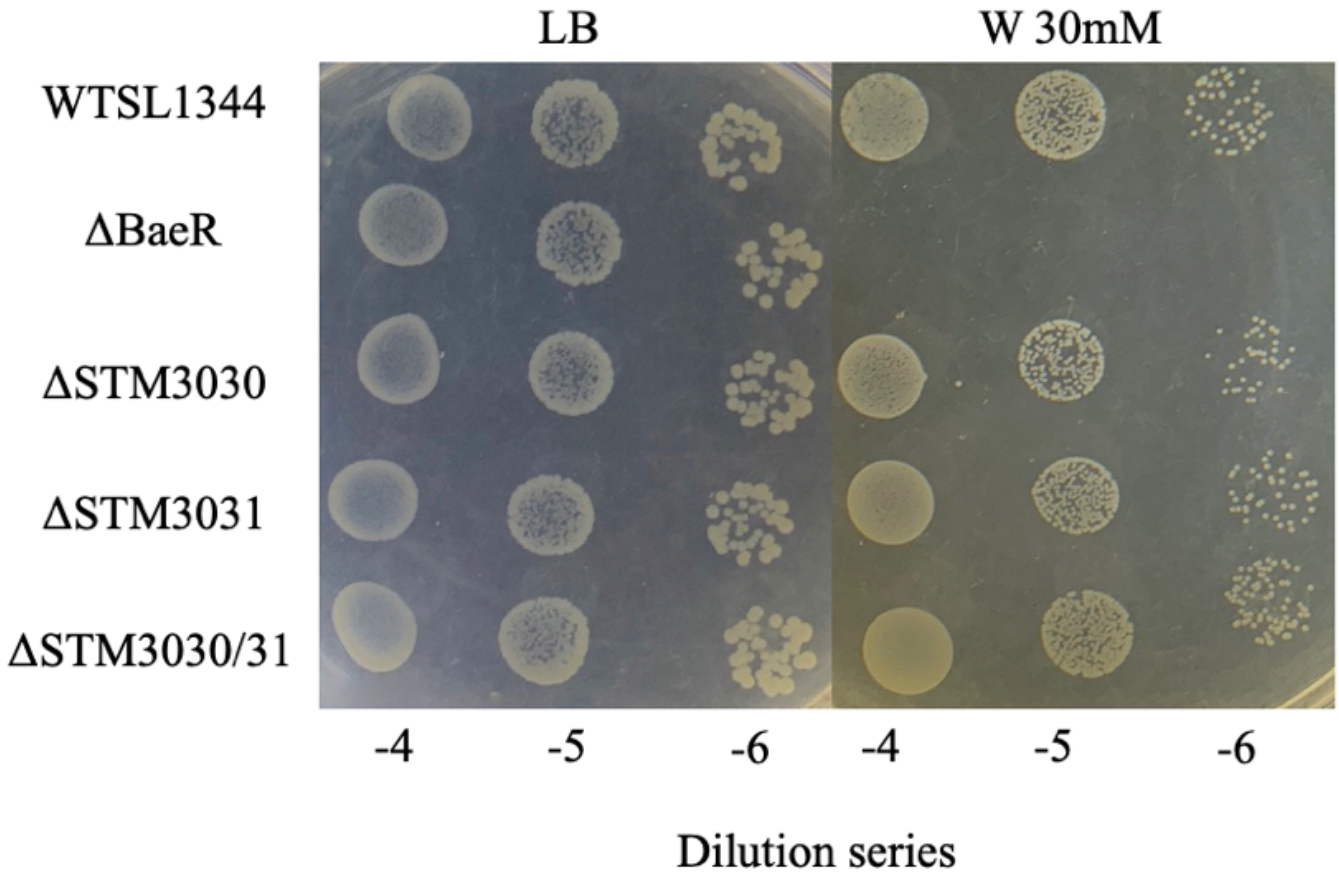

Figure 7. Tungstate sensitivity of several *Salmonella* Typhimurium strains. Growth of *baeR* mutant is impaired on media containing 30mM sodium tungstate; growth of WTSL1344 wildtype, and STM3030 and STM3031 single and double mutants is not impaired

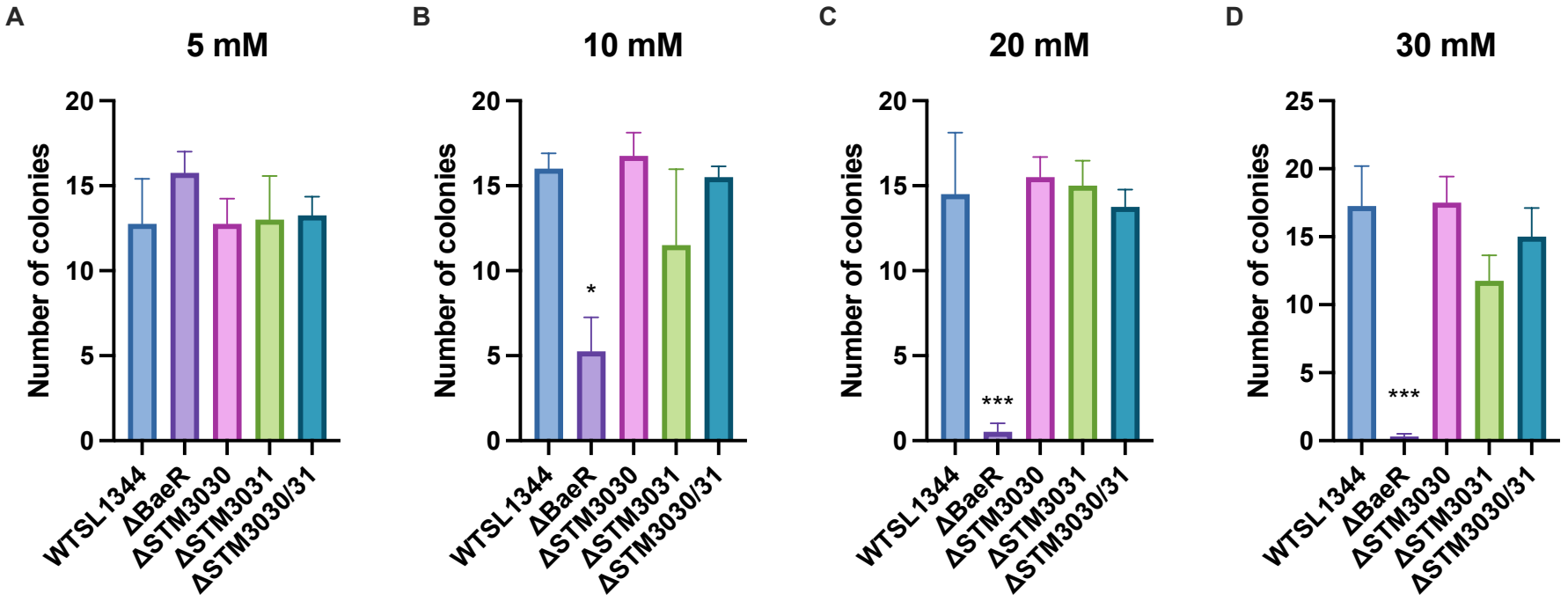

Figure 8. Sodium tungstate spot plate colony counts. At strain indicated in the figure statistical significance is indicated by the asterisks. Error bars in all graphs represent SEM from four repeats. *P<0.0332, **P<0.0021, ***P<0.002, ****P<0.0001.

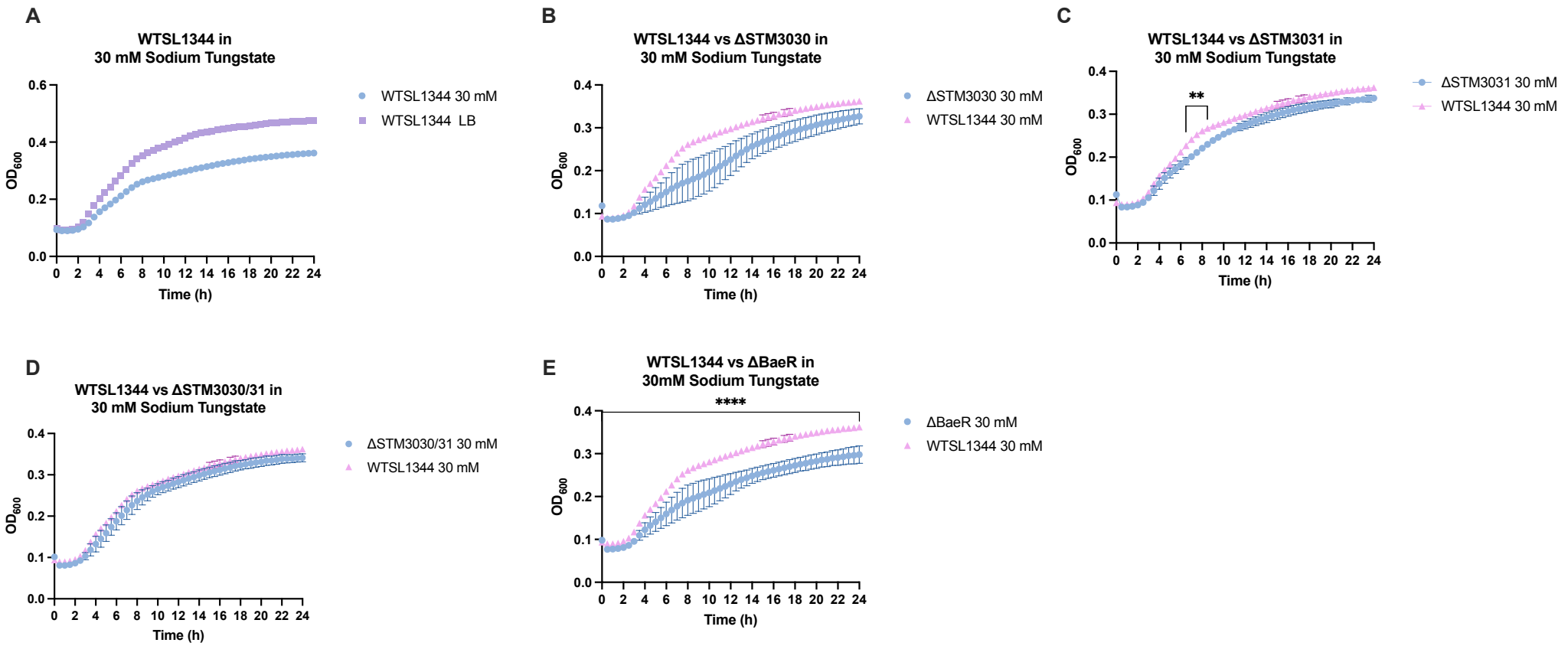

Figure 9. Sodium tungstate kill curves for all strains against the wildtype at 30 mM of sodium tungstate. A. Control kill curve for *S.* Typhimurium SL1344 wildtype strain in LB compared to 30 mM of sodium tungstate. **B**. Kill curves for *S.* Typhimurium ΔSTM3030 strain against the wildtype strain in 30 mM of sodium tungstate. **C**. Kill curves for *S.* Typhimurium ΔSTM3031 strain against the wildtype strain in 30 mM of sodium tungstate. **D**. Kill curves for *S.* Typhimurium ΔSTM3030/31 strain against the wildtype strain in 30 mM of sodium tungstate. **E**. Kill curves for *S.* Typhimurium ΔBaeR strain against the wildtype strain in 30 mM of sodium tungstate. At individual concentration indicated in the figure statistical significance is indicated by the asterisks. Error bars in all graphs represent SEM from three repeats. *P<0.0332, **P<0.0021, ***P<0.002, ****P<0.0001.

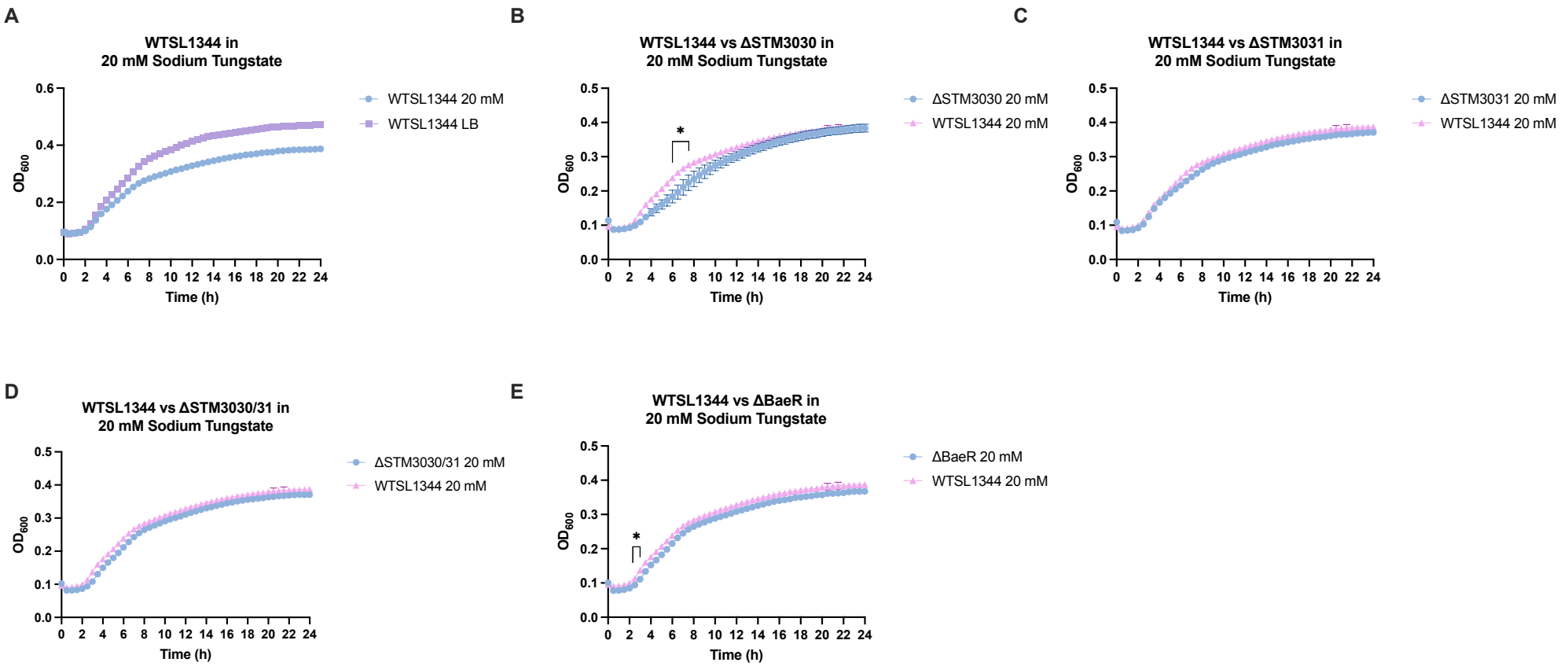

Figure 10. Sodium tungstate kill curves for all strains against the wildtype at 20 mM of sodium tungstate. A. Control kill curve for *S.* Typhimurium SL1344 wildtype strain in LB media compared to 20 mM of sodium tungstate. **B**. Kill curves for *S.* Typhimurium ΔSTM3030 strain against the wildtype strain in 20 mM of sodium tungstate. **C**. Kill curves for *S.* Typhimurium ΔSTM3031 strain against the wildtype strain in 20 mM of sodium tungstate. **D**. Kill curves for *S.* Typhimurium ΔSTM3030/31 strain against the wildtype strain in 20 mM of sodium tungstate. **E**. Kill curves for *S.* Typhimurium ΔBaeR strain against the wildtype strain in 20 mM of sodium tungstate. At individual concentration indicated in the figure statistical significance is indicated by the asterisks. Error bars in all graphs represent SEM from three repeats. *P<0.0332, **P<0.0021, ***P<0.002, ****P<0.0001.

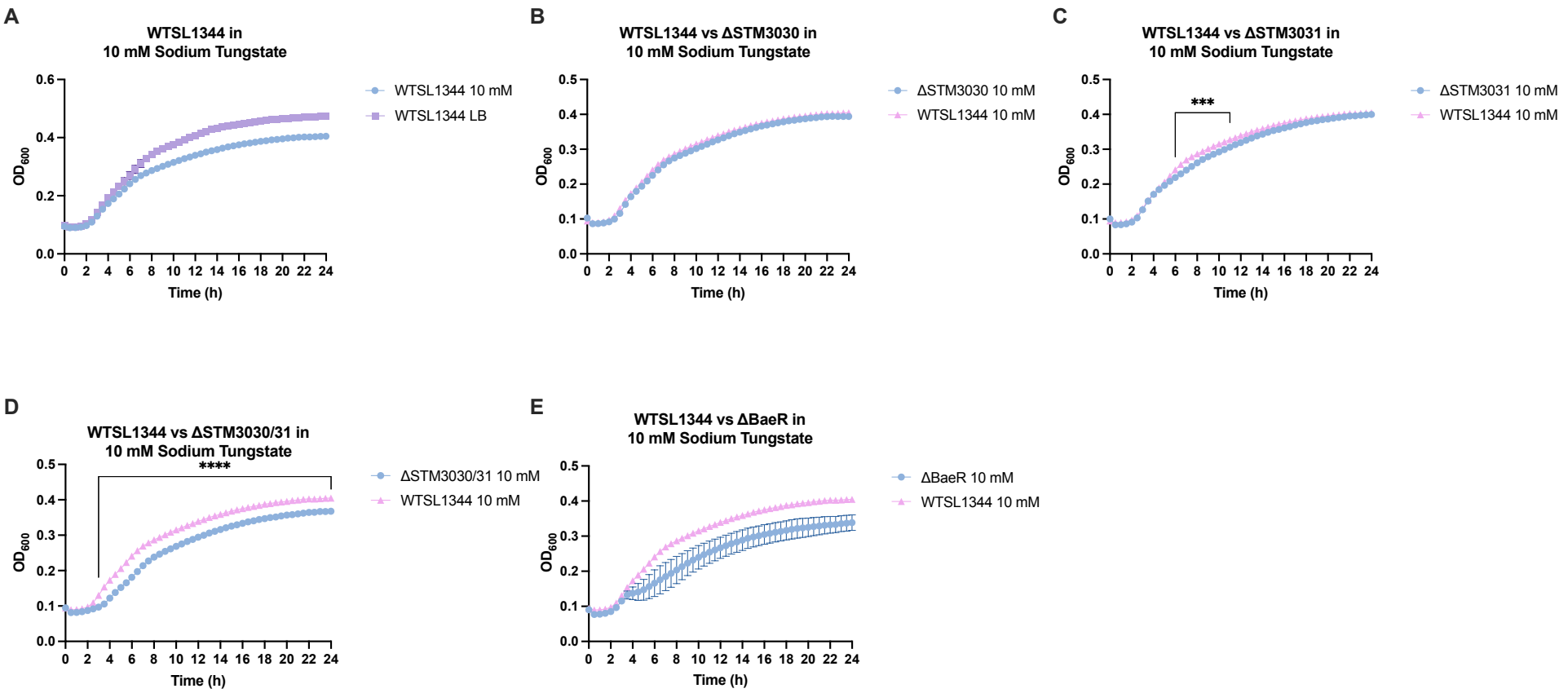

Figure 11. Sodium tungstate kill curves for all strains against the wildtype at 10 mM of sodium tungstate. A. Control kill curve for *S.* Typhimurium SL1344 wildtype strain in LB media compared to 10 mM of sodium tungstate. **B**. Kill curves for *S.* Typhimurium ΔSTM3030 strain against the wildtype strain in 10 mM of sodium tungstate. **C**. Kill curves for *S.* Typhimurium ΔSTM3031 strain against the wildtype strain in 10 mM of sodium tungstate. **D**. Kill curves for *S.* Typhimurium ΔSTM3030/31 strain against the wildtype strain in 10 mM of sodium tungstate. **E**. Kill curves for *S.* Typhimurium ΔBaeR strain against the wildtype strain in 10 mM of sodium tungstate. At individual concentration indicated in the figure statistical significance is indicated by the asterisks. Error bars in all graphs represent SEM from three repeats. *P<0.0332, **P<0.0021, ***P<0.002, ****P<0.0001.

3.3.4. STM3031 contributes to *Salmonella* resistance to cephalothin

MIC assays were conducted to verify cephalothin resistance phenotypes in ΔSTM3030 and ΔSTM3031 single and double mutants against the wildtype strain. Previous studies by Lin *et al.* reported involvement of both proteins in resistance to cephalothin (Lin, *et al*., 2019). For this, 96-well plates are used and the OD_{600} of each well is measured using a spectrophotometer plate reader after overnight incubation as described in 2.4.4.

Ten concentrations from 10 μg/mL to 0 μg/mL of the antibiotic cephalothin were used to test survival of all strains after a 24-hour incubation period. Results indicated decreased survival at higher concentrations, with the greatest decrease in survival being from 5 μg/mL to 10 μg/mL for all strains (Figure 12). ΔSTM3031 showed a statistically significant decrease in survival compared to the wildtype at all antibiotic concentrations (Figure 12B). On the other hand, ΔSTM3030 showed a statistically significant increase in survival compared to the wildtype only at the three lowest concentrations (Figure 12A). ΔSTM3030/31 showed statistically significant decrease in survival compared to the wildtype at higher concentrations of the antibiotic (Figure 12C).

It is important note that at the starting concentration of 0 μg/mL, there is a statistically significant difference between the OD_{600} values WTSL1344 strain and the ΔSTM3030 and ΔSTM3031 strains. Since the zero values for ΔSTM3031 are below that of the rest of the strains, this potentially lowers all ΔSTM3031 values at the following concentrations. There can be more confidence in results when the zero OD_{600} values are matched, therefore repeats for each antibiotic would be beneficial.

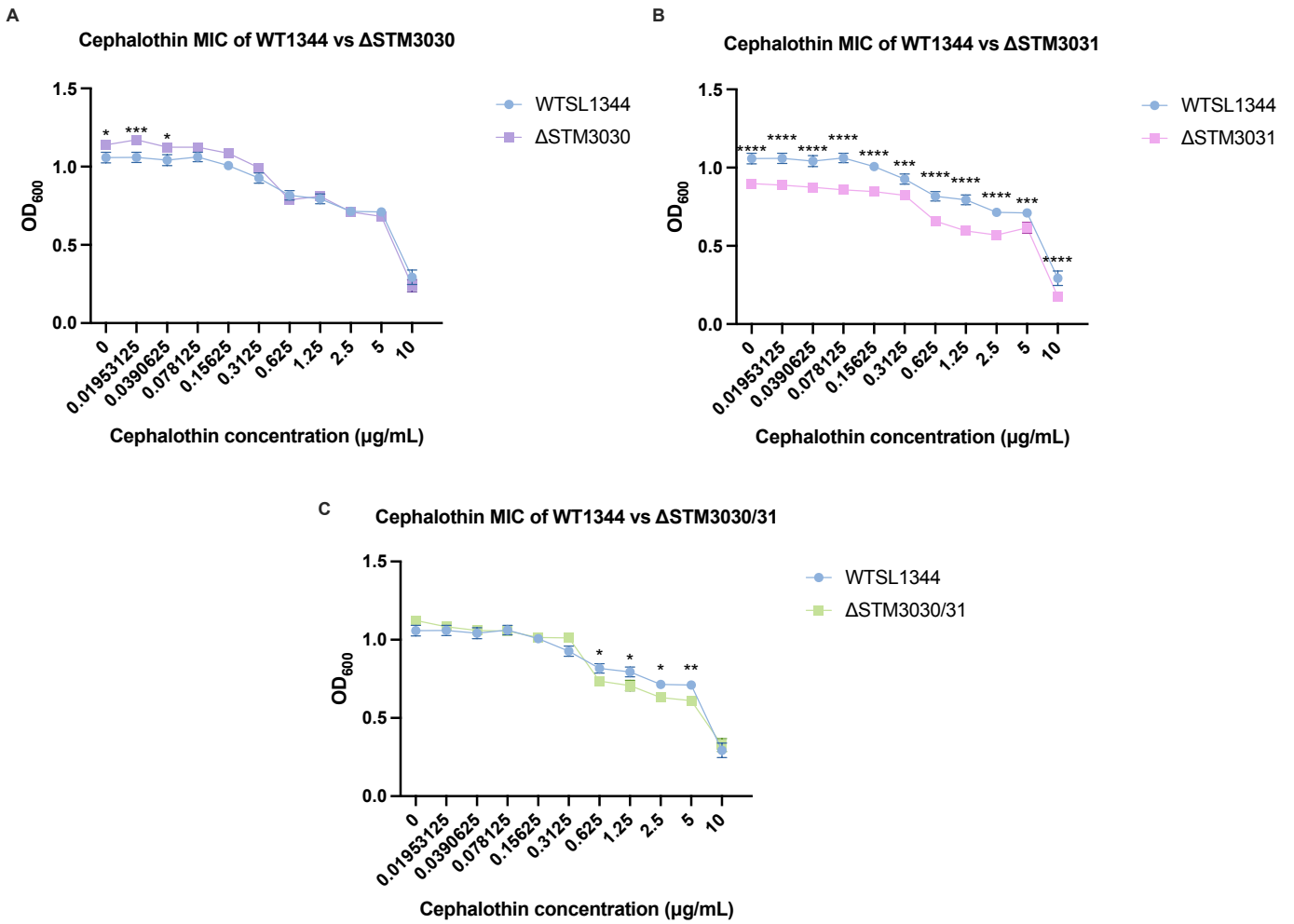

Figure 12. Cephalothin MIC graph for each strain against WTSL1344 with X-axis scale following each cephalothin concentration. A. Cephalothin MIC curve for *S.* Typhimurium WTSL1344 against ΔSTM3030. **B**. Cephalothin MIC curve for *S.* Typhimurium WTSL1344 against ΔSTM3031. **C**. Cephalothin MIC curve for *S.* Typhimurium WTSL1344 against ΔSTM3030/31. OD_{600} indicates bacterial cell growth by measurement of optical density of a liquid sample at a wavelength of 600 nm; the higher the OD_{600} value, the greater the growth of bacteria and vice versa; error bars represent standard deviation from 6 repeats. *P<0.0332, **P<0.0021, ***P<0.002, ****P<0.0001.

3.3.5. STM3030 and STM3031 do not affect *S.* Typhimurium's resistance to ceftriaxone

MIC assays were conducted to further verify ceftriaxone resistance phenotypes in ΔSTM3030 and ΔSTM3031 single and double mutants against the wildtype strain. Resistance to ceftriaxone has been shown to be mediated by STM3031 by Hu *et al.* (Hu, *et al*., 2009; Hu, *et al*., 2005). For this, 96-well plates are used and the OD_{600} of each well is measured using a spectrophotometer plate reader after overnight incubation as described in 2.4.4.

Ten concentrations of the antibiotic ceftriaxone from 3 μg/mL to 0 μg/mL were used to test survival against ceftriaxone. There was an overall gradual decline in survival of all strains, from the lowest to the highest concentration, which means that as the concentration of the antibiotic increases, survival of bacterial cells of all strains decreases (Figure 13). However, there was no overwhelming statistical significance between any mutant strains and the wildtype strain at all concentrations. A decrease in survival of the ΔSTM3031 strain compared to the wildtype was only observed at three concentrations, 0 μg/mL, 0.005859 μg/mL, and 0.375 μg/mL (Figure 13B). At 0.093750 μg/mL, ΔSTM3030 shows statistically significant increase in survival compared to the wildtype, otherwise there is no statistical significance at any concentration (Figure 13A). At three concentrations, between 0.046875 μg/mL and 0.1875 μg/mL, ΔSTM3030/31 had a statistically significant increase in survival compared to the WTSL1344 (Fig. 13C).

The results from this assay show that, at the two previously mentioned concentrations (Figure 13), growth of ΔSTM3031 is what is mostly negatively affected in comparison to the other mutant strains, but not compared to the wildtype. The increased survival of ΔSTM3030/31 compared to the wildtype (Figure 13C) is of interest as it contradicts previous studies that report a loss of the proteins impact resistance to ceftriaxone negatively.

Similar to results for the cephalothin MIC, there is a significant difference between the OD_{600} values WTSL1344 strain and the ΔSTM3031 strain in the ceftriaxone MIC. Since the zero values for ΔSTM3031 are below that of the rest of the strains, this potentially lowers all ΔSTM3031 values at the following concentrations.

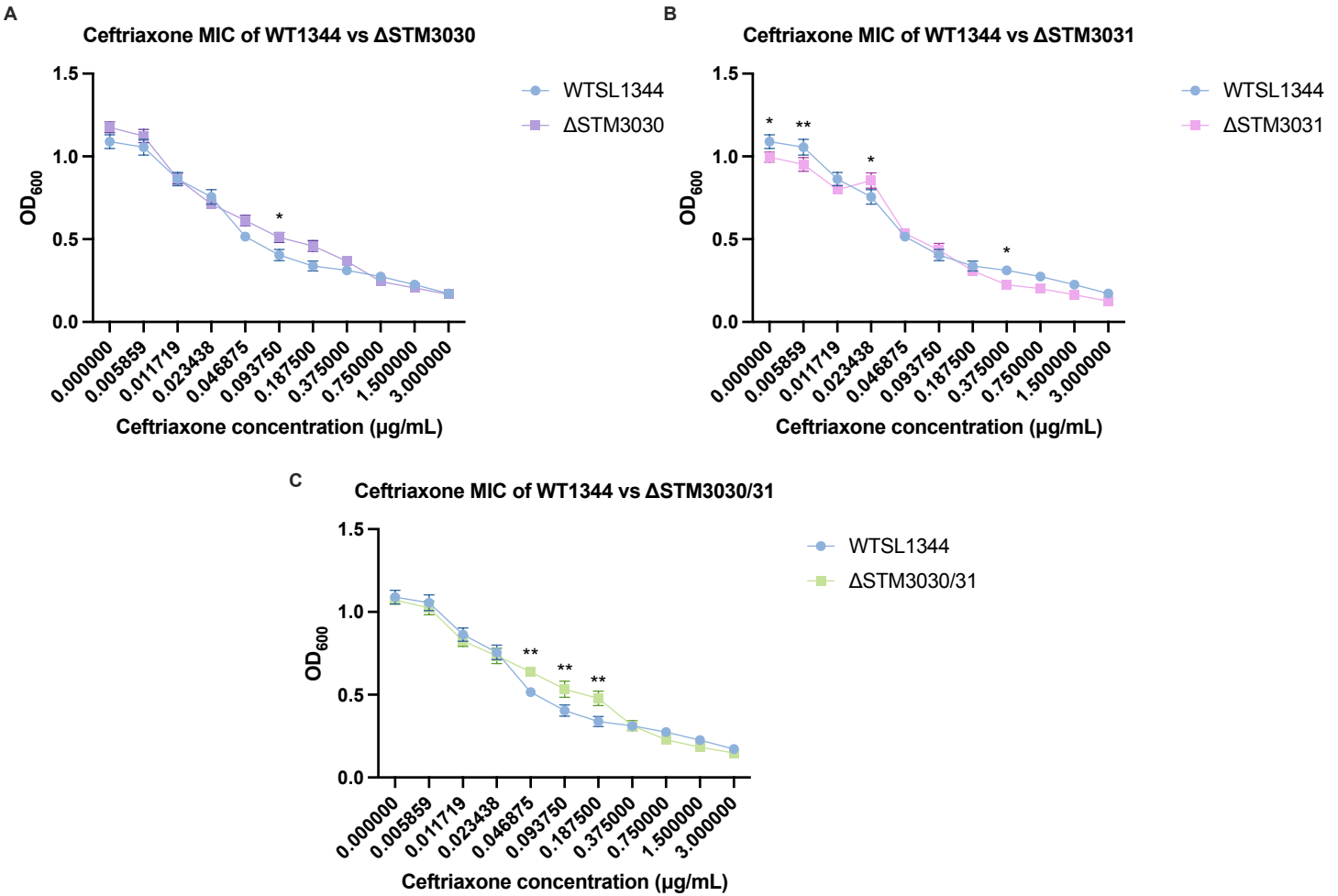

Figure 13. Ceftriaxone MIC graph for each strain against WTSL1344 with X-axis scale following each ceftriaxone concentration. A. Ceftriaxone MIC curve for *S.* Typhimurium WTSL1344 against ΔSTM3030. **B**. Ceftriaxone MIC curve for *S.* Typhimurium WTSL1344 against ΔSTM3031. **C**. Ceftriaxone MIC curve for *S.* Typhimurium WTSL1344 against ΔSTM3030/31. OD600 indicates bacterial cell growth by measurement of optical density of a liquid sample at a wavelength of 600 nm; the higher the OD600 value, the greater the growth of bacteria and vice versa; error bars represent standard deviation from 4 repeats. *P<0.0332, **P<0.0021, ***P<0.002, ****P<0.0001.

3.3.6. STM3030 and STM3031 have a complementary adhesion function in the *S.* Typhimurium SL1344 strain

To investigate whether claims by Chowdhury *et al.*, which stated that STM3031 has no role in adhesion of *S.* Typhimurium to human cells during infection, were applicable to the SL1344 *S.* Typhimurium strain, adhesion assays were conducted using human Caco-2 colorectal adenocarcinoma cells. These cells create a polarised confluent monolayer meant to replicate the human gut epithelium. Caco-2 cells present with typical enterocyte differentiation in laboratory settings and develop a microvilli brush border that is normally observed in the small intestine (Francis & Thomas, 1996).

Cells were infected with *S.* Typhimurium and incubated for a short period to allow bacterial cells to adhere to the human cells. Cells were then washed and lysed, the remnants of which were plated on LB agar plates and incubated overnight. Bacteria that grew on LB agar pates were bacteria that successfully attached to the Caco-2 cells using relevant adhesion mechanisms. Percentage adhesion and relative percentage adhesion denotes the percentage of *S.* Typhimurium cells that adhered to human cells compared to the overnight cultures used for each repeat and compared to the percentage adhesion of the wildtype strain, respectively.

Statistical analysis of the results from adhesion assays showed a significant difference between the wildtype and the double mutant strains in relation to percentage adhesion (Figure 14A) of bacterial cells to Caco-2 cells. On the other hand, there was no significant difference between wildtype and either of the single mutant strains. There also was no significant difference between any of the mutant strains. When adjusting the data to show relative percentage adhesion (Fig. 14B), where 100% equals the average percentage adhesion of wildtype bacterial cells, the mutant strains showed statically significantly less adhesion to host cells.

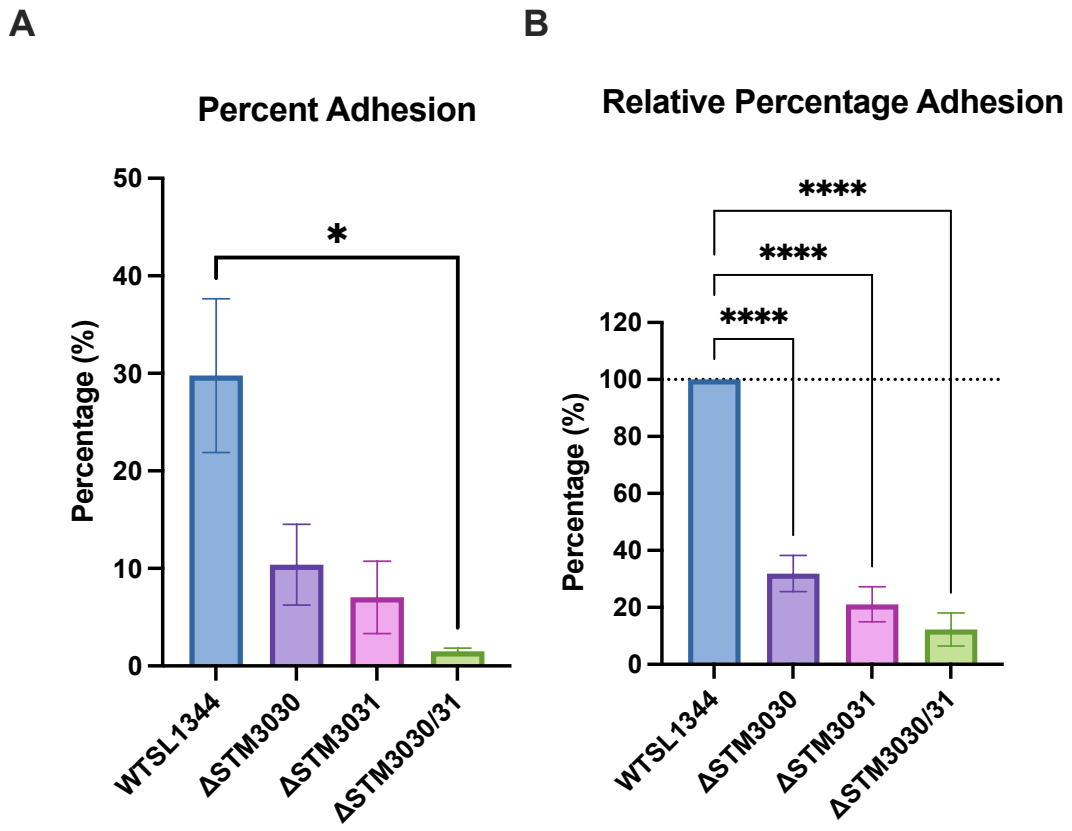

Figure 14. STM3030 and STM3031 have a complementary adhesion function in *S.* Typhimurium.

A. Percentage adhesion of wildtype WTSL1344, ΔSTM3030, ΔSTM3031, and ΔSTM3030/31 in human Caco-2 cells; significant difference ($p = 0.0261$) between WTSL1344 and ΔSTM3030/31 denotes a need for both proteins in adhesion to host cells

B. Relative percentage adhesion compared to wildtype; significant difference between WTSL1344 compared to mutants was found

Error bars represent SEM from at least 3 repeats. Statistical significance was calculated by one-way ANOVA or Kruskal-Wallis and Tukey's multiple comparisons test. *P<0.0332, **P<0.0021, ***P<0.002, ****P<0.0001.

3.3.7. Loss of STM3031 affects ability of *S.* Typhimurium SL1344 strain to invade cells after a 60-minute incubation period but not after a 24-hour incubation period

Further results from Chowdhury *et al.* also state STM3031 has no invasion function in *S.* Typhimurium infection, which required verification following the contradicting results from adhesion assays. Gentamicin protection assays, also known as invasion assays, were also conducted in Caco-2 cells, however, unlike in adhesion assays, longer incubation periods were implemented, and Caco-2 cells were then washed with the antibiotic gentamicin to kill any extracellular bacterial cells. This method ensures only intracellular bacteria that have successfully invaded human cells are cultured onto LB agar plates. Two incubation periods were required, one to allow the bacteria to invade cells and one to allow the gentamicin to kill any extracellular cells. Bacterial incubations of two different lengths of 60 minutes and 24 hours were trialled to investigate, whether this influenced *S.* Typhimurium invasion. Percentage invasion and relative percentage invasion denotes the percentage of *S.* Typhimurium cells that invaded human cells compared to the overnight cultures used for each repeat and compared to the percentage invasion of the wildtype strain, respectively.

Analysis of results from invasion assays with a 60-minute incubation (Figure 15) demonstrated a statistically significant decrease in bacterial invasion of human cells in the ΔSTM3031 strain compared to the wildtype strain both in terms of percentage invasion and relative percentage invasion, indicating a need for STM3031 to successfully invade host cells. There is no statistically significant difference between all mutant strains as well as the two other mutant strains compared to the wildtype strain. Intriguingly, the ΔSTM3030/31 double mutant showed a visual increase compared to the wildtype strain, which could mean that the STM3030 and STM3031 proteins are not needed in combination for invasion of human epithelial cells in a 60-minute period, unlike in adhesion.

Contrary to results gathered with a 60-minute incubation, analysis of results from the mutant strains with a 24-hour incubation (Figure 16) showed no statistically significant difference to the isogenic parent strain, both in terms of percentage invasion and relative percentage invasion. This points to STM3030 and STM3031 having no roles in invasion after a 24-hour incubation, either individually or together.

There is an overall decrease in the percentage invasion of bacterial cells of the wildtype strain between the 60-minute incubation and the 24-hour incubation, which would be of interest for further investigation. Hypothetically, the longer bacteria have to invade cells, which when applied to real-life circumstances would mean longer exposure to bacterial infection, the more the bacteria would successfully invade human cells. This is also observed in the ΔSTM3030 and ΔSTM3030/31 strains, however, in the case of ΔSTM3031, the percentage invasion increases slightly.

The large margin of error displayed by the error bars for the wildtype and double mutant strains in the results with the 60-minute incubation is an area of concern as this points to inconsistent and unreliable results. Error bars for all strains with the 24-hour incubation were all very large, except for the percentage invasion for the ΔSTM3030 strain.

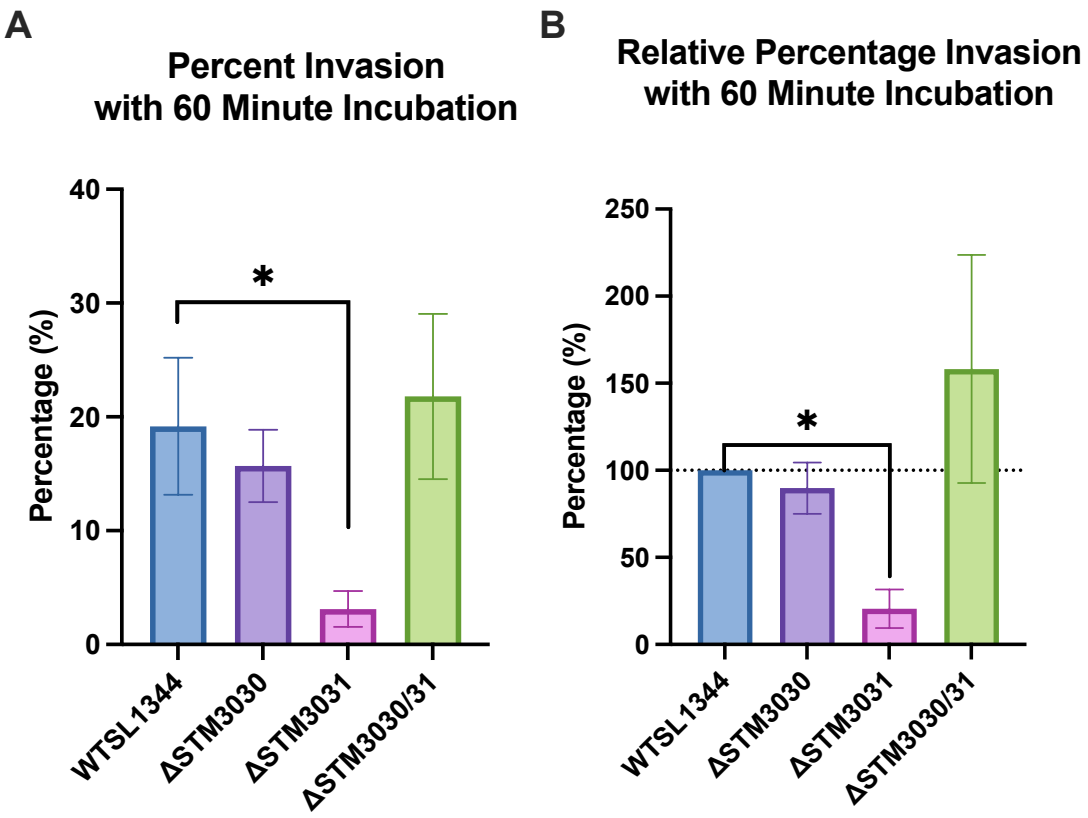

Figure 15. STM3031 has an invasion function in *S.* Typhimurium after a 60-minute incubation.

A. Percentage invasion of wildtype WTSL1344, ΔSTM3030, ΔSTM3031, and ΔSTM3030/31 in human Caco-2 cells; significant difference ($p = 0.0261$) between WTSL1344 and ΔSTM3030/31 denotes a need for both proteins in adhesion to host cells

B. Relative percentage adhesion compared to wildtype; significant difference between WTSL1344 compared to mutants was found

Error bars represent SEM from at least 3 repeats. Statistical significance was calculated by one-way ANOVA and Tukey's multiple comparisons test. *P<0.0332, **P<0.0021, ***P<0.002, ****P<0.0001.

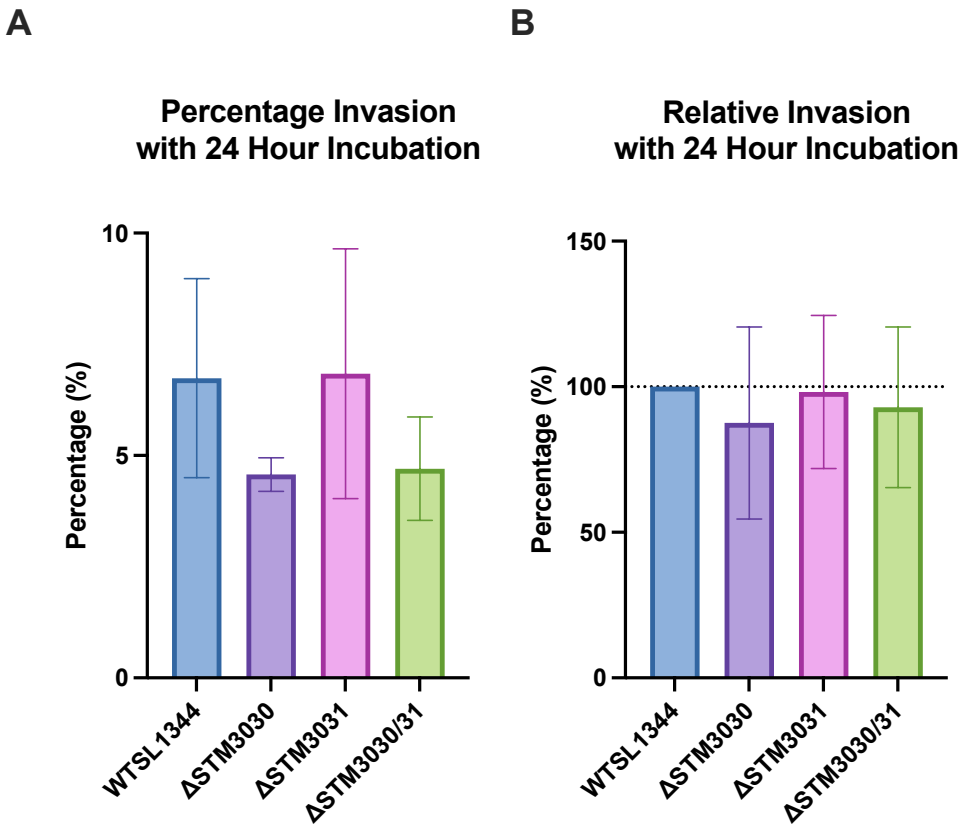

Figure 16. STM3030 and STM3031 have no invasion function in *S.* Typhimurium after a 24-hour incubation.

A. Percentage invasion of wildtype WTSL1344, ΔSTM3030, ΔSTM3031, and ΔSTM3030/31 in human Caco-2 cells; significant difference ($p = 0.0261$) between WTSL1344 and ΔSTM3030/31 denotes a need for both proteins in adhesion to host cells

B. Relative percentage adhesion compared to wildtype; significant difference between WTSL1344 compared to mutants was found

Error bars represent SEM from at least 3 repeats. Statistical significance was calculated by one-way ANOVA and Tukey's multiple comparisons test. *P<0.0332, **P<0.0021, ***P<0.002, ****P<0.0001.

3.4. Discussion and future work

STM3030 and 3031 have been associated with the BaeSR and CpxAR 2CSTs, as well as a role for the Typhi homologues in adhesion and invasion of host cells. This mechanistic study set out to phenotypically determine a role for these proteins in the ESR and host cell invasion using STM3030 and STM3031 single and double mutants of the *S.* Typhimurium SL1344 strain.

3.4.1. Aerobic growth on nutrient rich or minimal media is unaffected by loss of STM3030 and STM3031

Using single and double mutant strains, we grew the *S.* Typhimurium strains under normal laboratory setting growth conditions and with a minimal media intended to replicate actual growing conditions within potentially stressful environments. Though results did not uncover involvement in bacterial growth of STM3030 and STM3031, it allows us to effectively eliminate this facet of the proteins' putative functions.

Statistical analysis of growth curve data established that loss of STM3030 and STM3031, individually and together, had no significant effect on *Salmonella* growth, both in LB media and M9 minimal media, compared to the wildtype strain. However, a visual difference can be noted in the LB media growth curve (Figure 7), between the fourth and fifth hours, and in the M9 minimal media growth curve (Figure 9), in the eighth hour. Though statistical analysis does not support the visual finding, they could be potential timepoints of interest and require further investigation.

In the LB growth curve, the fourth to fifth hour timepoints are within the exponential growth phase, where bacteria grow fastest. The slower growth of ΔSTM3031 during that period suggests the mutant strain is unable to transition from the lag phase into the subsequent phase as quickly as the wildtype and other mutant strains. This could be due to the mutant bacteria not having the optimal growing capabilities when STM3031 is missing. After the fifth hour, the ΔSTM3031 strain catches up to the other strains and growth looks unaffected from the sixth to 24th hour. This pattern is not observed in the ΔSTM3030 strain, which indicates loss of STM3030 has no effect; however, this is contradicted by the unaffected growth in the ΔSTM3030/31 strain. It would be expected for the double mutant strain to lag like the ΔSTM3031 strain, though it doesn't. This could be due to an accumulation of the STM3030

protein when STM3031 is knocked out, negatively impacting growth. Conversely, the difference in growth throughout the early-exponential phase could indicate that STM3031 is normally expressed during that time. This would require use of promoter fusions to assess expression levels of both STM3030 and STM3031 during the different growth phases. Growth of the Δ*ompX* strain in *E. coli* was indistinguishable from that of the wildtype strain at either 30 or 42ºC in LB media (Mescas, *et al*., 1995). As STM3031 is the *S.* Typhimurium homologue for OmpX, the lack of a growth phenotype could be possible. Past studies therefore support the findings of this study.

For growth in M9 minimal media, growth of all strains compared to growth in LB media is overall is markedly slower. The final OD readings for all strains in M9 minimal media come to just below 0.8, whereas in LB media, they all reach 4. This is accounted for by the minimal growth nutrients offered in the M9 media. As observed in the LB growth curves, the ΔSTM3031 strain has a slower growth rate around the eighth hour, however in contrast, the ΔSTM3030/31 strain has a similar growth rate to ΔSTM3031. This could contradict the hypothesis of an accumulation of STM3030 negatively impacting growth in the ΔSTM3031 strain. Further investigation is required to assess the different growth rate of the double STM3030/31 mutant both in LB media and M9 minimal media. It would also be of interest to investigate protein expression levels in M9 media to see the effect of lack of nutrients.

It is to be noted that universal inconsistency of the components used for culture medias means variability in results is a common issue (Sridhar & Steele-Mortimer, 2016). In addition to the growth of mutant strains using standard culture media, the same can be done in a modified medium, as mentioned previously. Adjustment of culture media ingredients may induce an invasive profile in *Salmonella* Typhimurium cells; some studies have shown that changes in SPI-1 inducing conditions impact the ability of *Salmonella* to invade and replicate intracellularly using invasion and replication assays (Sridhar and Steele-Mortimer, 2016). These changes have been attributed to universal inconsistencies between commercial LB media components. This could explain the difference observed between growth of the ΔSTM3030/31 double mutant in LB media and M9 minimal media. In summation, a multitude of factors can affect *in vitro* bacterial growth.

Considering the visually slower growth of the ΔSTM3031 and ΔSTM3030/31 strains in M9 minimal media around the eighth hour compared to that of the wildtype strain, the next step

would be to gather data between the eighth and 24th hours. It would be of interest to identify firstly if their growth continues at a slower pace than the wildtype, and secondly when the two mutant strains recover and catch up to the wildtype. This would provide the basis for future experiments related to STM3030 and STM3031 protein expression during the various bacterial growth phases. This would be done through construction of *lacZ*-promoter fusions to be used in β-galactosidase assays. A promoter fusion consists of the fusion of a promoter region of a gene, in this case that of the *stm3030* and *stm3031* genes, to the coding sequence of a reporter gene, which in a *lacZ*-promoter fusion would be the *lacZ* gene (Ma, 2018). This was previously done with the STM3030/31 double mutant by Hannah Wells, a past PhD student from the Rowley lab, in 2015, however she was unable to make constructs of the two single mutants. Results from her study showed expression levels of STM3030/31 increased across all growth phases, as well as a ~5-fold increase in STM3030/31 promoter activity between the lag and stationary phases. Additionally, results showed a significant decrease in STM3030/31 expression in mutants lacking the CpxA and ZraS HSKs, and the CpxR, BaeR, and ZraR RRs. A future direction to take would be to construct STM3030 and STM3031 promoter fusions to assess the individual expression of the proteins to ascertain whether expression of the double mutant differs to that of the single mutants. It would be beneficial to carry these out in both LB media and M9 minimal media.

These results elucidate the effects of the loss of the STM3030 and STM3031 proteins, which is the primary purpose of reverse genetics. Following on from this, complementation studies would verify the phenotypes of deletion mutants. This would help determine if there is an observable phenotype recovery or unexpected gain of function. Doing this with the single and mutant strains could shed light onto the putative chaperone function of STM3030.

3.4.2. STM3030 and STM3031 do not contribute to sodium tungstate and ceftriaxone resistance, while STM3031 contributes to cephalothin resistance

These results detail MIC assays conducted on *S.* Typhimurium to assess the involvement of STM3030 and STM3031 in resistance to the antibiotics cephalothin and ceftriaxone by examining the effects of loss of the two proteins. The results also detail sodium tungstate sensitivity tests carried out through spot plates and kill curves. Using single and double mutant strains, we grew the *S.* Typhimurium strains under normal laboratory setting growth conditions

and tested the resistance of each strain to cephalothin, ceftriaxone, and sodium tungstate compared to the isogenic parent strain.

The inconsistent results observed in the sodium tungstate kill curves may be explained by a miscalibrated spectrophotometer, an event which was reported by other research groups. Further repeats should be conducted using a spectrophotometer that is known to be well calibrated, or inoculated LB control plates should be measured against known concentrations of cultures to be able to adjust for error.

The lack of apparent phenotype for sodium tungstate sensitivity does not exclude regulation of STM3030 and STM3031 by the BaeSR 2CST, and a simple explanation is that they may just not be involved in sodium tungstate resistance. These results aid in placing STM3030 and STM3031 in the BaeSR pathway in relation to their functions and regulation by the 2CST.

A decrease in survival of ΔSTM3031 compared to the wildtype would prove its role in cephalothin resistance, as when it is knocked out the bacterial cells do not grow as much. Results from the ΔSTM3030 strain point to STM3031 having the main resistance functions as loss of STM3030 has no detrimental effect on *S.* Typhimurium survival. Additionally, results indicate that no collaborative role in resistance between STM3030 and STM3031 exists as the ΔSTM3030/31 strain is not affected by loss of both proteins. This seems to be confirmed by the difference in survival between ΔSTM3030 and ΔSTM3031 at all concentrations, both of which suggest other components are induced in the absence of both STM3030 and STM3031 to restore resistance to cephalothin.

Importantly, as previously mentioned, the starting values at 0 μg/mL of the ΔSTM3031 were below that of the other strains in both the cephalothin and ceftriaxone MIC, which could have influenced the results from the successive concentrations. This deserves re-examining as any statistical significance could be due to this initial miscalibration. Had there been a growth phenotype associated with the loss of STM3031, a difference in STM3031 growth compared to the other strains could have been expected during the MIC, however since none was found, this can be excluded from the reasons behind this difference.

The results on STM3031 yielded in this study were aligned with previous literature that report the involvement of STM3031 in resistance to cephalosporin antibiotics like cephalothin (Lin,

et al., 2019). However, the same study reported a greater reduction in resistance to cephalothin for STM3030 than for STM3031, which was not observed in this study. Other studies by Hu *et al.* in 2005 and 2009 implicated STM3031 strongly in resistance to ceftriaxone, specifying that STM3031 contributes to antibiotic resistance through reduction of permeability by a decrease in OMPD porin levels and increase AcrD efflux pump activity to enhance export (Hu, *et al*., 2009; Hu, *et al*., 2005). It is possible that the strains used in the studies by Lin *et al.* in 2019 and Hu *et al.* in 2009, which utilised the same method, contribute to their observed phenotypes and why they were not replicated in this study. For their studies, a ceftriaxone susceptible *S.* Typhimurium strain was used and through a process of multistep resistance selection, a resistant strain was generated (Lin, *et al*., 2019; Hu, *et al*., 2009). Using this strain, STM3030 and STM3031 were deactivated through one-step inactivation of the chromosomal genes. This method casts doubt on the validity of the results as there is no comparisons with commonly used laboratory wildtype *S.* Typhimurium strains, like LT2, SL1344, or ATCC 14028 (Branchu, *et al*., 2018). Though in this study there was no phenotype linked to STM3030 and STM3031 mediated ceftriaxone resistance, conducting similar assays as to Lin *et al.* and Hu *et al.* could be of interest to see whether results are reproducible.

Further investigation is warranted to ascertain whether STM3030 indeed contributes to resistance, as reported by Lin *et al.*, or collaborates with STM3031 in any capacity. This could be done with more repeats of MICs but with a larger range of concentrations, particularly between 5 μg/mL and 10 μg/mL as it is between these two concentrations that the greatest decrease in bacterial survival was observed. Examining STM3030 and STM3031 protein expression levels would also be beneficial as results would indicate whether presence of antibiotic compounds induce expression. This would, as mentioned previously, be done through construction of *lacZ*-promoter fusions to be used in β-galactosidase assays. Additionally, alternative assays could be conducted to test the strains against antibiotics in different environments. For example, Kirby-Bauer disk diffusion susceptibility tests allow for a qualitative approach to antibiotic resistance evaluation. Disk diffusion assays are a visual way to assess antibiotic resistance as a ring of inhibition surrounding antibiotic-laden disks appears if a bacterium is susceptible (Bauer, *et al*., 1959). In the area closest to the disk, the antibiotic concentration is higher and the further away from the disk, the lower the antibiotic concentration through a logarithmic reduction in concentration. This would aid in quickly ruling out other antibiotics when assessing resistance for more quantitative MIC and minimum bactericidal concentration (MBC) assays to be done.

As antibiotic resistance is becoming an increasingly worrying issue, finding alternatives to antibiotics is becoming a pressing issue. Resistant bacterial strains, particularly enteric bacteria, effectively act as reservoirs that contain resistance genes that can easily be spread to surrounding bacteria within the same environment (Su, *et al*., 2003). Adaptation to toxic compounds and antibiotics is a crucial step in the development of resistance to these elements, which highlights the importance of understanding exactly what mechanisms influence these processes. These results highlight the need for more in-depth examination of STM3030 and STM3031 mediated resistance to antibiotics as results from this study and other studies are conflicting. Other antibiotics such as ciprofloxacin, a fluoroquinolone antibiotic, should be tested against STM3030 and STM3031 to check if they confer resistance. This study aimed to carry out MICs for ciprofloxacin but due to unforeseen circumstances caused by the COVID-19 pandemic, this could not be done. Furthermore, β-lactam antibiotics, such as penicillin G and carbenicillin, should be investigated as the YfdX homologue in *S.* Typhi, which is an ortholog for *S.* Typhi STY3178, was reported to be involved in their resistance (Lee, *et al*., 2019).

3.4.3. Adhesion to host cells requires STM3030 and STM3031, while invasion requires STM3031 only within a 60-minute incubation but not after a 24-hour incubation

These results detail adhesion and invasion assays conducted on *S.* Typhimurium to assess the roles of STM3030 and STM3031, individually and together, in host infection and the effects of their loss. Using single and double mutant strains, we grew the *S.* Typhimurium strains under normal laboratory setting growth conditions and tested the ability to adhere and invade of each strain in human Caco-2 cells compared to the isogenic parent strain.

Adhesion assays were performed using Caco-2 cells, human epithelial colorectal adenocarcinoma cells. *Salmonella*'s primary point of entry into human cells is through epithelial cells in the intestines, therefore Caco-2 cells, which form confluent monolayers *in vitro*, are a good model to assess the adhesion and invasion potential of strains. The assay involves infecting the Caco-2 cell with the strain of choice and leaving to incubate for 15 minutes to recreate *in vivo* infection. Cells are washed and subsequently lysed, and in theory, only bacterial cells that have successfully adhered to the Caco-2 cell surfaces remain. The substrate is plated onto agar plates in descending concentrations and incubated overnight. If

any bacterial cells adhered to the Caco-2 cells, they would grow, and the bacterial colony spots are counted and compared to the overnight cultures used in the experiment. This method allows for calculation of percentage adhesion of bacterial cells to human cells.

These results gathered in this study contradict the results from Chowdhury *et al.* in which they state STM3031 in *S.* Typhimurium does not have a role in adhesion and invasion. There are several conceivable explanations for the results gathered by Chowdhury *et al.*, varying from the *S.* Typhimurium strain used to the use of mice to assess *S.* Typhi pathogenesis. The greatest indicator that STM3031 may not be redundant in all *S.* Typhimurium strains is the use of the LT2 *S.* Typhimurium strain in their study. The LT2 strain is known to be a non-virulent strain, and in mouse models are found to be less virulent than other strains, such as SL1344 (Wiesner, *et al*., 2016; García-Quintanilla & Casadesús, 2011). The possibility that the results gathered by Chowdhury *et al.* are only applicable in an LT2 background allows for the possibility of STM3031 having role in pathogenesis in other *S.* Typhimurium strains.

Since the double mutant showed significant difference in percentage adhesion and relative percentage adhesion, it can be assumed that STM3030 and STM3031 are needed together for adhesion. As STM3030 is a putative chaperone for STM3031, their mechanism of action could be that of STM3030 assisting and directing STM3031 in carrying out its adhesion function when in contact with host cells. Contrarily, as only the ΔSTM3031 strain showed a significant decrease in the ability invade cells, it could mean that once STM3030 has carried out its chaperone function in adhesion to host cells, STM3031 no longer requires it and only STM3031 is involved in invasion. This would add credence to STM3030 being a chaperone for STM3031. However, a possible alternative occurrence is that STM3030 accumulates within bacterial cells in the absence of STM3031 and creates a toxic environment during invasion, causing bacteria to die, which is why the ΔSTM3031 has a lower level of invasion compared to both other mutants. This toxic accumulation of STM3030 is not seen in the double ΔSTM3030/31 mutant during invasion as STM3030 is knocked out. To verify this, RNA and protein expression of STM3030 should be checked by SDS-PAGE Western blots.

A method to assess whether these adhesion and invasion functions are in fact related to STM3030 and STM3031 and to verify the phenotype, complementation studies using a plasmid should be done. As mentioned previously in section 3.5.1., this would determine if there was an observable phenotype recovery or unexpected gain of function. The protein sequences

would be reintroduced into the knockout strains and both adhesion and invasion assays would be conducted again to check whether the ability to adhere to and invade cells would be reinstated. If their ability to adhere to and invade cells was not regained, it could mean that an additional mutation was gained upon knockout of the genes, which would not be rescued by complementation of the genes.

Another method to evaluate cellular adhesion and invasion is to use non-invasive *in vivo* imaging. Qualitative differential immunofluorescence (DIF) assays would permit visualisation of extracellular and intracellular bacteria. Through immunostaining of specific elements, the number of bacterial cells that adhere to host cells could be counted and invasion mechanisms could be assessed. It has been proposed that T3SS-independent invasion could be mediated through a different mechanism than the "trigger" mechanism, such as the "zipper" mechanism, and it would be of interest to know which invasion mechanism is employed by STM3031 (Chowdhury, *et al*., 2015).

Since many bacterial pathogens tend to infect mucosal tissues and adhesins have been primarily implicated as virulence factors, it would be beneficial to examine the adhesion and invasion of *S.* Typhimurium STM3030 and STM3031 mutant strains in a cell line that produces more mucus, such as the HT29-MTX cell line. The HT29-MTX cell line is a mucus-secreting cell line derived from the HT29 cell line, which are human colon adenocarcinoma cells that have been differentiated into mature goblet cells using methotrexate (Martínez-Maqueda, *et al*., 2015). As Caco-2 is a non-mucus producing cell line, the results could elucidate either an enhanced or a reduced phenotype. HT29-MTX cells are thought to be better for studying host-pathogen interactions as the mucus layer they form offer more physiologically relevant characteristics (Gagnon, *et al*., 2013). Studies have shown that although mucus is a defence mechanism employed by the host against pathogens, *Salmonella* is able to sabotage this to increase pathogenicity, therefore using high mucus producing cells could reveal stronger phenotypes (Gagnon, *et al*., 2013). According to other studies, the role that adhesins and invasins have in aspects like cell specificity also must be considered, therefore infection assays in professional phagocytes, like macrophages, would be beneficial to ascertain whether STM3030 and STM3031 have greater cell specificity in professional phagocytes or other cell types (Velge, *et al*., 2012). As more T3SS-1-independent adhesion and invasion mechanisms are being uncovered, it would also be beneficial to determine whether adhesion and invasion mediated by STM3030 and STM3031 are indeed independent of the T3SS-1.

Both adhesion and invasion of bacterial cells are affected by various factors, such as host cell type, host cell and bacterial cell viability, confluency of host cells, and bacterial growth conditions, which means there is experimental variability to be expected (Steele-Mortimer, 2008). There are many elements that could have been affected by human errors during this study, which could invalidate results and have been a partial cause of the large margin of error seen in the wildtype and double mutant error bars in all the invasion results, which warrant further investigation as the results could have been impacted negatively by an unknown factor with the large margin of error evidencing this.

On a wider scale, broader research is required to truly understand STM3030 and STM3031 functions. Neither protein structure in *S.* Typhimurium has been purified and all protein structure studies have so far been done using computational predictions and bioinformatic techniques, which limits the ability to understand the three-dimensional folded structure of the proteins, and how they interact with each other and other factors within the bacterial cell. To do this, purification of the proteins should be conducted to then determine their structure through X-ray crystallography. The protein-protein interactions of STM3030 and STM3031 should also be investigated either through protein affinity chromatography or immunoprecipitation. To assess the putative chaperon activity of STM3030, co-purification studies of the two proteins should be done, with additional *in vitro* screenings to verify the activity.

4. General discussion

As the results of the results chapter were discussed individually, this general discussion will focus on the broader conclusions of this study, wider context, and the potential impact of this research.

The results gathered in this study have enhanced our understanding of the YfdX family protein STM3030 and the Ail/OmpX-like protein STM3031 by using four separate *S.* Typhimurium strains, one isogenic parent strain and three mutant strains, providing insight into their contributions to *S.* Typhimurium stress response and virulence.

4.1. Context

Salmonella spp. is a Gram negative, rod-shaped bacterium, belonging to the Enterobacteriaceae family. *Salmonella* spp. are facultative intracellular pathogens that invade host epithelial cells and uses host phagocytes to replicate during infection (Gong *et al*., 2011). *Salmonella* taxonomy is complex and interestingly, ever evolving, whereby *Salmonella* strains can be classified into species and subspecies according to the similarity in genetic sequences (MacKenzie, *et al*., 2017). The diseases caused by the various serovars of medical relevance to humans makes *Salmonella* a prevalent source of mortality and morbidity worldwide, and the rise of multi-drug resistant (MDR) strains is posing an increasingly serious threat (Saleh, *et al*., 2019; Eng, *et al*., 2015). The *Salmonella* serotype and overall health status of the host greatly influences the severity of the infection (Eng, *et al*., 2015). Antibiotic resistance has the potential to take us back to a pre-antibiotic era as only a few new antibiotics have been introduced in the last years, compared to the Golden Age of antibiotic production, and there is a scarcity of new broad-spectrum antibiotics (Klemm, *et al*., 2010). This, added to the presence of iNTS strains associated with other illnesses such as HIV, malaria, and malnutrition in sub-Saharan Africa, puts a stress on the need for novel *Salmonella* therapies and prophylaxis. Though vaccines for *S.* Typhi exist, they are limited to infants and are not useful against NTS and Paratyphi infections (Eng, *et al*., 2015).

Salmonella spp. have a large variety of components that aid in processes such as adaptation to stress and pathogenesis in the host, with many of these elements overlapping and cooperating. The mechanisms that contribute to responding to any stressors encounters are ESR systems, which are tasked with countering signals though a myriad of cascade events to fix damage, maintain cell integrity, and maintain homeostasis. These systems are also involved in

pathogenesis, working to respond to any changes encountered within the host to promote survival.

Although the threat posed by emerging pathogens, such as the SARS-CoV-2 virus, is of global importance, the millions of cases caused by bacterial pathogens, such as *Salmonella* Typhimurium and Typhi, every year cannot be dismissed. The ability to cause disease in host animals is a fundamental aspect of a pathogen's life cycle and microbial pathogens are the cause of a quarter of all yearly deaths worldwide (Welch, 2015). Uncovering the mechanisms behind *Salmonella* survival, success, and proliferation outside and within the host remains of utmost importance in the effort to fight against these diseases. The added menace of antibiotic resistance is another reason why the search for novel and alternative therapies is imperative.

4.1.1. Why study *Salmonella* stress response and virulence?

As a Gram-negative bacterium, Salmonella's bacterial envelope is comprised, from the inside outwards, of the inner membrane (IM), the periplasmic space (PP) that contains a peptidoglycan (PG) layer, and the outer membrane (OM) (MacRitchie, *et al*., 2008). These structures are what separates and protects the bacterial cell from the extracellular environment. Salmonella encounters various stresses in its environments, both inside and outside of a host, to which it must adapt. Changes in heat and pH, antimicrobial compounds, and reactive oxygen and nitrogen species are some stressors that can cause damage to the bacterium, particularly to the cell wall (Runkel *et al*., 2013). Various envelope stress response (ESR) pathways have evolved to combat these conditions, which work to sense protein damage and consequently fix them, ultimately restoring homeostasis. Some of these pathways include the 2CSTs BaeSR, CpxAR, and ZraSR; the extracytoplasmic sigma factor σ^E (*rpoE*) pathway; the regulator of capsule synthesis (Rcs) phosphorelay system, and the phage shock protein (Psp). They all contain regulon members that effectuate the countering response to any stress encountered. Some of these ESRs have alleged roles in antibiotic resistance and are therefore potential targets for novel antibacterial therapies (Rowley, *et al.*, 2006). Furthermore, the ability to successfully invade and infect hosts is mediated by the methodical regulation of various virulence factors, many of which are reported to be envelope-localised (Hews, *et al*., 2019). ESR mechanisms have been investigated as therapeutic targets due to their critical roles in protecting Salmonella in the challenging environments it finds itself during host and non-host

lifestyles, as well as being essential for bacterial pathogenesis. The vital roles the components within systems have make them suitable and promising targets for laboratory research.

Investigating *Salmonella*'s molecular mechanisms of disease helps us to understand how pathogens adapt to their environment and is an important step in developing novel therapies, including vaccines, to combat antibiotic resistance. Prevention of disease is the ideal method in the fight against pathogens as it not only impedes a person from becoming ill but can also avoid spread of disease from one host to another. Factors that influence bacterial pathogenesis, such as adhesion, can be the perfect targets as they effectively inhibit a bacterium's ability to initiate disease. Adhesion has been described as the Achilles heel of *Salmonella* pathogenesis and is an attractive therapeutic target for vaccine development (Klemm, *et al*., 2010). Anti-adhesive vaccines have been trialled previously, though these have mainly been targeted at bacterial fimbriae. A successful anti-diarrhoea vaccine targeting the K88 fimbriae in Enterotoxigenic *E. coli* (ETEC) was developed for piglets, however other vaccines targeting fimbriae have failed due to genetic variation in fimbrial proteins (Klemm, *et al.*, 2010). A vaccine against the FimH adhesin in *E. coli* has been shown to be effective against cystitis, exposing adhesins as viable targets for vaccine development (Klemm, *et al.*, 2010). Bacterial adhesion is also imperative for *Salmonella* survival outside of the host, with a predominant function of biofilm formation. Biofilms allow bacteria to persist in harsh conditions and are extremely resistant to antimicrobials and disinfectants, which make them a serious issue as they can revert back to a planktonic state with the ability to create infection with no delay. Biofilms have been implicated in outbreaks in medical settings and in the food industry (Paytubi, *et al*., 2017). In a study by Bernbom *et al.*, it was uncovered that coating surfaces with α-tropomyosin from fish muscles inhibited the adhesion of various strains of *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *E. coli*, *Vibrio anguillarum*, and *Aeromonas salmonicida* to various inert surfaces for at least 7 days while also reducing their ability to form biofilms, showing promise for the development of anti-adhesive products against *Salmonella* and other biofilm-forming pathogens (Bernbom, *et al*., 2006).

4.2. STM3030 and STM3031 – contribution to *S.* Typhimurium stress response and virulence

This study set out to further characterise STM3030 and STM3031 functions as homologues in other *Salmonella* serovars and other bacterial species have been reported to have roles in antibiotic resistance, pathogenesis, virulence, and biofilm formation. The proteins are also thought to be regulated by two 2CSTs, CpxAR and BaeSR, as functional overlaps between them have been reported, implicating the proteins in *Salmonella* stress response.

Results from a previous study by a former PhD student of the Rowley lab, Dr Hannah Wells, reported a biofilm phenotype associated with the loss of STM3030 and STM3030/31 in a single STM3030 mutant strain and a double STM3030/31 mutant strain (Wells, 2015). As biofilm formation is heavily linked to adhesion, the roles of the proteins in adhesion to human host cells were of research interest. Though a study by Chowdhury *et al.* reported STM3031 as being redundant for adhesion and invasion, these results were conducted in the minimally virulent LT2 *S.* Typhimurium strain, which sparked our interest. This study sought to compare the results of adhesion and invasion assays in a more virulent strain of *S.* Typhimurium, SL1344.

A hypothesis of at least having STM3031 be involved in adhesion or invasion of human host cells was put forward, seeing as OmpX homologue in *Y. pestis* is involved bacterial adhesion and invasion of mammalian cells, as well as virulence, and the SL1344 strain is more virulent than the LT2 strain (Vogt & Schulz, 1999). Results from this study confirmed STM3031 involvement in adhesion and invasion of Caco-2 cells, as well as implicating STM3030 in adhesion. The proposed mechanism of these protein functions is that as STM3030 is a putative chaperone for STM3031, and adhesion requires both proteins, STM3030 is responsible for chaperoning STM3031 for correct interaction with the host cell surface. As only STM3031 is needed in host cell invasion, it can be assumed that STM3030 has completed its role of chaperone and only STM3031 is required beyond that point.

The biofilm functions of both STM3030 and STM3031 were planned for this study and assays using CFA plates were prepared, however due to interruptions caused by the COVID-19 pandemic and subsequent lockdown, they were pushed aside. There is potential for the proteins' involvement in biofilm formation, as they have been implicated in host cell adhesion.

The substantiation of a role in biofilm formation could potentially classify STM3031 as a *S.* Typhimurium adhesin, coupled with definitive confirmation of STM3031 being involved in host cell attachment.

4.3. Concluding remarks

The wider implications of this study are far reaching, when being ambitious. Starting from a continuation of this study, further characterisation of STM3030 and STM3031 would be greatly beneficial as it would establish their function in relation to stress response through regulation by the CpxAR and BaeSR 2CSTs, as well as their involvement in bacterial pathogenesis and biofilm formation, in addition to their previously documented roles in antibiotic resistance. Understanding how the proteins function and interact can then lead to them being investigated as potential therapeutic targets. As previously mentioned, targeting bacterial adhesion has been successful in preventing attachment to abiotic surfaces and as a vaccine target. Another aspect would be to examine how alternative therapies, aside from vaccines, could block adhesion and invasion in host cells. Agents that can either inhibit the STM3030 chaperone function could limit the ability for STM3031 to attach to cells. Uncovering what STM3031 binds to during host cell attachment would also be a viable therapy target. These options would ease clearance of bacteria from host tissues as well as impede any damaging factors, such as toxins, being released by bacteria and averting disease (Stadler & Dersch, 2016). The development of drugs that modify bacterial behaviour rather than kill them, like many standard antibiotics do, could be a worthwhile alternate as these drugs may lead to less bacterial resistance (Klemm, *et al*., 2010).

As many *Salmonella* species have many genetic sequences in common, the corresponding homologous proteins for STM3030 and STM3031 in a wide range of *Salmonella* should be investigated for their roles in antibiotic resistance and virulence. As there are currently no vaccines available against other NTS strains and *S.* Paratyphoid, there is the potential for these proteins to be vaccine targets.

These results reveal investigative options that are worthy of being examined. Many *Salmonella* genes remain uncharacterised, limiting our understanding and our ability to control *Salmonella* mediated diseases. The future of bacterial pathogen research is bright but has to quickly catch up to the immense threat caused by antibiotic resistance.

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