Investigating Resistance to Antibody–Dependent Complement–Mediated Lysis in Invasive *Salmonella* Typhimurium

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Von

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LIST OF ACRONYMS AND ABBREVIATIONS

ARV	Anti–retroviral
ESBL	Extended–Spectrum Beta–Lactamase
GMMA	Generalized Modules for Membrane Antigens
HIV	Human Immunodeficiency Virus
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LPS	Lipopolysaccharide
MAC	Membrane Attack Complex
MHC	Major Histocompatibility Complex
MLST/ST	Multi–Locus Sequence Type/Sequence Type
NK	Natural Killer [Cell]
NTS/iNTS	Nontyphoidal *Salmonella*/invasive Nontyphoidal Salmonellosis
PMN	Polymorphonuclear [Leukocytes]
SCV	*Salmonella*–Containing Vacuole
SNP	Single–Nucleotide Polymorphism
SPI	*Salmonella* Pathogenicity Island
SSA	Sub–Saharan Africa
T3SS	Type–III Secretion System
TCS	Two–Component System
TLR	Toll–Like Receptor
TNF	Tumour Necrosis Factor

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Mwandani wangu Muthamia: Kwa nyakati zote zile uliponiamini na uwezo wangu wa kuimaliza kazi hii kuliko nilivyojiamini mwenyewe; sina kingine cha kukupa ila moyo wangu.

To mama, papa, Paul, Sandra, Joan, Brandon, and Jeff; Thank you for being the brightest spots of encouragement along this long path: your love and support have seen me through.

All the glory and honour to Him who gives the power to think and do.

DEDICATION

This thesis is dedicated to

my parents William and Hellen,

to whom I owe my existence, and untiring desire to learn;

and to Prof. Conrad D. Clausen and the memory of Venus E. Clausen,

whose dedication, selflessness, and sacrifice have inspired me to look above and beyond.

SUMMARY

Salmonella Typhimurium is a common cause of self–limited foodborne gastroenteritis globally. In contrast, epidemics of fatal, invasive nontyphoidal *Salmonella* (iNTS) disease have long been reported in sub–Saharan Africa (SSA), accounting for most of the iNTS disease burden worldwide. Despite this, the significance of this bacterium as a cause of disseminated infections, and the specific mechanisms of pathogenesis have only received considerable attention within the last 15 years or so.

Nontyphoidal *Salmonellae*, particularly serovars Typhimurium and Enteritidis are a leading cause of bacterial bloodstream infections in SSA, associated with high mortality, particularly among young children and HIV–infected adults. High rates of malnutrition, malaria, sickle–cell disease, poor sanitation, and unavailability of clean water are other known contributing factors. Importantly, acquisition and spread of multiple drug resistance genes have led to the emergence of a unique pathovar, S. Typhimurium ST313. Drug resistance is also a challenge for disease management efforts, which are further confounded by a lack of distinguishing clinical features, that makes diagnosis and adequate treatment difficult. In the imminent dearth of treatment and diagnostic options for iNTS disease, it is imperative that an effective vaccine is developed.

Central to the establishment of systemic infection and invasive disease is the ability of the bacteria to overcome host defenses, first by surviving within phagocytic cells, where they mostly reside during extraintestinal infection. *Salmonellae* also disseminate between phagocytic cells during a transient extracellular phase in the bloodstream, where they encounter potent antibody responses that effect opsonophagocytosis and complement lysis. Antibody and complement are important for immunity to NTS by preventing this extracellular bacterial growth. Promising vaccine candidates based on surface antigens, particularly LPS O–polysaccharide glycoconjugates, confer protection primarily by eliciting antibody responses. Understanding the modalities of these responses, and the characteristics and important determinants of pathogen virulence that may affect antibody immunity among epidemic strains is, therefore, an important consideration for vaccines.

Characterisation of epidemic African S. Typhimurium ST313 strains thus far reveals some traits of adaptation to human infection, and a predilection for extraintestinal disease such as decreased enteric

inflammation, increased rates of systemic spread, and have a higher tolerance to complement bactericidal activity than gastrointestinal isolates.

The aim of this study, therefore, was to identify genetic determinants of resistance to antibody–dependent complement–mediated lysis among *Salmonella* Typhimurium strains and investigate the impact of this phenotype on virulence and potential to contribute to invasive disease. To achieve the study objectives, whole genome sequencing, phylogenetic analyses, saturated transposon mutant library screens in immune human serum coupled with insertion–site sequencing (TraDIS), targeted mutagenesis, transcriptome profiling and phenotypic characterization by serum bactericidal and broth macrodilution assays, in–vivo and in–vitro invasion assays were performed in both laboratory–adapted strains and invasive S. Typhimurium ST313 clinical isolates with stable complement resistance or susceptibility profiles.

No direct correlation between increased resistance to complement lysis and virulence was found in this study. While S. Typhimurium SL1344 *sapA* and *yfgA* null mutants were found to be more resistant to complement lysis than the wild–type parent strain, overall virulence measured by growth in vivo, tolerance to detergent (SDS) and osmotic (high NaCl) stress, and invasion of epithelioid cells, was not enhanced, and was observed to be at lower levels in the *yfgA*$^{-}$ strain. When measured in invasive strains, tolerance to detergent and osmotic stress was not higher among complement–resistant strains. These observations suggest that complement resistance can occur independently of other virulence traits.

Similarly, no genotypic features (at DNA sequence level) were identified as being associated with natural variation in complement resistance among the invasive strains in this study. Phylogenetic analyses of 32 S. Typhimurium ST313 isolates established that fully resistant isolates (with net growth following 3–hour exposure to serum) were found in both ST313 lineages, indicating independent instances or mechanisms of complement resistance. Whole genome analyses of a subset of these strains did not identify a common feature (SNP, indel, rearrangement, or other mobile genetic element) that discriminated resistant and susceptible strains. Characterisation of polymorphisms in individual strains predicted in–silico to have an impact on protein function did not affect viability in immune human serum when transferred to D23580. Since the vast majority of ST313 isolates studied were serum–susceptible, these observations suggest that unlike other traits of ST313 that have co–

evolved with multiple drug resistance, complement resistance does not seem to be sustained at a high level among these populations, but instead seems to be selected against in favour of susceptible isolates, and indicates absence of selection due to natural immunity against S. Typhimurium ST313.

Requirements for survival and growth of invasive S. Typhimurium strains in immune human serum were also identified by identifying differentially expressed genes and counter–selected mutants from a D23580 saturating mutant library screen. Genes involved in iron scavenging and metabolism were the most significantly represented common genes in all the six strains profiled, demonstrating the critical role of iron during growth in serum. Additionally, genes involved in extracellular polysaccharide synthesis, particularly of colanic acid were also found to be significantly up–regulated, although colanic acid mutants were not significantly affected when exposed to serum.

Cluster analysis identified transcriptional profiles that distinguished resistant and susceptible strains, with the highest variance seen in genes associated with LPS and colanic acid synthesis. While fold upregulation of colanic acid genes was higher in susceptible strains, the *fepE* gene, associated with LPS O–antigen modular chain length of over 100 (very long) repeating units, was on average 2–fold more highly upregulated in resistant strains. Other genes known to be associated with complement resistance in S. Typhimurium, such as *pgtE*, *rck*, and *traT* were all present and intact in both susceptible and resistant strains, did not differ in expression between the two phenotypes, and did not affect viability in serum when disrupted. Since no change in DNA sequence was directly associated with complement sensitivity phenotypes, this thesis postulates an epigenetic effect causing differences in expression of membrane components, particularly of extracellular polysaccharides as a mechanism for complement resistance among invasive strains.

CHAPTER 1: INTRODUCTION

1.1. The genus *Salmonella*

Salmonella is a genus of the Enterobacteriaceae family, consisting of Gram–negative, facultative anaerobic, flagellated bacilli that infect and cause disease in humans and animals. Serological typing has distinguished over 2500 *Salmonella* variants (serovars) based on flagellar (H), capsular (Vi) and somatic/lipopolysaccharide (O) antigens. *Salmonellae* are classified into two species: *Salmonella bongori* and *Salmonella enterica*. *S. bongori* serovars are mainly commensals in poikilotherms, although they can also infect humans (Giammanco *et al.*, 2002). *S. enterica* is further sub–divided into six subspecies, designated subspecies I–VI: *enterica* (subsp. I), *salamae* (subsp. II), *arizonae* (subsp. IIIa), *diarizonae* (subsp. IIIb), *houtenae* (subsp. IV), and *indica* (subsp. VI) (Desai *et al.*, 2013).

About 99% of *Salmonellae* occurring in humans and other homeotherms are subspecies I (*S. enterica enterica*) serovars (Brenner *et al.*, 2000). Of these, a relatively small number causes salmonellosis in humans and animals. Typhoidal serovars, namely, Typhi, and Paratyphi A, B, and C are human–restricted. In the year 2000, Typhoid fever was estimated to cause 27 million illnesses and over 2 million deaths, and paratyphoid fever estimated to cause 5.4 million illnesses (Crump *et al.*, 2004). Other salmonelloses (nontyphoidal *Salmonellae*, henceforth referred to as NTS), are caused by any other serovar, which typically infect a range of hosts, with varied pathogenic potential and clinical outcomes (Fierer and Guiney, 2001).

1.2. Epidemiology of nontyphoidal salmonellosis

Globally, NTS were estimated to cause 93.8 million cases of gastroenteritis and 155,000 deaths in 2006 (Majowicz *et al.*, 2010), and in 2015, mortality was estimated to be 90,000 (Troeger *et al.*, 2017). The decline is attributed to improved access to safe water and sanitation. In developed countries and most parts of the world, NTS are a leading cause of foodborne illness, with outbreaks most commonly being associated with commercial food production and distribution (Majowicz *et al.*, 2010). In these settings, infection typically presents as acute, self–limited gastroenteritis, with symptoms including fever, vomiting, diarrhoea, abdominal pain, and muscle cramps. Systemic disease is rare (occurring in approximately 6% of NTS patients) and often limited to immune–compromised individuals, children under 5, and elderly patients (Hohmann, 2001; Crump *et al.*, 2015). In sub–Saharan Africa

(SSA), however, severe, extraintestinal disease (invasive NTS/iNTS disease) is the dominant manifestation of nontyphoidal salmonellosis (Berkley *et al.*, 2005; Graham, 2009; Reddy *et al.*, 2010), although NTS are increasingly being recognized as a significant cause of diarrheal disease globally (Troeger *et al.*, 2017).

1.3. Invasive nontyphoidal salmonellosis in sub–Saharan Africa

NTS are the most common *Salmonellae* isolated from bacteremic patients in sub–Saharan Africa (Reddy *et al.*, 2010). They are a significant cause of invasive bacterial illness and death, particularly in the context of the implementation of vaccines against *Hemophilus influenzae* b and *Streptococcus pneumoniae*, respectively, which were once dominant causes of bacteraemia in this region (Graham and English, 2009; Tapia *et al.*, 2015). A combination of prevailing host, environmental, and pathogen factors define the distinct epidemiology of iNTS disease in SSA, where they are endemic. There is a distinct bimodal age–associated incidence of iNTS disease in this region, being highest among children aged 6–18 months, and adults between 24–40 years in whom the highest rates of HIV infection occur (Crump *et al.*, 2015; Gordon, 2011) (Fig. 1.1). Incidence rates of iNTS disease from the year 2010 were estimated to be 175–388 cases per 100,000 in children aged 3–5 years and 2,000–8,500/100,000 for HIV–infected adults per year (Feasey *et al.*, 2012), and 49/100,000 overall (Ao *et al.*, 2015). iNTS disease resulted in approximately 3.4 million cases and over 680,000 deaths worldwide in 2010, 57% (or approximately 390,000) of which occurred in Africa (Ao *et al.*, 2015).

Disseminated NTS disease (including bacteraemia, sepsis, and meningitis) is often fatal, with case fatality rates of up to 25%, even where blood culture facilities are available. The dominant circulating serovars are Enteritidis and Typhimurium, which account for between 80 and 95% of invasive NTS infections in Africa (Feasey *et al.*, 2012; Feasey *et al.*, 2016; Kingsley *et al.*, 2009; Okoro *et al.*, 2012; Kalonji *et al.*, 2015). Other common serovars include S. Stanleyville (more common in West Africa), S. Isangi (DRC and South Africa) S. Concord (Ethiopia) S. Dublin (Mali), S. Bovismorbificans (Malawi) and S. Infantis (Krubwa *et al.*, 1976; Tennant *et al.*, 2010; Ngandjio *et al.*, 2012; Beyene *et al.*, 2011; Kruger *et al.*, 2004; Bronowski *et al.*, 2013).

1.4. Factors associated with invasive NTS disease in sub–Saharan Africa

1.4.1. Host factors

Fatal iNTS disease is virtually exclusive to individuals with weakened or under–developed immunity (Dhanoa and Fatt, 2009). While it could occur as a primary infection, iNTS disease in SSA is predominantly an opportunistic disease associated with a higher proportion of either immune–naïve or otherwise predisposed individuals inherently at higher risk of acquiring other infections.

1.4.1.1. Young age

Children between six months and three years of age are at the highest risk for iNTS disease (Graham and English, 2009). NTS mortality is high in children, with case fatality rates of up to 27% (Brent *et al.*, 2006; MacLennan *et al.*, 2017; Graham and English, 2009) (Fig 1.1) and is speculated to be higher due to many unreported deaths of children occurring outside health facilities (Berkley *et al.*, 2005; MacLennan, 2012). This age bracket coincides with waning maternally acquired immunity in infants, and undeveloped *Salmonella*–specific antibody immunity in toddlers (MacLennan *et al.*, 2008). Exclusive breastfeeding is another plausible explanation for the relative sparing of children below four months (Morpeth *et al.*, 2009), as they presumably have less contact with infected individuals, contaminated food, water, and fomites, unlike older, weaning children. Conversely, the presence of these antibodies in older children is significantly associated with protection from fatal NTS bacteraemia (Gondwe *et al.*, 2010; MacLennan *et al.*, 2008), underscoring the importance of antibody in protection against iNTS disease.

1.4.1.2. Malnutrition

Severe malnutrition is a chronic problem in many parts of Africa, exacerbated by diarrheal diseases and parasite infections. Undernourished children are highly susceptible to infections including severe NTS bacteraemia (Keddy *et al.*, 2017a; Friedland, 1992; Noorani *et al.*, 2005; Brent *et al.*, 2006; Coetzee and Pretorius, 1956). The risk of death in malnourished children with NTS infection has been found to be as high as 49% (Berkley *et al.*, 2005; Brent *et al.*, 2006). Malnutrition reduces overall immune function by specifically altering the structure of the gastrointestinal tract mucosa, reducing lymphocyte count and IgA secretion in gut lymphoid tissue (Beisel, 1996), which could facilitate NTS proliferation in the intestine. Pathogenic bacterial infection, in turn, causes nutrient malabsorption in the gut (Rosenberg *et al.*, 1977).

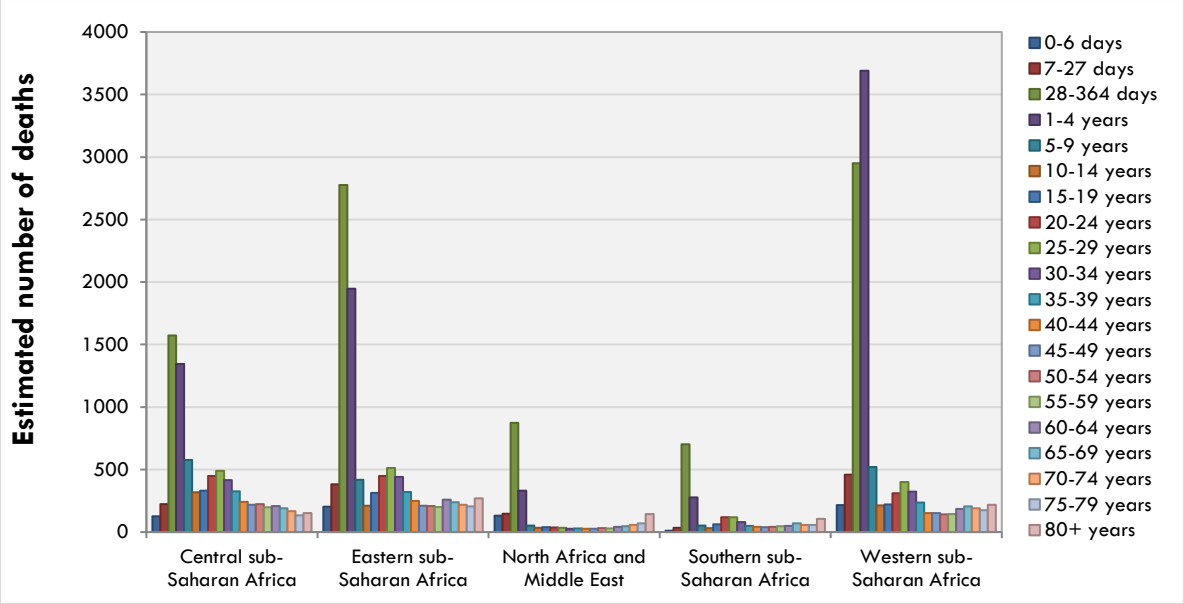

Figure 1.1. Estimates of deaths attributed to nontyphoidal *Salmonellae* in Africa in 2010, by age and region.
NTS disease prevalence and mortality, occurs in two age peaks, predominantly in young children, and around reproductive age in adults. Note: Although case fatalities in SSA are mainly due to invasive infections, these data do not distinguish between gastroenteritis and systemic NTS–related deaths. Data source: Institute for Health Metrics and Evaluation Global Burden of Disease 2010 project.

1.4.1.3. Sickle–cell disease

Approximately 80% of children with sickle–cell disease live in sub–Saharan Africa (Williams *et al.*, 2009), and are at a significantly higher risk for developing iNTS disease (Brent *et al.*, 2006; Williams *et al.*, 2009). Sera from individuals homozygous for the sickle cell trait are deficient in complement components and complement–activating capacity (Wilson *et al.*, 1979), hence impaired bactericidal activity and opsonization of *Salmonella* (Hand and King, 1977; Luo and Rowland, 1986). Furthermore, sickle–cell anaemia potentially increases the severity of iNTS disease (discussed in section 1.4.2.2).

1.4.2. Co–endemic pathogens

1.4.2.1. HIV

Observations of NTS bacteraemia as a feature of HIV/AIDS date as far back as the onset of the HIV/AIDS pandemic (Fischl *et al.*, 1986). Subsequently, invasive NTS had become a significant emergent disease with the spread of HIV in Africa by 1990 (Gilks *et al.*, 1990; Levine *et al.*, 1991), and is currently used in stage IV AIDS case definition. Because of its high prevalence, HIV is the

primary predisposing factor for iNTS disease among adults in SSA. It is also a significant risk factor for iNTS disease among children (Brent *et al.*, 2006; Feasey *et al.*, 2010; Feasey *et al.*, 2012; MacLennan *et al.*, 2017). HIV infection increases the risk of NTS bacteraemia by 20–100 fold (Gordon, 2008; Guerrant *et al.*, 2011; Meremo *et al.*, 2012). On average, 95% of adults with iNTS disease are HIV–positive (Mabey *et al.*, 2013). Recurrence rates of HIV–associated iNTS disease are high, with a mortality rate of between 20–50% (Gordon *et al.*, 2002; Mabey *et al.*, 2013). Individuals with lower $CD4^+$T cell counts are at higher risk of invasive NTS disease and death.

Defective cellular immunity during HIV infection is the primary risk factor for opportunistic infections such as NTS. HIV–induced damage to an individual's immune system favours invasive NTS pathogenesis by specifically: a) depleting T_H17 cells which are essential for maintaining the gut epithelial barrier and mucosal immunity thereby allowing the bacteria to penetrate the intestinal lining more efficiently and enter systemic sites (Dandekar *et al.*, 2010; Raffatellu *et al.*, 2008), b) causing dysregulated cytokine production, which allows persistent NTS infection (Gordon *et al.*, 2007), c) triggering dysregulated production of high anti–LPS IgG titers, which impair killing of *Salmonella* (Goh *et al.*, 2016; MacLennan *et al.*, 2010; Rowley, 1968), and d) depletion of $CD4^+$ T–cells necessary for clearance of bacteria from tissues during the systemic phase of NTS infection (Griffin and McSorley, 2011).

1.4.2.2. Malaria, malarial anemia, and anaemia

Malaria is a well–recognised risk factor for invasive salmonellosis, having first been identified early in the 20th century (Giglioli, 1929). The first known report from the African continent of the link between iNTS disease and falciparum malaria was made in 1987, where 42% of children in the Gambia with NTS bacteraemia also had malaria (Mabey *et al.*, 1987). Subsequent studies have found evidence for demographic, clinical, geographical, seasonal, and immunological associations of iNTS disease with *P. falciparum* malaria co–infection (Park *et al.*, 2016; Takem *et al.*, 2014). Malaria and conditions that cause severe hemolytic anaemia, are associated with substantially increased risk of NTS bacteraemia and death in co–infected children, as well as in murine models of infection (Brent *et al.*, 2006; Bronzan *et al.*, 2007; Kaye *et al.*, 1967; Roux *et al.*, 2010).

Several mechanisms of immune modulation during *P. falciparum* infection that predisposes to and exacerbates iNTS disease have been demonstrated, and include impairment of phagocyte, intestinal barrier, and humoral immune function (Takem *et al.*, 2014). Malaria increases plasma complement consumption, which possibly reduces complement–mediated bactericidal activity (Dulaney, 1948; Nyakoe *et al.*, 2009; Williamson *et al.*, 1978). It also decreases the availability of L–arginine, which induces inflammation, reduces the synthesis of antibacterial reactive nitrogen species, and weakens the intestinal barrier making it more permeable to *Salmonella* (Chau *et al.*, 2013). Phagocytosis of red blood cells by macrophages (both uninfected and parasitized) reduces their ability to kill *S.* Typhimurium (Gill *et al.*, 1966; Kaye *et al.*, 1967). Accumulation of heme and hemozoin in phagocytes and serum during parasite–induced haemolysis impairs phagocyte function (Schwarzer *et al.*, 1998), and increases the amount of iron available to the bacteria which enhances their survival (van Santen *et al.*, 2013). Elimination of reactive oxygen species by heme oxygenase 1 (HO–1) to mitigate heme–induced cytotoxicity during parasitic haemolysis impedes oxidative burst killing of *Salmonella* (Cunnington *et al.*, 2011). HO–1 also causes premature release of granulocytes with reduced oxidative burst capability from bone marrow (Cunnington *et al.*, 2011).

P. falciparum infection also dysregulates cytokine expression, and subsequently the response to iNTS. In mice, it diminishes levels of IL–12, which reduces phagocyte function (Roux *et al.*, 2010). In humans, it triggers secretion of IL–10, which also inhibits anti–*Salmonella* phagocytic activity (Lokken *et al.*, 2014), and down–regulates secretion of pro–inflammatory cytokines necessary for the mucosal response to NTS (Mooney *et al.*, 2014; Peyron *et al.*, 1994). Suppression of heterologous antibody production (Cunnington and Riley, 2010) and disorganization of splenic architecture during *falciparum* malaria infection (Gomez-Perez *et al.*, 2014) have also been hypothesized to impair functional responses to NTS infection.

1.4.3. Environmental factors

As with any pathogen transmitted via the faecal–oral route, ingesting contaminated food and water (common in resource–poor areas), significantly increases attack rates of *Salmonella*. These high infection rates are often a consequence of inadequate clean water, poor sanitation and drainage, crowded and squalid living conditions, and unsafe food–handling practices. NTS incidence peaks at the onset of the rainy season (Brent *et al.*, 2006; Tapia *et al.*, 2015), presumably due to increased

surface runoff carrying faecal organisms into water sources, and a surge in malaria transmission. In rural and peri–urban areas, rearing livestock within homesteads and unprotected water sources have been identified as potential risk factors for NTS infection (Morpeth *et al.*, 2009). However, while contact with infected animals and contaminated animal food products is a definite mode of transmission in developed countries, the contribution of zoonotic transmission in SSA has long been a subject of debate, since a lack of association between NTS genotypes isolated from humans and animals living closely together has been reported (Dione *et al.*, 2011; Kariuki *et al.*, 2006b).

1.4.4. Pathogen factors

NTS serovars cause disease at different frequencies, with some inherently being more invasive than others (Jones *et al.*, 2008; Chiu *et al.*, 1999; Andino and Hanning, 2015), albeit depending on geographical region (Langridge *et al.*, 2009). Serovars Typhimurium, Enteritidis, Dublin, and Choleraesuis, which most frequently cause invasive disease in animals and humans, all have the *spv* locus (Guiney *et al.*, 1995; Guiney and Fierer, 2011). *spv* genes are essential for survival within macrophages and extraintestinal dissemination of iNTS in mice (Guiney and Fierer, 2011). SpvB induces apoptosis and prevents polymerization of actin, which disrupts the infected macrophage's cytoskeleton hence the release and proliferation of bacteria (Lesnick *et al.*, 2001; Tezcan-Merdol *et al.*, 2001). The bacteriophage ST64B is also strongly associated with S. Typhimurium bloodstream isolates, and the presence of this locus increases the ability of bacteria to survive in blood, albeit by a mechanism that is as yet unidentified (Herrero-Fresno *et al.*, 2014a).

Analyses of iNTS strains have identified genetic and phenotypic characteristics that may explain why NTS strains dominantly cause invasive disease in Africa, which include unique genomic features, and phenotypes that could potentially be associated with a gradual adaptation to infecting to a human host, and to causing invasive disease.

The multi–locus sequence type 313 (ST313), a relatively diverse sub–clade of S. Typhimurium is strongly associated with iNTS disease in SSA (Okoro *et al.*, 2012; Leekitcharoenphon *et al.*, 2013; Kingsley *et al.*, 2009). Although initially thought to be exclusive to SSA, reports indicate that they account for approximately 3% of clinical S. Typhimurium isolates in the UK (Ashton *et al.*, 2017). UK isolates are mostly genetically distinct from African ST313, and isolation of invasive isolates is

strongly associated with travel to Africa (Ashton *et al.*, 2017). A study from Brazil also reported about 10% of S. Typhimurium strains isolated from as early as the 1980's as being ST313 (Almeida *et al.*, 2017), albeit with a different antibiotic resistance profile from African isolates. ST313 strains causing invasive disease in SSA are predominantly multi–drug resistant (Akullian *et al.*, 2018; Kingsley *et al.*, 2009; Okoro *et al.*, 2012). The spread of HIV and acquisition of resistance to chloramphenicol are factors postulated to have influenced two sequential clonal expansions that led to the emergence and spread of the ST313 pathovar in Africa (Okoro *et al.*, 2012). The two ST313 lineages/clades have been estimated to emerge approximately 52 and 35 years ago, with the latter represented among the majority of the current invasive S. Typhimurium isolates (Okoro *et al.*, 2012).

ST313 strains have undergone genome degradation and accumulated nonsense mutations resulting in loss of genes, including those that facilitate survival in diverse hosts and environments. This is a feature seen extensively in the genomes of many host–restricted or host–adapted *Salmonella* serovars such as Typhi (human), Choleraesuis (swine), Pullorum and Gallinarum (wild fowl/poultry), and S. Typhimurium DT2 (feral pigeon) (Foley *et al.*, 2013; Guard *et al.*, 2011; Langridge *et al.*, 2015; Sabbagh *et al.*, 2010; Kingsley *et al.*, 2013). Compared to reference genomes, invasive S. Typhimurium ST313 isolate D23580 is 15kb shorter than SL1344 and has a total of 77 pseudogenes, 23 of which are unique to D23580 (Kingsley *et al.*, 2009), compared to 39 identifiable pseudogenes in strain LT2.

These genomic events are hypothesised to be features of adaptation to human infection (Kingsley *et al.*, 2009). The extent of genome alterations that influence the unique pathogenicity and clinical phenotypes of S. Typhimurium ST313 is not yet fully characterised, although some loci unique to ST313 among S. Typhimurium strains associated with virulence factors have been identified. ST313 possesses unique insertion sequences and a distinct repertoire of prophage elements (Kingsley *et al.*, 2009; Owen *et al.*, 2017), which have the potential to modify virulence in these strains. An example is the modification of O–antigen length in D23580 and phage resistance conferred by a putative endorhamnosidase gene, *gtr*C, present on the ST313 prophage BTP1 (Kintz *et al.*, 2015). *st313–td*, a gene dominant among ST313 but absent in animal and gastrointestinal S. Typhimurium isolates, was associated with intramacrophage survival, invasiveness and systemic infection in mice (Herrero-Fresno *et al.*, 2014b; Leekitcharoenphon *et al.*, 2013). *st313–td* has homologs in other invasive

Salmonellae such as S. Dublin, which suggests that virulence gene acquisition may have had a role in the emergence of the ST313 pathovar as an agent of invasive bacterial disease.

Adaptation of ST313 strains to human infection is partly evident in the loss of genes that specifically enable bacterial survival outside the human host. This includes genes such as *bcsG*, and *katE*, resulting in phenotypes such as reduced biofilm formation and loss of bacterial aggregation, necessary for the survival of extreme environments (Ramachandran *et al.*, 2016; Singletary *et al.*, 2016), which suggests adaptation to a more restricted mode of transmission of this genotype. While there is growing evidence for their adaptation to human infection, the reservoir for S. Typhimurium ST313 is still undetermined, and successful infection of various animal species has been demonstrated with D23580, an index ST313 strain (Parsons *et al.*, 2013).

Besides host adaptation, S. Typhimurium ST313 phenotypes also indicate evolution to systemic, rather than localised gastrointestinal infection. Compared to *Salmonellae* causing gastroenteritis, they induce less IL–1β–mediated inflammation associated with the acute phase of the innate immune response by reduced expression of flagellin, which not only decreases their motility, but blunts immune responses, reduces levels of apoptosis, and caspase–1–dependent macrophage death (Carden *et al.*, 2015). ST313 strains are also less enteropathogenic, and have lost the ability to utilize some nutrient sources available in the gastrointestinal tract, such as allantoin and tartrate (Okoro *et al.*, 2015), are better adapted to survival within macrophages (Almeida *et al.*, 2017; Ramachandran *et al.*, 2015), and less susceptible to antibody and complement–mediated bactericidal activity (Goh and MacLennan, 2013). ST313 strains also disseminate more efficiently to extraintestinal sites, evidenced by being isolated more rapidly and in higher loads from submucosal lymphoid tissue and the spleen than the gastrointestinal laboratory strain SL1344 (Parsons *et al.*, 2013; Yang *et al.*, 2015). This hyper–dissemination has been shown to be facilitated by loss of the SPI–2 effector SseI, (Carden *et al.*, 2017), which otherwise restricts dendritic cell–mediated systemic spread in gastrointestinal isolates (McLaughlin *et al.*, 2009). *sseI*$^{-}$ dependent hyper–dissemination, however, is not a pre–requisite for invasive S. Typhimurium disease, since isolates from Californian bacteremic HIV–positive patients all had an intact copy of the gene (Preziosi *et al.*, 2012).

More recently, a C to T nucleotide substitution occurring in the *pgtE* gene promoter (12 bases upstream of the *pgtE* transcsriptional start site) of the has been identified among ST313 lineage II isolates (Hammarlöf *et al.*, 2018). This polymorphism significantly increased transcription and activity of the PgtE protein in representative lineage II strains when compared to representative gastrointestinal (ST19) isolates. Subsequently, virulence in vivo was enhanced, as well as complement resistance through proteolytic cleavage of complement factor B (Hammarlöf *et al.*, 2018).

1.5. Diagnosis and clinical features of iNTS disease

Invasive NTS disease lacks a definitive clinical presentation (Peters *et al.*, 2004). Fever is the most commonly observed symptom in iNTS disease patients (Fig. 1.2), and blood culture is the gold standard for confirming NTS bacteraemia. Blood culture diagnosis, however, is confounded by poor sensitivity (about 50%), and the low blood concentrations of bacteria in iNTS disease patients, which can be up to 1cfu/mL (Gordon *et al.*, 2010; Tennant *et al.*, 2011). Co–infection with pathogens that cause febrile illness such as *P. falciparum* malaria or other Gram–negative bacteraemia, and a lack of laboratory capacity in many rural areas also make diagnoses and appropriate treatment exponentially difficult. PCR–based diagnostic assays for S. Typhimurium have also been developed and tested even in African settings (Akiba et al., 2011; Tennant et al., 2010), but are also largely limited in their use in areas without such facilities.

Progression of invasive NTS disease is rapid, and deaths due to undiagnosed NTS often occur (MacLennan, 2012). Although ST313 strains can cause gastroenteritis (Kariuki *et al.*, 2006a), diarrhoea and other symptoms typical of gastroenteritis are often absent (Brown and Eykyn, 2000; Gordon *et al.*, 2002; Kariuki *et al.*, 2006b; MacLennan *et al.*, 2017), (Fig1.3), which in individuals co–infected with *P. falciparum* could partly be the result of dampening of the mucosal inflammatory response by IL–10 secreted during malaria (Mooney *et al.*, 2014). NTS meningitis, common among HIV–infected adults and neonates, has a higher fatality rate than other bacterial causes of meningitis (Keddy *et al.*, 2015; Milledge *et al.*, 2005). Other complications of iNTS disease such as pneumonia and other respiratory symptoms, septic arthritis, and osteomyelitis have been reported (Graham and English, 2009; MacLennan *et al.*, 2017; Molyneux *et al.*, 2009; Zaidi *et al.*, 1999).

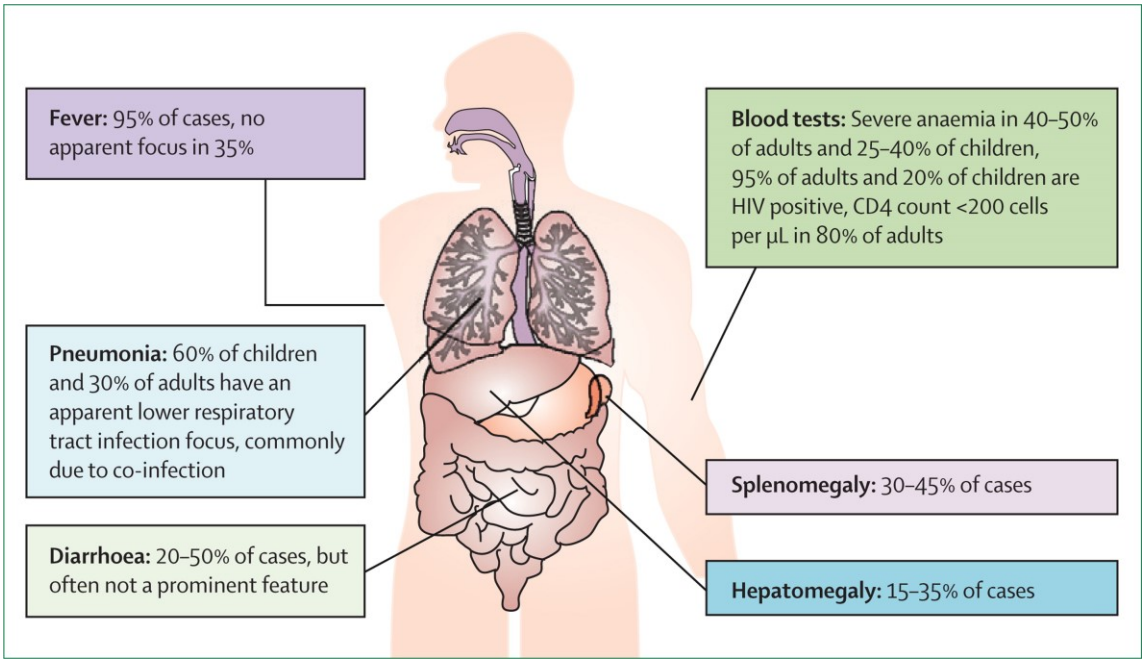

Figure 1.2. Common clinical presentations of iNTS in patients.
Figure reproduced from Feasey et. al., 2012.

1.6. NTS disease management

Due to the opportunistic nature of NTS progression to invasive disease, iNTS disease could be minimised by controlling primary risk factors. For instance, the control of HIV has significantly reduced the occurrence of AIDS–related opportunistic infections and deaths (Feasey *et al.*, 2014; Keddy *et al.*, 2017b), and has been successful for iNTS disease in developed countries (Hung *et al.*, 2007). Likewise, proper sanitation, improved nutrition, ARV therapy and measures to control associated diseases could alleviate the risk of iNTS disease in developing countries. Currently, however, the mainstay for specific iNTS disease management in predominantly resource–poor settings is antibiotics, with intensive research efforts offering the prospect of effective vaccines.

1.6.1. Antibiotics

The spread of multiple resistance to affordable antibiotics among disease–causing strains has significantly limited the treatment options available for iNTS disease. Infections with multi–drug resistant *Salmonella* strains result in higher frequencies of bacteraemia, hospitalizations (Varma *et al.*, 2005), and death (Helms *et al.*, 2002). iNTS disease outbreaks and epidemics have been attributed to horizontally acquired multi–drug resistance genes (Kariuki *et al.*, 2015; Kingsley *et al.*, 2009). Two examples are the rapid spread of *S.* Typhimurium strains in Malawi, where multi–drug–resistant

strains have accounted for up to 90% of NTS bloodstream isolates (Gordon *et al.*, 2008; Feasey *et al.*, 2015), and the expansion of ESBL–producing S. Isangi in South Africa (Kruger *et al.*, 2004). The emergence of multi–drug resistant NTS strains is common in many sites in SSA (Muthumbi *et al.*, 2015; Oneko *et al.*, 2015; Kalonji *et al.*, 2015; Kwambana-Adams *et al.*, 2015).

A high level of resistance to drugs such as ampicillin, chloramphenicol, streptomycin, ciprofloxacin, azithromycin, and co–trimoxazole is widespread among S. Typhimurium ST313 iNTS strains in SSA, (Kariuki *et al.*, 2006b; Gordon *et al.*, 2008; Kingsley *et al.*, 2009; Kalonji *et al.*, 2015), and cases of resistance to third–generation cephalosporins such as ceftriaxone have also been reported (Feasey *et al.*, 2015). Appropriate antibiotic management is further complicated by diagnosis and treatment of clinically indistinguishable co–endemic febrile illnesses such as malaria, which are ineffective for NTS. Fluoroquinolones and cephalosporins, the more effective drugs currently used to treat drug–resistant NTS (Mabey *et al.*, 2013), are also under the threat of resistance, and fluoroquinolone–resistant *Salmonellae* are currently a global priority for research into novel antibiotics (WHO, 2017). The relative unavailability and high costs of effective drugs, and impending widespread resistance warrants alternative ways to manage the disease. In the current shortage of new antibiotic chemical entities, it is imperative that a vaccine for preventing NTS disease be developed.

1.6.2. State of vaccine development

No licensed vaccines are available for preventing NTS in humans as yet. A vaccine for iNTS disease in SSA needs to be safe, affordable, and effective for use in children and HIV–infected adults. NTS serovars lack polysaccharide capsules that coat other bacteria that commonly cause invasive disease such as S. Typhi, *H. influenzae* b, *S. pneumoniae*, or *N. meningitidis,* which have been exploited successfully for use in glycoconjugate vaccines against these bacteria. A few other strategies and antigens, therefore, are currently being explored for designing vaccines for NTS.

Salmonella infection elicits antibody and T–cell responses to several antigens that include outer membrane proteins such as porins, heat shock proteins, flagella and fimbriae (Mastroeni *et al.*, 2001), and O–antigens (Colwell *et al.*, 1984). Historically, killed whole cell vaccines have been used with some success in poultry and cattle, and a similar approach was employed for S. Typhi vaccines in humans (Levine *et al.*, 1989; Zhang-Barber *et al.*, 1999). Their inability to elicit adequate T–cell

immunity (Hormaeche *et al.*, 1990a) or confer protection against invasive disease makes them less attractive as vaccines for iNTS disease.

Current efforts for development of NTS vaccines for use in humans are variations of four main strategies: live attenuated vaccines, O–antigen glycoconjugates, recombinant protein vaccines, and Generalized Modules for Membrane Antigens (GMMA) bacterial particle delivery systems.

Numerous vaccines using chemically or genetically attenuated *S.* Typhimurium have been produced and evaluated in preclinical studies or developed into veterinary vaccines (Tennant and Levine, 2015). Their advantage is that besides having a broad range of antigens that can confer protection against several strains, they elicit strong mucosal and T–cell immunity and are relatively easier to develop than subunit vaccines, with simpler purification processes. Whole cell vaccines, however, are more reactogenic compared to subunit vaccines, and retention of virulence in some vaccine strains presents vaccine safety concerns (Hone *et al.*, 1988; Nnalue and Stocker, 1986; WHO, 2008), thus a practical challenge for their use against invasive disease in predominantly immune–vulnerable individuals.

Sub–unit vaccines take advantage of dominant, immunogenic antigens that can elicit robust protective responses. The LPS O–antigen and its potential as a vaccine has long been documented (Robbins *et al.*, 1992). Active immunisation with O–antigen conjugates or passive transfer of immunised sera or monoclonal antibodies protects against *S.* Typhimurium challenge in mice (Svenson and Lindberg, 1981; Watson *et al.*, 1992). This response has similarly been demonstrated for an invasive NTS strain D23580, which elicited bactericidal antibodies in mice (Rondini *et al.*, 2013).

As T–independent antigens, extracted, purified polysaccharides alone do not cause class switching, elicit weak, short–lived antibody immunity with no immunological memory and are ineffective for children under two years of age (Siegrist, 2013). Conjugation of O–antigens with protein carriers has the potential to solve some of these problems. These include highly immunogenic toxoids (such as tetanus (TT) and diphtheria (DT), recombinant mutant diphtheria toxin CRM_{197}) or flagellin (Micoli *et al.*, 2011; Simon and Levine, 2012; Simon *et al.*, 2011). Glycoconjugation has yielded promising vaccine candidates for invasive African *S.* Typhimurium thus far (Rondini *et al.*, 2015). As the majority of the O–antigen immune response is serovar specific, adequate coverage of dominant

circulating serovars might involve multivalent combinations of two or more O–polysaccharide types (Li *et al.*, 2017b; Li *et al.*, 2018) or with other conserved antigens.

Other antigens also induce protective immune responses, albeit at relatively lower levels than O–antigens (Dougan *et al.*, 2011; Mastroeni *et al.*, 2001). The porin protein OmpD, for instance, has been shown to elicit strong antibody responses in mice (Gil-Cruz *et al.*, 2009). An advantage of membrane proteins over O–polysaccharide–based vaccines is that they are more conserved antigens, hence capable of conferring cross–protection against a broader range of serovars (Nandre *et al.*, 2015; Liu *et al.*, 2016).

A relatively recent strategy applied to nontyphoidal *Salmonella* vaccine design is the use of bacterial outer membrane vesicles (OMV), released naturally by Gram–negative bacteria (Kuehn and Kesty, 2005; Meloni *et al.*, 2015). Generalized Modules for Membrane Antigens (GMMA) are OMV's derived from bacteria engineered by deleting membrane–anchoring proteins to facilitate hyper–vesiculation (Berlanda Scorza *et al.*, 2008), circumventing detergent extraction conventionally used in OMV production. GMMA have advantages of both subunit and whole–cell vaccines, as they can deliver both O–polysaccharide and protein antigens in their native conformations, eliciting broader protection with potentially lower reactogenicity than whole–cell vaccines. The success of GMMA for vaccines such as that under development for *S. sonnei* is an important prospect for S. Typhimurium and S. Enteritidis vaccines (Gerke *et al.*, 2015; Rossi *et al.*, 2016). The high yields, simpler production processes and relatively lower costs of production than other subunit vaccines (Meloni *et al.*, 2015; Berlanda Scorza *et al.*, 2012) make it an attractive platform for the development of cost–effective vaccines for prevention of iNTS disease in resource–limited settings.

1.7. Natural history and pathogenesis of NTS infection and disease

Nontyphoidal *Salmonellae* are adapted to withstand harsh conditions such as non–physiological pH, osmolarity and temperature, nutrient starvation, different host niches, antibiotics, and a range of host immune responses. They possess over 75 virulence genes, many of which are absent in host–adapted S. Typhi, which specifically enable them to successfully colonise, invade, survive, and be transmitted between these environments and the different hosts that they infect. Most *Salmonella* virulence genes are within horizontally acquired, conserved gene clusters, known as *Salmonella*

pathogenicity islands (SPI). More than 15 SPI's have been identified in *Salmonella* with some being found only found in certain serovars, which influence their ability to infect and cause disease in hosts.

Much of what is known about NTS infection and pathogenesis is from studies of S. Typhimurium in mice and cultured cells, with some data from human infections. Models have been invaluable for unravelling *Salmonella* disease, but their differences from humans could limit the extent to which they recapitulate human infection (Mestas and Hughes, 2004; Siggins *et al.*, 2011).

1.7.1. Infection and systemic spread of NTS

Ingestion of bacteria and their survival in acidic conditions in the stomach are the first steps in a typical *Salmonella* infection. The infectious dose for *Salmonella* in humans is $>10^5$ organisms (Kothary and Babu, 2001), but can be as low as 100 bacteria depending on the physical and immunological status of the individual, the physiological state of the contaminating bacteria, infecting strain, and gastric pH (Álvarez-Ordóñez *et al.*, 2011; Kothary and Babu, 2001). Bacteria then progress into the small intestine, where they adhere to the epithelial lining via their long polar fimbriae and multiple adhesion factors (Young *et al.*, 2002) (Fig 1.3), then replicate to sufficient numbers before invasion.

Uptake of *Salmonella* by M cells within Peyer's patches is the primary mode of entry, but the bacteria can also invade resident macrophages, dendritic cells, non–phagocytic epithelial cells, or passively traverse weakened epithelial tight junctions (Garcia-del Portillo and Finlay, 1994). *Salmonellae* actively invade through activation of Type–III Secretion System (T3SS–1) proteins that assemble into a "needle complex", which delivers other SPI1–encoded proteins into the host cell (Fig. 1.3). These proteins induce cytoskeletal reorganization through activation of host Rho GTPases RhoG, Rac1, and Cdc42 (Haraga *et al.*, 2008), leading to ruffling of the host cell membrane, that allows it to wrap nonspecifically around the bacteria, facilitating their uptake (Ginocchio *et al.*, 1994; Coombes *et al.*, 2005). The bacteria move freely within the cytosol to the basal side of the epithelium. Direct delivery by M cells or traversing non–phagocytic epithelial cells both result in uptake by phagocytic cells in the submucosa (resident macrophages, dendritic cells, and $CD18^+$ phagocytes), through which the bacteria are transported to mesenteric lymph nodes (Fig. 1.3). *Salmonellae* can manipulate the rate of phagocyte motility to enhance dissemination from the gut mucosa into systemic sites (Bueno *et al.*, 2007).

Besides mediating T3SS invasion of cells, SPI1–encoded proteins have several functions which trigger: macrophage toxicity and caspase–1–mediated macrophage apoptosis, cytokine secretion from macrophages, inflammation and secretion of protein–rich fluid into the ileum, and also prevent apoptosis of epithelial cells (Fig 1.3) (Hersh *et al.*, 1999; Monack *et al.*, 1996). IL–1β and IL–18 cause infiltration of neutrophils through the gut epithelium, a process that occurs within 1–3 hours of infection. The host's response during this early acute phase ultimately manifests as gastroenteritis. In immune–competent individuals, NTS infection is usually controlled upon entry of the bacteria into mesenteric lymph nodes via innate defences (Maskell, 2006) (Fig 1.3), and the flushing action of diarrhoea and vomiting which reduces bacterial load in the gut. Immune– suppression or co–infection with malaria, however, suppresses mucosal immunity. Diarrhoea or gastrointestinal symptoms, therefore, are often absent during invasive NTS disease (Brown and Eykyn, 2000; Mooney *et al.*, 2014).

If sufficient numbers of bacteria can avoid mucosal defences in the intestine, they can travel either directly or intracellularly into lymphatics and eventually into the reticuloendothelial system (Mastroeni *et al.*, 2009), which marks the onset of systemic *Salmonella* infection. Both SPI1 and SPI2 have a role in facilitating systemic infection. SPI1 proteins cause dissemination of bacteria by inducing macrophage apoptosis, and SPI2 proteins allow the bacteria to survive the harsh intra–macrophage environment (Fig. 1.3). *Salmonellae* within host cells occupy self–assembled membrane–bound compartments (*Salmonella*–Containing Vacuoles/SCV). They can also live within the cytoplasm, where SPI2–encoded proteins allow the bacteria to avoid killing by reactive nitrogen intermediates (RNI), and reactive oxygen species (ROS) (Fig. 1.3, discussed in section 1.7.2.1). The SPI–2 effector protein SseI, in particular, enables chronic systemic murine infection by inhibiting migration of dendritic cells and macrophages (McLaughlin *et al.*, 2009), and its absence seems to contribute to the ability of *S.* Typhimurium ST313 to cause invasive disease (Discussed in section 1.4.4) *Salmonellae* are predominantly found within phagocytic cells in the bloodstream, and are much less frequently found freely within the blood, with counts around 1 cfu/mL in bacteremic iNTS disease patients (Gordon *et al.*, 2010). To disseminate, however, bacteria are released from phagocytic cells and enter, replicate and briefly survive in the bloodstream before infecting new macrophages, causing transient bacteraemia.

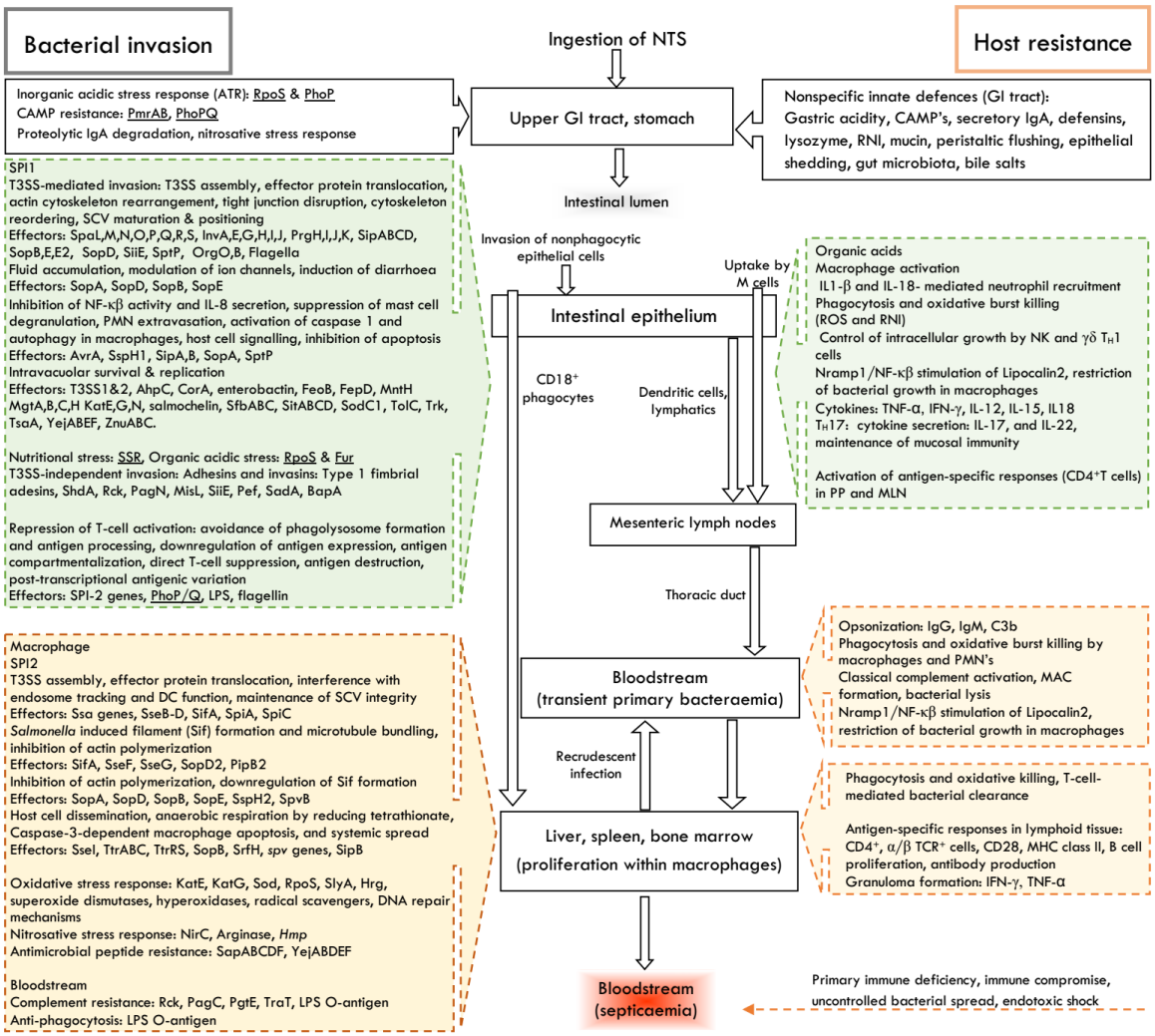

Figure 1.3. The course of *Salmonella* infection and major mechanisms of bacterial invasion, immune evasion and spread, and host immunity.

Abbreviations: ATR: Acid Tolerance Response, CAMP: cationic antimicrobial peptides, DC: dendritic cell, GI: Gastrointestinal, SSR: Starvation stress response, PMN: Polymorphonuclear leukocytes, T3SS: *Salmonella* Type III secretion system, SCV: *Salmonella*–Containing Vacuole, ROS/RNI: Reactive Oxygen Species/Reactive Nitrogen Intermediates, PP: Peyer's Patches, MLN: Mesenteric Lymph Nodes, MAC: Membrane Attack Complex, SSR: Starvation Stress Response. Two–component systems/gene regulons are underlined. Figure redrawn from (Siggins, 2012).

Entry into the bloodstream triggers potent innate responses that are rapidly established to clear bacteria. Antibody–mediated activation of the complement pathway and bacterial opsonization and phagocytosis are known to be important during this transient bacteraemic phase, and protect individuals from fatal invasive NTS disease (MacLennan *et al.*, 2008; Gondwe *et al.*, 2010). Importance of complement–mediated bactericidal activity and opsonophagocytosis in protection against iNTS disease is further underscored by the fact that individuals with primary immune

deficiencies resulting in impaired in antibody, complement or oxidative burst activity (such as chronic granulomatous disease patients) are prone to recurrent bacterial infections (MacLennan *et al.*, 2004).

Bacteria are also distributed in the liver, spleen and bone marrow, residing and multiplying within macrophage–rich pathological lesions, as observed in mice (Mastroeni *et al.*, 2009). Here, both innate and adaptive responses check bacterial growth. Occasional relapsing bacteraemia could occur if the bacteria are not cleared from host tissues. If uninhibited by host immune responses or antibiotics, uncontrolled bacterial replication and spread into blood and tissues ensues. This unchecked bacterial growth causes hyperactivation of host immune responses that lead to systemic inflammation, tissue injury, endotoxic shock, and eventually, death (van der Poll and Opal, 2008; de Jong *et al.*, 2010)..

1.7.2. Host defences in systemic NTS

As mentioned in section 1.7.1, most invading bacteria will be cleared by innate defences before entering the bloodstream or reaching systemic sites in an immune–competent host. This initial phase of infection allows development of more sustained, *Salmonella*–specific adaptive responses that enable clearance of bacteria and prevent lethal or secondary infections.

1.7.2.1. Phagocytic cells

Phagocytic cells have a major function in containing bacterial spread from the intestine, clearance of bacteria from the bloodstream, and inhibiting proliferation of bacteria in the liver and spleen. As most bacteria are located within macrophages and polymorphonuclear phagocytes during systemic NTS infection, the ability to live and multiply inside these cells is critical for virulence (Fields *et al.*, 1986). Inhibition of growth, if not killing of bacteria by phagocytes, therefore, is an essential component of the host's response in iNTS disease.

Phagocytes recognise bacteria through surface Toll–like receptors TLR2, TLR4, and TLR5, which associate with bacterial pathogen–associated molecules such as DNA, LPS, peptidoglycan and flagellin (Rhen, 2007). This interaction activates macrophages and triggers signalling pathways that lead to the production of pro–inflammatory cytokines such as IL–1β, IL–6, IL–8, IFNγ, and TNFα. Cytokine secretion facilitates infiltration of activated inflammatory cells, neutrophils, and dendritic cells, to the site of infection, where together with resident macrophages, they engulf bacteria. Uptake of bacteria is the primary function of phagocytes and is enhanced by opsonization of bacteria with

antibody and complement, which attach to Fcγ and complement receptors on phagocytic cells. Phagocytosis can enable dissemination and systemic survival of bacteria if they can evade killing. Within phagocytes, bacterial growth is limited by antimicrobial peptides and lysozyme (Hancock and Scott, 2000), and, more importantly, reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) through oxidative burst killing.

NADPH/phagocyte oxidase enzyme subunits are assembled during macrophage activation and phagocytosis (DeLeo *et al.*, 1999). Vesicles containing NADPH oxidase localize and fuse to phagosomes/SCV, where oxygen is reduced to oxygen radicals. Inducible nitric oxide synthase (iNOS), mediates production of nitric oxide from NADPH, oxygen and L–arginine (Maskell, 2006). Both nitric oxide and superoxide radicals are highly toxic to cells, effectively killing bacteria (Mastroeni *et al.*, 2000b; Vazquez-Torres *et al.*, 2000). *Salmonellae* can survive killing by phagocytes through several mechanisms, including SPI–2–mediated blockage of SCV fusion with lysosomes and thus avoiding processing for presentation to MHC molecules, neutralization of reactive species, and oxidative damage repair mechanisms (Fig 1.3).

1.7.2.2. Cytokines

Recognition, uptake of bacteria and subsequent activation of macrophages trigger secretion of cytokines that play a significant role in both the early stages of infection as well as contributing to the development of *Salmonella*–specific adaptive responses.

Macrophages, NK cells, NKT, and γδ–T cells secrete IFNγ during the innate–adaptive response to *Salmonella* infection (Nyirenda *et al.*, 2010; Janssen *et al.*, 2002). IFNγ activates macrophages and enhances phagocytosis and bactericidal activity (both oxidative and non–oxidative) as well as that of NK cells (Foster *et al.*, 2003; Eckmann and Kagnoff, 2001; Gordon *et al.*, 2005). IL–12/IL–23 and IL–18 are also secreted by macrophages and are essential for stimulating IFNγ production by NK cells and T–helper cells. Moreover, IL–12 polarizes T–helper cells to a T_H1 response (Mastroeni *et al.*, 2001). Patients with primary defects in either IL–12 or IL–23 signalling are significantly more susceptible to *Salmonella* infection (MacLennan *et al.*, 2004). Macrophages and monocytes secrete IL–15, which activates NK cells, NKT, and γδ–T cells (Ashkar *et al.*, 2009; Lapaque *et al.*, 2009).

TNFα is secreted by T_H1 cells early during the innate response to *Salmonella* but has major functions both at the onset and through the course of infection. In mice, both IFNγ and TNFα are essential for sequestering bacteria within granulomas, which prevents further spread into tissues (Mastroeni *et al.*, 2009). TNFα mediates localisation of lytic vesicles to SCV's. The absence of TNF receptor p55 in mice significantly reduces their ability to control systemic *S.* Typhimurium infection (Maskell, 2006). IL–10 and IL–4 are anti–inflammatory macrophage–secreted cytokines that regulate the activity of IFNγ (Eckmann and Kagnoff, 2001). Cytokine profiles indicative of fatal iNTS disease have been identified in Malawian children, where individuals with fatal outcomes had higher expression of cytokines involved in acute inflammation and neutrophil recruitment, particularly of IL–1, HGF, and IL–8 than convalescents (Gilchrist *et al.*, 2016).

1.7.2.3. T cells

T cells may not have a significant role during the initial stages of primary salmonellosis (Hormaeche *et al.*, 1990b). In mice, however, they can be activated early during infection, mainly by antigen–presenting dendritic cells in gut–associated lymphoid tissue (Dougan *et al.*, 2011; Valdez *et al.*, 2009; Hormaeche *et al.*, 1990b). T_H–17 cells secrete IL–17, and T_H–17–associated cytokines IL–21, IL–22, and IL–26, which regulate and maintain mucosal immune defences (Gordon *et al.*, 2011; Schulz *et al.*, 2008).

Clearance of a primary *Salmonella* infection and development of immunity to subsequent challenge depends on *Salmonella*–specific T–cell responses, specifically via $CD4^+$–αβ T_H–1 cells. Activation of $CD4^+$T cells is CD28–dependent and under MHC class II control (Dougan *et al.*, 2011; Mastroeni *et al.*, 2001). It begins as soon as bacteria enter the reticuloendothelial system (Peyer's patches then mesenteric lymph nodes), which in mice is 3–9 hours into an infection (Griffin and McSorley, 2011). Surface proteins such as flagellin, pili, and porins are major *Salmonella* T–cell antigens (Bergman *et al.*, 2005; Mastroeni *et al.*, 2001). Natural exposure to NTS is associated with the acquisition and development of T–cell responses in children (Nyirenda *et al.*, 2014).

$CD4^+$ T cells mediate immunity against *Salmonella* by secreting T_H1–like cytokines such as IFNγ, TNFα, and IL–12, which stimulate and amplify macrophage–dependent effector functions, and hence clearance of bacteria from tissues (Moon and McSorley, 2009; Ravindran and McSorley, 2005).

$CD4^+$ T cells also control the activation, differentiation and affinity maturation of *Salmonella*–specific B–cell subsets, and isotype switching of antibodies to LPS and *Salmonella* protein antigens, and establish T_H1 immunological memory, necessary for protection against secondary infection (Mittrücker and Kaufmann, 2000).

Salmonella–specific $CD8^+$T cells are also produced during infection, but their role in the early stages is not well understood, and they may be dispensable in primary salmonellosis in mice (Dougan *et al.*, 2011; Wijburg *et al.*, 2002). Depletion of $CD8^+$T cells, however, reduces the ability of mice to control virulent *Salmonella* infection (Mittrücker and Kaufmann, 2000). $CD8^+$ cytotoxic T cells and facilitate protection by releasing intracellular S. Typhimurium from infected macrophages, thus exposing them to extracellular killing by antibody and complement (Kaufmann, 1988). $CD8^+$T cells are also essential for resolving the primary infection and immunological memory, particularly following vaccination (Lee *et al.*, 2012; Salerno-Goncalves *et al.*, 2002).

1.7.2.4. B cells

Secretion of specific antibody is the primary function of B cells, necessary for protection resistance against secondary infection in both mice and humans (Mittrücker *et al.*, 2000). B cells recognise LPS and other bacterial TLR4 ligands and mediate antigen presentation and subsequent development of T_H1 responses such as T_H1–type cytokine secretion (Griffin and McSorley, 2011; Mastroeni *et al.*, 2001). B cells also mediate protective immunity against virulent oral primary *Salmonella* infection in mice (Mittrücker and Kaufmann, 2000). However, B cell deficiency in mice does not seem to affect the ability to control primary infection with attenuated *Salmonella* (Mittrücker *et al.*, 2000; Mastroeni *et al.*, 2000a).

1.7.2.5. Antibody

*Salmonella*e are facultatively intracellular pathogens that primarily reside within macrophages during systemic infection. Systemic spread, however, depends on the ability of the bacteria to survive their transient release from macrophages to infect new ones. Antibodies have an important role in resistance to invasive *Salmonella* infection by facilitating the clearance of extracellular bacteria.

In iNTS–endemic regions, acquisition of NTS–specific antibodies in young children increases with age and correlates with increased protection from potentially fatal bacteraemia (MacLennan *et al.*,

2008). O–polysaccharides (O–antigens) constitute a significant target for NTS–specific antibody responses. The absence of O–antigen–specific antibodies from both mouse serum immunised with O–antigen–deficient bacteria (Rondini *et al.*, 2013) and human sera pre–absorbed with LPS (Trebicka *et al.*, 2013) inhibits serum bactericidal activity. Outer membrane proteins and flagellin also elicit antibodies with NTS bactericidal activity (Gil-Cruz *et al.*, 2009; Simon *et al.*, 2011).

Together with T cells, antibodies have a dual role in preventing severe, virulent NTS bacteraemia (McSorley and Jenkins, 2000). They opsonize bacteria for uptake by phagocytic cells and subsequent oxidative burst killing (Gondwe *et al.*, 2010), and activate the classical complement pathway (MacLennan *et al.*, 2008), which results in rapid bacterial killing.

1.7.2.6. Complement

The complement system comprises at least 30 heat–labile plasma proteins and is a major effector of innate and adaptive humoral immunity. These proteins, consisting of proteases, activated products, complement regulators and inhibitors, and membrane receptors, work in a highly regulated manner to mediate attack on bacteria (Zipfel and Skerka, 2009). Upon recognition of invading bacteria, a series of enzymatic activation and amplification steps is initiated, which eventually leads to opsonization of bacteria, the release of inflammatory factors, and bacterial lysis by forming a membrane attack complex pore on the surface of the bacterium (Fig. 1.4).

The complement cascade is activated in three ways. The classical pathway is initiated by complement complex C1 recognizing and binding to IgM, IgG1, IgG2 and IgG3 antibodies bound to the bacterial surface. Activation of the classical complement cascade differs by IgG subclass, although different studies have yielded varied results (Bindon *et al.*, 1988; Dillman *et al.*, 1995). IgG3 is the most potent activator of complement, followed by IgG1, then IgG2 (Dillman *et al.*, 1995). IgG4 does not bind C1q, hence does not activate the classical complement pathway (Dillman *et al.*, 1995). Binding of C1q to C–reactive protein (CRP), nucleic acids, LPS and immune complexes can also activate the classical complement pathway (Nauta *et al.*, 2002; Loos, 1982). The lectin pathway is activated by bacterial polysaccharides binding to lectins in plasma such as mannose–binding lectin (MBL) and ficolins, which are structurally homologous to and serve the same function as C1r and C1s subunits of the C1 complex. Both the classical and lectin complement pathways activate the cleavage of C4 and C2 (Fig 1.4). The alternative pathway is activated from spontaneous lysis of C3 to C3b and

requires factors B, D, and Mg^{2+}. All three pathways result in the formation of C3 convertase, which in the classical and lectin pathways consist of C4bC2a, and C3bBb in the alternative pathway (Fig 1.4).

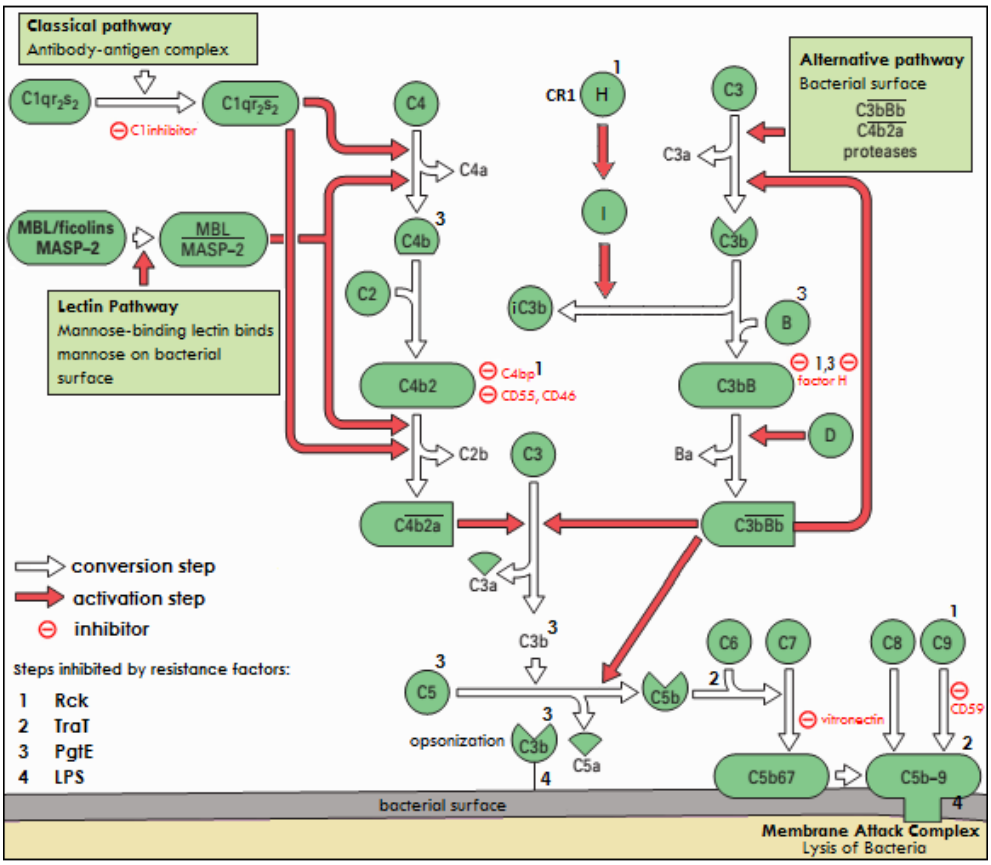

Figure 1.4. The complement pathway.
Activation, regulation, function of the complement cascade in response to bacterial infection, and steps that are inhibited by known complement resistance factors in S. Typhimurium and S. Enteritidis. Figure modified from (Male *et al.*, 2013).

Central to the complement cascade is cleavage of C3 and formation of C5 convertase, which comprises C3b and C3 convertase. C5 convertase (C4b2a3b or C3bBbC3b) then cleaves C5 into C5a and C5b, which gives way to the lytic pathway. C5b is deposited on the bacterial surface, and C6, C7, and C8 sequentially recruited to form the C5b678 complex, which inserts into the bacterial membrane. This sequence then catalyses of the polymerization of between 3 and 20 C9 subunits, which are attached to the C5b678 complex to form a pore on the bacterial envelope called the membrane attack complex (C5b–C9).

The precise events leading to cell death following insertion of the MAC into the bacterial membrane possibly involve the presence of a non–specific transmural pore that disrupts the cell wall, allowing the free release of cytoplasmic contents such as ATP, ions, amino acids, and entry of water and lysozyme through the bacterial membrane. The loss of cellular energy, transmembrane proton gradient, and osmotic balance all contribute to cell lysis and death (Dankert and Esser, 1986; Taylor and Kroll, 1983). The action of serum lysozyme, which breaks down peptidoglycan, has been shown to contribute to the lytic process, and deletion of bacterial lysozyme inhibitors significantly enhances the efficiency of serum bactericidal activity (Derbise *et al.*, 2013; Vanderkelen *et al.*, 2012), although bacterial killing can also occur in the absence of lysozyme (Schreiber *et al.*, 1979).

Peptides C4a, C5a, and C3a are pro–inflammatory mediators that facilitate recruitment of antimicrobial serum factors and phagocytes to the site of infection. C3b remains attached to the bacterial cell surface and acts as an opsonin for uptake by PMN's. Phagocytes only recognise bound IgM associated with C3b. Factors H and I mediate processing of C3b into peptides iC3b, C3c, and C3dg, which are also ligands for complement receptors on leukocytes, enhancing opsonization and phagocytosis of bacteria (van Lookeren Campagne *et al.*, 2007).

Soluble and host cell surface–bound regulatory proteins inhibit complement to terminate the cascade and further complement activity, thereby minimizing complement damage to host cells. These regulatory proteins include complement factors C4bp, factor H, factor S, C1 inhibitor, properdin (factor P), clusterin, and vitronectin. *Salmonellae* can recruit complement regulatory proteins, which interfere with normal complement function, allowing them to avoid killing by complement (discussed in section 1.8.3).

1.8. Factors determining susceptibility to antibody–mediated complement–dependent bactericidal activity in *Salmonella* Typhimurium

Resistance to the bactericidal activity of immune serum is a well–studied virulence factor in nontyphoidal *Salmonellae* and is a commonly observed feature of strains isolated from immune–competent bacteraemic patients (Joiner *et al.*, 1982a). Strains which are less susceptible to complement killing have been demonstrated to be more virulent and more pathogenic than

antibody–sensitive ones (Joiner *et al.*, 1982a). Invasive disease associated with antibody resistant Gram–negative bacteria has been observed even after successful vaccination (Williams *et al.*, 2001).

Early studies recognized a failure in the terminal complement pathway, characterized by expulsion of the MAC before bacterial killing occurs, and the inability of the MAC to assemble correctly into the bacterial membrane in *Salmonellae* resistant to complement attack (Joiner *et al.*, 1982a; Joiner *et al.*, 1982b). These studies defined the role of bacterial surface factors in protection from complement killing. It is currently recognized, however, that full expression of resistance to killing mediated by antibody and complement in *Salmonella* is complex, and is regulated and determined by multiple factors associated with, but not limited to the bacterial membrane.

Extracellular survival, replication, and spread of NTS outside of the gastrointestinal tract depend on the ability of the bacteria to avoid immune recognition and clearance that acts through the action of antibody and complement. Several factors influence in–vitro and in–vivo antibody–mediated complement–dependent bactericidal activity and bacterial susceptibility to the bactericidal activity of normal human serum, which include bacterial growth phase, genetic factors, and bacterial growth conditions (Taylor, 1983).

Much of the available data on in–vitro antibody susceptibility of invasive African NTS is from studies on D23580, isolated from a 26–month–old bacteraemic child in Malawi (MacLennan *et al.*, 2008). The strain is typical of invasive, epidemic S. Typhimurium ST313 (Kingsley *et al.*, 2009), and is thus far the most extensively described.

Depending on experimental conditions, D23580 undergoes a $\log_{10}$ kill between 1 and 3 after 180 minutes of incubation in whole serum from healthy human adult donors. In children, higher serum levels of *Salmonella*–specific antibodies correlate with increased bactericidal activity against D23580 (MacLennan *et al.*, 2008). Killing of D23580 is reduced or abrogated in serum lacking antibody against S. Typhimurium LPS O–antigen (Rondini *et al.*, 2013), or altogether devoid of *Salmonella*–specific antibody (Goh and MacLennan, 2013). Conversely, S. Paratyphi A is readily lysed in neat serum lacking anti–*Salmonella* antibody. These variations in susceptibility suggest different abilities of the two serovars to activate the alternative complement pathway (presumably determined by O–

antigen structure), may allow survival of invasive S. Typhimurium in individuals with undeveloped antibody responses against NTS (Grossman *et al.*, 1990).

D23580 is refractory to killing in complement–deficient serum. Heating serum at 56°C for 30 minutes denatures heat–labile complement components C1, C2, and C3, which inactivates the complement pathway, and abrogates killing of D23580. Serum depleted of C9 or diluted by 10–fold limits bactericidal activity against D23580, indicating a requirement for the membrane attack complex for bactericidal activity (MacLennan *et al.*, 2008), contrary to what was previously thought.

Sufficient amounts of both antibody and complement are therefore necessary for the in–vitro killing of S. Typhimurium D23580, and the classical complement pathway resulting in direct bacterial killing via formation of the membrane attack complex, an important function of protection against disease. These two components of normal human serum are also important for opsonization and phagocytosis of invasive S. Typhimurium. This requirement seems higher for invasive NTS strains than enteric Typhi or Paratyphi, which under similar conditions show relatively higher susceptibility to serum killing (Boyd *et al.*, 2014). In–vitro rates of opsonization and phagocytosis of D23580 with serum antibody and complement have been observed to be higher than the rate at which the C5b–C9 complex is formed on the bacterial surface (Siggins *et al.*, 2014). Decreased rates of C3 convertase activity and subsequently reduced C3b deposition on the bacterial surface have also been associated with resistance to complement–mediated killing in an invasive, unencapsulated *Haemophilus influenzae* b strain following vaccine failure (Williams *et al.*, 2001). If this indeed reflects what happens in–vivo, bacterial uptake preceding complement attack provides a means for bacterial escape from antibody and complement–mediated killing during systemic NTS. Among invasive strains with varying levels of susceptibility to cell–free killing, increased susceptibility correlates with higher blood phagocyte killing (O'Shaughnessy, 2013), which suggests a generalised mechanism of resistance to antibody defences (both complement and opsonophagocytic killing).

1.8.1. LPS O–antigen

Lipopolysaccharides form the outermost portion of most Gram–negative cell envelopes. They consist of three chemically and biologically distinct regions; lipid A (endotoxin), the bioactive portion of LPS embedded in the outer membrane of the envelope, a core oligosaccharide, and an O–polysaccharide

(O–antigen) side chain, which forms the distal portion. In S. Typhimurium, the LPS O–antigen side polymerizes into between 1 and about 100 4–sugar–residue monomer units (Fig. 1.5). O–antigens are dominant pathogen–associated surface molecules and therefore major targets for host antibody responses, and also act as receptors for bacteriophages, mediate protection against host immunity and environmental stress, thus being indispensable for virulence.

A trimodal distribution of O–antigen occurs on the *Salmonella* bacterial envelope, as short–chain, low molecular weight O–antigen (<16 repeating units), long chain (about 16–35 repeats) and very long chain (>100 repeats) (Murray *et al.*, 2003). Two O–antigen chain length determinants, *wzzB* (wzz_{ST}) and *fepE* (wzz_{fepE}), have been identified as responsible for the synthesis of long and very long O–antigen chains respectively (Murray *et al.*, 2003). *Salmonella* resistance to complement–mediated killing is mainly determined by the structure and distribution of the bacterial outer membrane structures, primarily via LPS O–antigen chain length (Grossman *et al.*, 1987; Murray *et al.*, 2006).

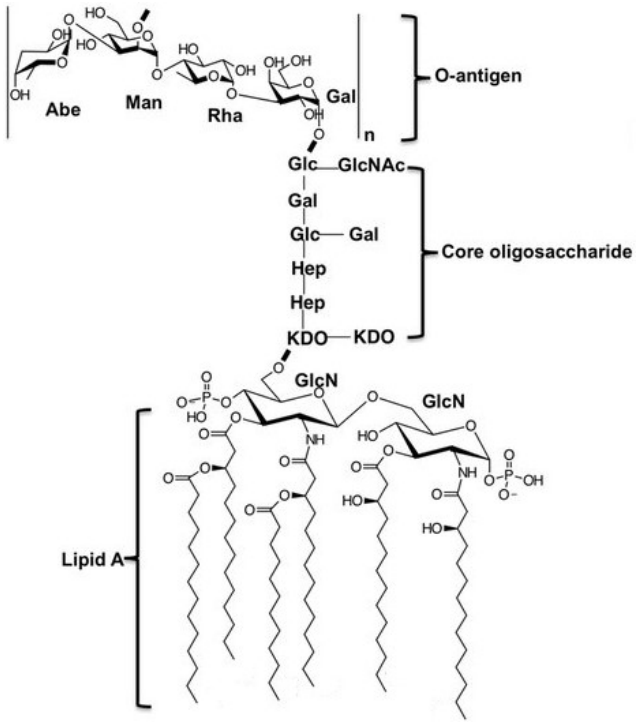

Figure 1.5. *Salmonella* Typhimurium str. LT2 lipopolysaccharide.
Figure reproduced from (Li *et al.*, 2015b).

O–antigen interferes with the complement pathway by preferentially binding C3, thus hindering complement activation. It also forms a physical barrier that prevents proper insertion of the membrane attack complex into the bacterial wall, inhibits opsonization and phagocytosis, and shields

the bacteria from antimicrobial peptides (Murray *et al.*, 2006). *Salmonella* strains incapable of LPS O–antigen synthesis, or rough mutant derivatives of virulent strains are highly sensitive to antibody and complement killing, and typically avirulent (Rowley, 1968). O–polysaccharide structure and composition can also determine alternative complement pathway activation independently of chain length. These variations can influence the rate and extent to which bacteria are opsonized and ingested by macrophages or killed by complement (Grossman *et al.*, 1990; Grossman and Leive, 1984; Liang-Takasaki *et al.*, 1983; Liang-Takasaki *et al.*, 1982).

Being a major antigenic determinant, diversity in O–antigen structure and composition, besides chain length, provides a reservoir for variation to avoid immune pressure (Reeves and Wang, 2002). Variations, or within–host modifications in LPS O–antigen composition like those in lipid A, can enable the escape of host recognition and immunity, facilitating bacterial persistence and chronic infection (Maldonado *et al.*, 2016). These changes occur through epigenetic processes such as phase variation and DNA methylation, differential gene expression, and horizontal gene transfer, that modify O–antigens by fucosylation, glucosylation, and acetylation (Slauch et al., 1995). O–antigen acetylation in S. Typhimurium occurs through the addition of an acetyl group on abequose or rhamnose through the *oafA* gene product and confers the O5 serotype (Slauch et al., 1995). Phage–derived phase–variable *gtr* genes both acetylate and glycosylate S. Typhimurium O–antigens (Davies *et al.*, 2013; Kintz *et al.*, 2017). The extent of O–antigen acetylation and glycosylation levels enable intra–strain cross–environment diversity.

These modifications can affect the immunological properties of *Salmonella* O–antigens, particularly of mucosal immunity (Slauch et al., 1995). In S. Typhi, O–antigen glycosylation decreased complement binding and enhanced survival in immune serum (Kintz *et al.*, 2017), a possible result of these additional groups modifying major epitopes. A survey of invasive clinical isolates of S. Enteritidis and S. Typhimurium from Kenya (including ST313 isolates), however, established no direct effect of O–antigen modifications on immunogenicity or levels of complement sensitivity (Onsare et al., 2015).

1.8.2. Other Enterobacterial surface polysaccharides: Enterobacterial common antigen, colanic acid, and Group IV (O–antigen) capsules

In addition to LPS, other constituents of the glycocalyx such as the enterobacterial common antigen (ECA), colanic acid (CA) and the group IV O–antigen capsule can cause considerable variation of the bacterial surface in response to external physiological stress. While they may not all be present or necessary for virulence in all Enterobacterial pathogens, their role in conferring protection against lytic factors in the host suggest they might have a role in complement resistance.

The enterobacterial common antigen is a highly conserved polysaccharide, and unlike O–antigen capsules or colanic acid, a constitutive feature of wild–type Enterobacteria (Kuhn *et al.*, 1988). Its precursor and many biosynthetic genes are shared with LPS (Fig. 1.6). An intact *wec* operon, with genes for ECA biosynthesis, is necessary for oral infection of mice and bile resistance in *Salmonella* (Ramos-Morales *et al.*, 2003), and seems to contribute to complement resistance in pathogenic *E. coli* (Phan *et al.*, 2013), but its role in serum resistance in *S.* Typhimurium is as yet undescribed.

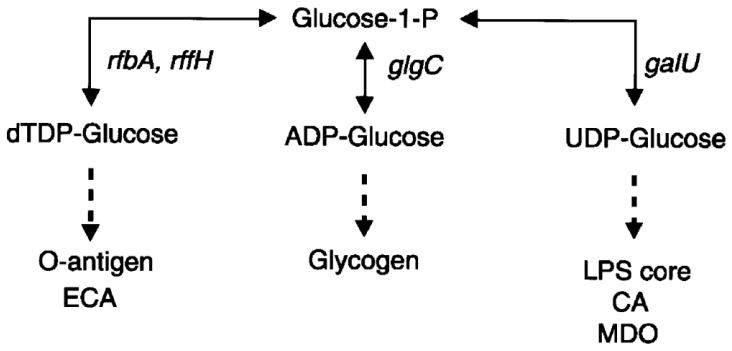

Fig 1.6. **Major pathways for synthesis of surface polysaccharides in Enterobacteriaceae from the glucose–1–phosphate precursor.**
ECA, enterobacterial common antigen; LPS, lipopolysaccharide; CA, colanic acid; MDO, membrane–derived oligosaccharide (also called osmoregulated periplasmic glucan/OPG). Figure modified from (El-Kazzaz *et al.*, 2004).

Colanic acid is an exopolysaccharide common among the Enterobacteriaceae, that is necessary for withstanding multiple envelope stress signals under control of the Rcs phosphorelay (Laubacher and Ades, 2008). It also forms a scaffold for biofilms (Ledeboer and Jones, 2005), and maintains membrane potential (Pando *et al.*, 2017). Colanic acid locus genes are significantly up–regulated in response to exposure to serum bactericidal activity in pathogenic *E. coli* (Miajlovic *et al.*, 2014; Phan *et al.*, 2013), but deletion of CA genes do not seem to have an impact on virulence in vivo in *S.* Typhimurium (Wang *et al.*, 2013).

Group IV capsules (G4C) have been described in many enteric Gram–negative bacteria (Peleg *et al.*, 2005; Caboni *et al.*, 2015; Nakhamchik *et al.*, 2007; Snyder *et al.*, 2006). Capsules found in strains within conventionally or predominantly acapsular serovars such as S. Typhimurium have highly similar monomer units to O–antigens (Marshall and Gunn, 2015). Because of the minimal distinction between group IV capsular polysaccharides and lipopolysaccharides, however, the prevalence of capsules or their contribution to disease in clinically relevant NTS strains is still poorly defined (Cartee and Yother, 2006). Unlike O–antigens which are always present on the bacterial envelope, G4C appear to be differentially regulated during infection (Marvasi *et al.*, 2013). G4C biosynthesis genes in *Salmonella* are highly conserved, within the *ysh*A–*yih*V locus, regulated by *agfD* (Gibson *et al.*, 2006; Marshall, 2013). G4C polysaccharides are often directly ligated to the outer core as opposed to being linked to lipid A as is with O–antigens and are of higher molecular weight, assembled into units containing up to 3000 monomers (Marshall, 2013).

G4Cs have a role in environmental survival (Gibson *et al.*, 2006) and pathogenic traits of Enterobacteria, such as resistance to antimicrobial peptides (Thomassin *et al.*, 2013), dissemination of bacteria into systemic sites (Caboni *et al.*, 2015), and complement resistance (Caboni *et al.*, 2015; Marshall and Gunn, 2015). They are co–expressed with other components of the S. Typhimurium glycocalyx such as colanic acid, and do not interfere with LPS O–antigen expression (Marshall and Gunn, 2015). Although the presence of an O–antigen capsule was shown to be necessary for full complement resistance in S. Typhimurium (Marshall and Gunn, 2015), its contribution to this trait in the presence of other components of the bacterial membrane is not clear.

1.8.3. Membrane proteins

Salmonellae express surface and membrane proteins associated with resistance to killing by complement–dependent antibody defences. These proteins mediate resistance by a) actively degrading complement components b) interfering with either complement deposition, assembly or incorporation of the membrane attack complex (MAC) into the bacterial outer membrane or both, or c) binding complement regulatory proteins, which disrupts the complement cascade.

1.8.3.1. Rck

Rck ("resistance to complement killing") is 17kDa protein in the Ail/Lom family that includes complement resistance–associated membrane proteins in Enterobacteriaceae such as Ail, OmpX, and

PagC (Heffernan *et al.*, 1992a). Rck is encoded on S. Typhimurium and S. Enteritidis virulence plasmids and has a role in both antibody resistance and bacterial invasion of epithelial cells (Cirillo *et al.*, 1996; Mijouin *et al.*, 2012). Rck–mediated resistance to antibody–dependent complement–mediated killing is associated with the outer membrane C–terminal portion of the protein, which also functions in host cell invasion (Cirillo *et al.*, 1996). In S. Typhimurium, Rck decreases surface deposition of polymerised C9, thus preventing complement killing by interfering with MAC assembly (Heffernan *et al.*, 1992b).

Rck also blocks the complement cascade by binding to complement regulatory proteins. It can recruit factor H (fH), which, in the presence of factor I amplifies C3b by processing it to iC3b, C3c, and C3dg in the alternative pathway (Fig. 1.4). Inhibition of factor H by Rck consequently reduces C3b, and Bb effector functions hence decreased bacterial killing (Ho *et al.*, 2010). The *Neisseriae* also exploit factor H binding for evading complement attack, and the *N. meningitidis* factor H binding protein (fHbp) has been used successfully in the multi–component meningitis B vaccine (Gorringe and Pajón, 2012). Besides factor H, Rck can bind to the inhibitor C4 binding protein (C4bp), which promotes degradation and inactivation of complement factor C4b and C4b2a convertase, blocking the classical and lectin pathways (Ho *et al.*, 2011). Rck–mediated resistance to antibody–mediated killing in S. Enteritidis and Typhimurium can occur independently of O–antigen length (Heffernan *et al.*, 1992b).

The invasive S. Typhimurium strain D23580 has an intact copy of the *rck* gene on the pSLT–BT plasmid (Kingsley *et al.*, 2009), but *rck* does not prevent the killing of D23580, nor confer high–level resistance in normal immune human serum (MacLennan *et al.*, 2008). As antibody is necessary for the killing of invasive African NTS strains, it is possible that Rck has a minimal role during classical complement activation and subsequent lysis, but could confer resistance to complement attack occurring via antibody–independent complement activation pathways.

1.8.3.2. PgtE

PgtE is a conserved outer membrane protease in *Salmonella*. It cleaves multiple complement components, including C3b, C4b, C5, factors B and H thereby disrupting complement cascade activity (Ramu *et al.*, 2007; Riva *et al.*, 2015). It also confers resistance to antimicrobial peptides (Guina *et al.*, 2000). A SNP identified in the *pgtE* promoter has recently been demonstrated to have

a significant impact on the virulence and resistance to complement among ST313 lineage II strains (Hammarlöf *et al.*, 2018)(discussed in section 1.4.4). PgtE expression and complement proteolytic activity are significantly increased upon release of bacteria from macrophages, and contingent on the presence of a short O–antigen on the bacterial surface (Ramu *et al.*, 2007). Since intracellular *Salmonellae* truncate their O–antigens in order to avoid activating macrophages (Murray *et al.*, 2006), this increase in PgtE activity has been hypothesized to be an adaptation to extracellular survival, and compensation for decreased fitness that accompanies intramacrophage downregulation of O–antigen expression (Ramu *et al.*, 2007). Modification of pgtE among ST313 isolates thus adds to evidence of the evolution of this pathovar to causing bloodstream, instead of gastrointestinal disease.

1.8.3.3. TraT

TraT is an outer membrane lipoprotein encoded on S. Typhimurium and S. Enteritidis virulence plasmids. Its primary function is surface plasmid exclusion (Harrison *et al.*, 1992; Sukupolvi *et al.*, 1990), and also has a role in bacterial growth within liver macrophages and antibody resistance (Rhen and Sukupolvi, 1988). Mutations in the *traT* gene can affect membrane permeability (Sukupolvi and O'Connor, 1990). TraT effects antibody resistance by inhibiting the formation of the C5b6 complex, and preventing insertion of the MAC into the bacterial cell envelope (Pramoonjago *et al.*, 1992; Rhen and Sukupolvi, 1988).

1.8.3.4. PagC

PagC and Rck share the Ail domain, which is responsible for the invasion and antibody resistance phenotypes mediated by these proteins (Miller *et al.*, 2001). PagC is essential for bile resistance (Prouty *et al.*, 2004) and the survival of S. Typhimurium in macrophages, and is required for full expression of antibody resistance in S. Choleraesuis (Nishio *et al.*, 2005). The mechanism by which it causes resistance to antibody–mediated killing, however, is still unclear (Nishio *et al.*, 2005).

1.8.3.5. Rsk

The rsk ("reduced serum killing") locus is a 66bp region on a 95–kb plasmid identified in S. Typhimurium. Integration of this plasmid into the chromosome increased serum sensitivity, but curing the plasmid did not seem to affect serum susceptibility (Vandenbosch *et al.*, 1989a). Overexpression of the 66bp fragment on a multi–copy plasmid increased resistance to killing by antibody and complement (Vandenbosch *et al.*, 1989b). The *rsk* locus is not a protein–coding or

promoter region but is hypothesised to regulate disruption of suppressors of antibody resistance and virulence gene expression on the plasmid (Vandenbosch *et al.*, 1989b).

1.8.4. Bacterial growth phase

Bacteria in early exponential growth are observed to be most susceptible to in–vitro serum killing and virtually resistant to before log growth or in stationary phase (Taylor, 1983). This phenomenon is partly due to the substantial increase in the density of O–antigens induced during the late log and early stationary phase in bacterial growth cycle in culture (Bravo *et al.*, 2008). While this factor has implications for in–vitro assays, its role in influencing pathogenicity or survival within the host is still unclear. However, the initial phase of *Salmonella* infection requires active bacterial replication to sufficient numbers before entry into "sanctuary sites" such as phagocytes and lymphoid tissue where they persist and are more dormant. This implies that during much of the bloodstream phase (Fig. 1.3), multiplying bacteria will be more susceptible to host responses.

1.8.5. Regulation of complement resistance factors in *S.* Typhimurium

To adapt and survive in diverse environments, host niches, evade immune responses, and persist in the host, S. Typhimurium expresses a diverse array of virulence factors (Fig. 1.3). Many of these factors pose a high metabolic cost to the bacterium, and the pathogen will need to mount an appropriate set of responses to each condition it encounters. These virulence factors are therefore tightly regulated in response to survival within a particular environment. The wzz_{fepE} gene, for instance, is dispensable during much of an S. Typhimurium infection, but is significantly up–regulated in serum and bile–exposed bacteria, which increases very–long O–antigen chain density (Crawford *et al.*, 2012; Murray *et al.*, 2005). Very long O–antigen synthesis is costly. wzz_{fepE}–mediated resistance to killing by antibody and complement seems to be a specific adaptation in occupying the extracellular niche (Murray *et al.*, 2005).

Differential expression of genes associated with surface polysaccharides associated with resistance to antibody killing in S. Typhimurium is highly dynamic, regulated by several virulence genes, and mediates processes of infection such as adhesion, responses to several environmental stressors that breach the bacterial cell membrane, such as antibiotics, or host immunity. In Enterobacteria, most surface polysaccharides have a common precursor (Fig. 1.6), which can be synthesised through

different pathways in response to varying environmental cues or conditions. The primary virulence factor with a direct impact on complement resistance thus regulated is the O–antigen, through three main mechanisms, phase variation, DNA methylation, and two–component systems.

1.8.5.1. Phase variation

Phase variation is a heritable, reversible, bimodal, stochastic switch in the expression state of a gene that results in variation in protein expression levels between two cells of a bacterial clone, giving rise to a heterogeneous population (van der Woude and Bäumler, 2004). It occurs via epigenetic events (such methylation of DNA through DNA adenine methyltransferase/Dam, (Discussed in Section 1.8.5.3) or frameshift alterations in DNA sequence that result in gene inactivation. Phase variation changes surface modification exposed structures by altering the expression of membrane proteins, or proteins involved in cell envelope synthesis and modification, allowing expression of phenotypes that maximize bacterial fitness.

Modification of O–antigen through phase variation can affect *S.* Typhimurium resistance to complement. For instance, DNA methylation causes phase variation of the *opvAB* locus, which encodes inner membrane proteins that alter O–antigen chain length. When unmethylated, the locus is locked in an ON state, and expression of the *opv* genes diminishes O–antigen polymerisation, which impairs complement resistance and the ability of S. Typhimurium to replicate in macrophages (Cota *et al.*, 2012).

O–antigen–modifying genes in the *gtr* and *wzz* operons are also influenced by phase variation (Kintz *et al.*, 2017; Broadbent *et al.*, 2010). In S. Typhi *gtr*–mediated glucosylation interferes with bacterial recognition of *Salmonella* by antibody, complement binding, subsequently enhances resistance to serum bactericidal activity (Kintz *et al.*, 2017). *Gtr* locus genes also modify *S.* Typhimurium LPS, although their impact on complement resistance in this serotype has not been studied. *Wzz* polymerase modulates O–antigen chain length and serum resistance influenced by phase variation acting via DNA methylation (Broadbent *et al.*, 2010; Sarnacki *et al.*, 2009).

1.8.5.2. Two–component regulatory systems

Survival of bacterial within the diverse environments they encounter is influenced in part by two–component systems, in response to environmental stimuli such as osmolarity, temperature, pH,

nutrient availability or starvation (due to sequestration of growth factors such as iron and zinc which is heightened during inflammation), antibody and complement responses, and antimicrobial peptides, to enable adaptation to the diverse host microenvironments they occupy. The exit of *Salmonellae* from macrophages into the extracellular space in the bloodstream, for instance, requires adjustment from a highly oxidative/nitrosative environment into encountering potent antibody and complement–mediated immunity, hence alteration of antigen expression or metabolic activity.

Two–component systems (TCS), are the prokaryotic sensory–response units. Each typically consists of a membrane–bound histidine kinase sensor/receptor, coupled to a response regulator protein, which functions through a signal transduction system, resulting in changes in gene transcription. Most *Salmonella* virulence genes are regulated by two–component systems (Fig. 1.3), which typically control the synchronised expression of several genes.

In response to different physiological signals, three major TCS that modify LPS O–antigen chain length have been characterised in *S.* Typhimurium, which mostly regulate expression of wzz_{ST} and wzz_{fepE} genes, responsible for altering the *Salmonella* O–antigen chain length, and hence complement resistance.

1.8.5.2.1. PhoP/Q

In *Salmonella* Typhimurium, the *phoPQ* two–component system activates and represses more than 40 different genes during various stages of infection (Guo *et al.*, 1997). It is responsible for virulence in vivo, resistance to cationic antimicrobial peptides, survival of the harsh intramacrophage environment, and inorganic acid stress tolerance (Kidd, 2011). The PhoPQ TCS effects evasion of host immunity by regulating several genes involved in remodeling of the bacterial envelope through modification of lipid A (Guo *et al.*, 1997), and regulating expression of membrane–bound complement resistance genes such as *pag*C (Miller *et al.*, 1993), and *pgtE* (Guina *et al.*, 2000). PhoPQ also, directly and indirectly, causes changes in the O–antigen composition of LPS. Constitutive expression of PhoPQ in a $PhoP^{C}$ strain decreases average O–antigen chain length (Guo *et al.*, 1997), and modifies O–antigen indirectly through activation of the PmrAB TCS (Gunn and Miller, 1996).

1.8.5.2.2. PmrA/B

All PmrAB–regulated genes characterised to date participate in the modification of LPS (Chen and Groisman, 2013; Farizano *et al.*, 2012). The PmrA/B TCS is activated under high Fe^{3+}, low pH conditions, and is necessary for S. Typhimurium resistance to cationic antimicrobial peptides such as polymyxin B, and for virulence in mice (Richards *et al.*, 2010). PmrAB is activated either through PhoPQ, via the PmrD shunt, or via direct environmental signals activating the TCS through the PmrB sensor on the bacterial surface (Richards *et al.*, 2010). The PmrAB TCS has direct effects on genes responsible for modification of lipid A (Chen and Groisman, 2013; Farizano *et al.*, 2012) and O–antigen, through activation of the chain–length determinants wzz_{ST} and wzz_{fepE} (Farizano *et al.*, 2012; Delgado *et al.*, 2006; Pescaretti Mde *et al.*, 2011). It increases the fraction of LPS molecules with long and very long O–antigen chains, causing heightened resistance to serum bactericidal activity in S. Typhimurium (Pescaretti Mde *et al.*, 2011).

1.8.5.2.3. RcsCBD/YojN

The RcsCBD system is induced by stresses to the bacterial envelope such as desiccation, high osmolarity, low temperatures, and exposure to β-lactam antibiotics or cationic antimicrobial peptides (Richards *et al.*, 2010). It regulates expression of multiple genes involved in the synthesis of membrane proteins, polysaccharides, and exopolysaccharides, such as colanic acid capsules, Vi antigen, and promotes biofilm maturation (Richards *et al.*, 2010). Although the environmental cues are different, the RcsC/YojN/RcsB phosphorelay has a similar effect to that of the PmrAB system. It enhances resistance to serum complement bactericidal activity in S. Typhimurium by promoting transcription of wzz_{ST}, and hence proportions of long O–antigen subunits (Delgado *et al.*, 2006).

1.8.5.3. DNA adenine methylation

The epigenetic activity of the DNA adenine methyltransferase gene (*dam*) is an essential component of γ–Proteobacteria physiology. By methylation of adenine at the N^6 position at 5'–GATC–3' sites, Dam differentially controls several phenotypes and physiological processes such as chromosomal replication, methyl–directed mismatch repair, restriction modification, and transcription (Heusipp *et al.*, 2007), by changing promoter–binding activity. Dam–dependent regulation is crucial for both in vivo and in vitro virulence in *Salmonellae*, controlling the expression of at least twenty virulence genes induced during infection (Heithoff *et al.*, 1999), evident in the significant attenuation and loss

of multiple virulence traits in *dam*–deficient mutants (Balbontín *et al.*, 2006; Dueger *et al.*, 2001; García-Del Portillo *et al.*, 1999).

In *S.* Typhimurium and Enteritidis, *dam* activity is specifically involved in either directly regulating transcription of the O–antigen synthesis gene *wzz* (Sarnacki *et al.*, 2009) or indirectly acting via the PmrA/PmrB and RcsC/RcsD/RcsB two–component regulatory systems (Sarnacki *et al.*, 2013; Delgado *et al.*, 2006). Induction of these two systems increases proportions of long O–antigen chains, and in effect, increased resistance to serum bactericidal activity (Delgado *et al.*, 2006). Inheritance of these methylation patterns is known to control the expression of complement resistance, in addition to other virulence phenotypes (Heithoff *et al.*, 1999).

1.9. Thesis Rationale and Hypotheses

The significance of invasive nontyphoidal *Salmonella* disease in sub–Saharan Africa is a consequence of multiple host, pathogen and environmental factors. While host and environmental factors are well characterised, we are only beginning to understand precise mechanisms by which NTS serovars, predominantly *S.* Typhimurium ST313, cause fatal disseminated disease. Lateral transfer of multiple antibiotic resistance genes has perhaps had the single most significant pathogen–associated influence on *S.* Typhimurium ST313 epidemics, and thus largely been at the centre of intensive research efforts, both at the epidemic and molecular level.

Vaccination as a means to counteract the challenge to disease control posed by increasing antibiotic resistance is currently a research priority. Central to natural and potentially vaccine–induced immunity against the transient extracellular phase of *Salmonella* infection causing extraintestinal disease is the acquisition of specific antibodies, which facilitate rapid bacterial clearance from the bloodstream, preventing life–threatening sepsis. The complement cascade, one arm of the humoral response to blood–borne bacteria, is an important effector of *Salmonella*–specific immunity. The classical complement pathway mediated by *Salmonella*–specific antibodies, in particular, has been shown to have a dominant role over the other complement–activating pathways in protecting Africans from severe NTS bacteraemia (MacLennan *et al.*, 2008), and is thus the focus of this study. With the current shift in research on novel iNTS disease interventions towards vaccines, modalities of protection and the reservoir of other bacterial virulence factors mediating the characteristic

invasive pathogenesis of S. Typhimurium ST313, especially those capable of modulating antibody immunity, come into focus.

The ability of bacteria to spread beyond the gastrointestinal tract to cause invasive disease depends, in part, on their capacity to survive transient release into the extracellular compartment including the bloodstream. Natural variability in the resistance to antibody–dependent, complement–mediated killing has been observed in invasive nontyphoidal *Salmonella* isolates against a backdrop of minimal genetic variation (Msefula et al., 2012). This provides a finite number of discrete features that can be investigated for their impact on complement susceptibility. The approach used here, therefore, unlike most studies on invasive ST313 strains, was to compare variation within the genotype, rather than to non–ST313 strains or gastrointestinal isolates.

The success of S. Typhimurium ST313 as a pathogen has depended on the expansion of multi drug–resistant clones that have been the subject of intensive research. Likewise, successful vaccines that elicit bactericidal antibodies could benefit from characterising determinants of such 'high–risk' clones to antibody immunity, which might enable survival in the face of natural or vaccine–induced immunity. It is now known from studies of representative strains that gene flux, either due to the acquisition of novel phage elements or nonsense mutations resulting in pseudogene formation, has impacted the characteristics of the ST313 pathovar. This combination of pathogen traits, together with a predominantly susceptible host population manifests as a significantly human–adapted bacterium with a predilection for extraintestinal infection. Selectable genetic features under vaccine pressure and mechanisms by which bacteria evade natural, and potentially protective vaccine immunity are therefore important considerations for vaccine implementation.

This thesis, therefore, tests the central hypotheses that differences in genotype account for the variability in the susceptibility to antibody–dependent complement–mediated serum killing, and are capable of modifying virulence in invasive S. Typhimurium.

1.10. Aim and Objectives

The aim of this project was to establish the contribution of genomic variation on the responses of S. Typhimurium, particularly those of invasive ST313 strains, to antibody–dependent complement–mediated bactericidal activity of immune human serum, and identify means by which complement–

resistant strains express this phenotype, in the context of the expansion and dominance of the ST313 pathovar and its adaptation towards invasive human disease.

The hypotheses of this thesis were tested experimentally by way of five specific objectives, which were:

1. To analyse the relationship between increased complement resistance and virulence traits in S. Typhimurium,
2. To describe the genetic diversity and phylogenetic relationships among invasive S. Typhimurium ST313 strains with respect to their various responses to antibody–dependent complement–mediated bactericidal activity,
3. To determine the association between genomic variation and complement resistance phenotypes among S. Typhimurium ST313 strains,
4. To describe the transcriptome and full complement of genes required for the survival and growth of invasive S. Typhimurium strains in immune human serum,
5. To identify putative mechanisms for serum resistance among invasive S. Typhimurium strains.

Antibody is key to living with invasive African *Salmonella* Typhimurium

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Potential conflicts of interest

CAM and EMO are former employees of the Novartis Vaccines Institute for Global Health. FM is an employee of GlaxoSmithKline. CAM is the recipient of a clinical research fellowship from GlaxoSmithKline. The authors declare no other conflicts of interest.

ABSTRACT

Many *Salmonella* Typhimurium in sub–Saharan Africa belong to a clade, ST313, that causes life–threatening bloodstream infections among infants in sub–Saharan Africa. A large proportion of African S. Typhimurium blood isolates are multi–antibiotic resistant. Vaccine strategies aim to induce the *Salmonella*–specific antibodies that naturally protect older children and adults. We show that although mutations causing resistance to control by antibody develop sporadically, there is selection against such mutants. S. Typhimurium was isolated from the blood of 301 consecutive Malawian children, of whom 59 died during their admission. Of these isolates, 5% were resistant or moderately resistant to antibody–induced killing, while 94% were multi–antibiotic–resistant. Phylogenetic analysis shows that resistance to antibody–induced killing evolves independently in different S. Typhimurium isolates, without the emergence of resistant clades. This suggests that natural selection acts against the mutations that cause resistance to antibody–induced bacterial killing, contrasting with a strong positive selection of S. Typhimurium resistance to antibiotics. Therefore, a vaccine inducing protective antibodies against S. Typhimurium, unlike current antibiotics, could have a long–term role in preventing childhood mortality from *Salmonella* in sub–Saharan Africa.

INTRODUCTION

Salmonellae are a major cause of community–acquired generalized bacterial infections in Africans (1). The nontyphoidal *Salmonella*, S. Typhimurium, predominates in sub–Saharan Africa, with a significant minority of S. Enteritidis (1–4). Many invasive S. Typhimurium belong to a distinct clade, ST313 (5, 6), that causes life–threatening invasive disease, particularly among infants. This contrasts with diarrhoea–inducing S. Typhimurium infections in Europe and North America (7). A large proportion of African S. Typhimurium blood isolates are multi–antibiotic resistant (2, 5) and no vaccine is available against nontyphoidal *Salmonellae* (8). *Salmonellae* can colonize and persist in macrophages, and T lymphocytes play a key role in eliminating these bacteria from within macrophages (9, 10). Nevertheless, extracellular spread is a key factor resulting in fatal infection, and this can be prevented by naturally–induced antibodies that kill *Salmonella* and protect older children and adults (11, 12). Defective cellular function, associated with failure of the IL–12/IFNγ axis, is characterized by clusters of S. Typhimurium–infected macrophages forming granulomata (13). These are unpleasant lesions, but typically not life–threatening (14, 15).

The considerable impact on public health of the S. Typhimurium pandemic in Africa is in large part due to the spread of these bacteria into the blood and meninges. Case–fatality rates for S. Typhimurium bacteraemia are consistently around 20–25% in children (16). The non–specific clinical presentation of invasive S. Typhimurium disease, lack of blood culturing facilities and widespread emergence of multi–antibiotic–resistant isolates are major challenges to early diagnosis and effective management (17–19). These factors make a strong case for introducing a vaccine that can induce protective anti–S. Typhimurium antibodies in infants. Here we test whether the rapid appearance and dominance of multiple–antibiotic resistant S. Typhimurium in Africa will be mirrored by the emergence of strains resistant to antibody–induced killing following the introduction of a vaccine.

RESULTS AND DISCUSSION

We studied 301 primary blood culture isolates of S. Typhimurium from children admitted to Queen Elizabeth Central Hospital, Blantyre, between August 2003 and July 2004. The median age of children was 13 months (range 1 to 156 months). Each isolate was assessed for susceptibility to antibody–induced complement–mediated killing by serum pooled from ten healthy Malawian adults. 95% underwent a reduction in bacterial numbers by a factor of 25 or more, and for 19% there was a 1000–fold reduction or more (Figure 1A). Only two S. Typhimurium showed continued growth in the presence of antibodies and complement. By contrast, 94% of isolates were multi–antibiotic resistant (Figure 1B). We classified isolates into groups resistant, moderately resistant, susceptible and highly susceptible to antibody–induced complement–mediated killing. The susceptible group includes S. Typhimurium D23580, an ST313 isolate, which has been extensively studied (5, 11, 12).

We tested by flow cytometry whether fewer antibodies from the serum pool bound to the antibody–resistant isolates. The quantity of antibodies bound by resistant and sensitive isolates is similar and exceeds the threshold required to kill S. Typhimurium D23580 (Figure 1C) (11). When each of the ten constituent sera of the pool was tested individually, the pattern of killing of resistant and sensitive isolates was reproduced (Figure 1D). Further flow cytometric analysis shows that the amount of complement deposited on susceptible and resistant strains incubated in the serum pool is similar for both the C3 complement component (Supplemental Figure 1A) and membrane attack complex (C5b–9) (Supplemental Figure 1B). The amounts of C3 and C5b–9 deposited on each isolate greatly

exceeds the levels required to kill *S.* Typhimurium D23580 (11). There is no correlation between C3 or C5b–9 deposition and sensitivity to antibody–induced killing of isolates.

Persistent susceptibility of circulating *S.* Typhimurium to killing by antibody and complement would be important for the continued effectiveness of a vaccine that induces protective antibodies against *S.* Typhimurium in African children. To probe this, we determined the phylogenetic distribution of the antibody–resistance phenotype in field isolates using whole genome sequencing. Has this phenotype emerged independently on multiple occasions or does it represent a successful clonally–expanding clade? The majority of invasive *S.* Typhimurium Malawian isolates since 2002 share with the index strain D23580 the same ST313 multilocus sequence type, plasmid profile and XbaI–digest pulsed–field gel electrophoresis (PFGE) pattern (5, 11). Also, 94% (121/129) of invasive African *S.* Typhimurium studied to date belong to ST313 (6). Based on these findings, we assessed the high–resolution phylogenetic relationship of the six *S.* Typhimurium isolates representative of the range of serum susceptibilities, along with a further 29 invasive Malawian *S.* Typhimurium isolated between 1997 to 2006 (5, 6).

Fifteen of the *S.* Typhimurium have genome sequences highly–related to D23580 (Figure 2). The numbers of single nucleotide polymorphisms (SNPs) range from 13 to 20 among the fifteen sequences with reference to D23580. Fewer than eight SNPs are specific to the sequence of each isolate and they share the same plasmid profile (profile A, Supplemental Figure 2A) consisting of four plasmids of approximately 117 kb (the virulence plasmid pSLT–BT), 84 kb, 2.6 kb and 1.4 kb (5), and an indistinguishable PFGE pattern (pattern I). These isolates span all four antibody susceptibility/resistance groups. Thus *S.* Typhimurium that are extremely closely related genotypically, can differ markedly in susceptibility to antibody–induced complement–mediated killing. Importantly, this phylogenomic analysis indicates that the resistance shown by D25352 and D23005 emerged independently.

The remaining *S.* Typhimurium isolate (D26104) from Figure 1D is resistant to antibody–mediated killing and has a genome sequence closely related to the antibody–susceptible invasive Malawian *S.* Typhimurium isolate, A130. A130 was isolated in 2007 and, together with the remaining 17/34 African isolates from 1997 to 2006, falls into a ST313 lineage distinct from D23580 (5, 6). Forty–seven SNPs distinguish D26104 from *S.* Typhimurium A130. The three plasmid profiles (profile B)

and PFGE pattern (pattern II) of D26104 are indistinguishable from A130. Both the D23580 and A130 clades belong to the MLST sequence type, ST313, but fall into different lineages (5). Thus this third resistant S. Typhimurium isolate also emerged independently of D25352 and D23005. Two further isolates in this clade, A16083 and D15040, have a resistant/moderately–resistant phenotype, but again, have evolved independently of each other and the other resistant phenotypes.

Outer cell wall lipopolysaccharide (LPS) is an important determinant of resistance to complement–mediated killing of *Salmonella*. We have previously demonstrated the presence of long–chain polysaccharide (the O antigen) on invasive Malawian S. Typhimurium (11). We have also shown that truncation of LPS, by disruption of the *galE* gene, increases the sensitivity of D23580 to complement–mediated killing (from 'intermediate sensitivity' to 'high sensitivity'), with killing possible in the absence of antibody (11). We therefore examined the LPS content of the selected S. Typhimurium isolates (Table and Supplemental Figure 2B). Separation of crude LPS preparations by PAGE demonstrates the presence of long–chain LPS separated in the form of ladders that are similar for all isolates. O–antigen production is similar for all isolates, both in relation to total quantity and O–antigen average molecular weight, as determined by HPLC–SEC. The structure of the O–antigen repeating unit is identical for each isolate. Although there is variation in glucosylation and O–acetylation, there is no obvious correlation between these variations and resistance/sensitivity to antibody–mediated killing (Table 1). This suggests that differences in the lengths of LPS molecules and fine specificities do not account for the differences in sensitivity to antibody–induced killing.

We previously showed that invasive African S. Typhimurium resistant to cell–free antibody–dependent complement–mediated killing, can be killed by blood phagocytes following opsonization with antibody and complement (12). When the S. Typhimurium from the resistant groups of the current series were opsonized with the serum pool, they were phagocytosed as well as those from the susceptible groups (Figure 3A). They also induced an oxidative burst in phagocytes to the same extent (Figure 3B). Although *Salmonellae* resistant to antibody–induced complement–mediated killing were killed less effectively by blood phagocytes than were susceptible isolates (Figure 3C) there was either some killing or, for D26104, a bacteriostatic effect. Thus, even for the few strains that are resistant to complement–mediated killing, bloodstream infection may be controlled by phagocytes, antibody and complement.

Our phylogenomic studies suggest a clear difference in the selective pressures acting on S. Typhimurium strains that are antibiotic–resistant from those resistant to being killed by antibody and complement. The former, over a matter of a few years, have come to dominate S. Typhimurium populations (2, 16). By contrast, the phylogenomic analysis suggests that while resistance to killing by antibody and complement develops sporadically, the resistant strains are not becoming dominant. Since antibody against these bacteria is induced through natural exposure in Africans (11), selective pressure must have acted on mutants resistant to antibody–induced killing for millennia.

The biology of S. Typhimurium infection in sub–Saharan Africa may explain the apparent selective advantage of S. Typhimurium that can be killed by antibody and complement (20). *Salmonellae* reside in macrophages and have mechanisms that resist killing within such cells (21–25). Clearance of intracellular *Salmonellae* from the macrophage pool by T cells takes several weeks (9, 10), and in some circumstances, infections appear to become chronic (26). While intracellular infection persists, transmission may occur to other individuals, as has been documented for carriers of S. Typhi (27). A fatal outcome due to resistance to the extracellular killing of bacteria will stop host to host transmission, which is critical, since available evidence indicates that transmission of the African clade is between humans rather than zoonotic (28, 29).

Whatever the explanation for the failure of resistance to antibody–induced killing to become dominant, our findings suggest that a vaccine inducing antibodies to S. Typhimurium in infants is likely to have continued value for the prevention of morbidity and mortality from this bacterium in sub–Saharan Africa.

METHODS

Serum aliquots from ten healthy Malawian adults were stored at –80°C. S. Typhimurium were isolated from blood of children admitted to Queen Elizabeth Central Hospital, Blantyre, Malawi, as previously described (2) and antibiotic susceptibility determined by disc diffusion. Serum bactericidal assays; FACS–based anti–*Salmonella* IgG, C3 and MAC deposition assay; blood cell killing, phagocytosis and oxidative burst assays are as described (11, 12).

Bacterial DNA sequence reads from the Illumina platform were aligned against the reference D23580. A maximum–likelihood phylogenetic tree was constructed with RAxML v7.0.4 using the generated SNP alignment and running 100 independent bootstrap replicates. Sequence data were

submitted to the European Archive (http://www.ebi.ac.uk/ena/). Accession numbers are in Extended Data Table 2. Pulsed–field gel electrophoresis was performed on *Salmonella* XbaI endonuclease digests (30). Plasmids were prepared as by Kado and Liu (31).

LPS was visualized by silver staining as in (11). O–antigen extraction was by direct hydrolysis with acetic acid (32). Extracted total sugar was quantified by phenol sulfuric assay. O–antigen was purified by size exclusion chromatography and characterized by HPLC–SEC, 1H NMR and HPAEC–PAD.

STUDY APPROVAL

Ethical approval was from the College of Medicine Research and Ethics Committee, University of Malawi. Peripheral blood samples were obtained following informed, written consent from each subject.

Table 1.
Characterization of O–antigen purified from six selected invasive Malawian S. Typhimurium isolates

Sample	Susceptibility to killing by antibody	avgMW OAg (kDa)	µg of OAg extracted per mL culture (OD 35)	%Glc	OAc position	% OAc
D24545	highly susceptible	35.8 + shoulder at 85.7	844	30	OAc on C2/C3 Rha and on C2 Abe	163
D23580	susceptible	27.0 + shoulder at 88.0	736	39	OAc on C2/C3 Rha and on C2 Abe	142
D24871	susceptible	25.9	725	14	OAc mainly on C2/C3 Rha	76
D25352	moderately resistant	27.6	693	51	OAc on C2/C3 Rha and on C2 Abe	142
D26104	resistant	27.0	773	17	OAc on C2 Abe only	97
D23005	resistant	24.1	796	28	OAc on C2/C3 Rha and on C2 Abe	147

avgMW average molecular weight
OAg O–antigen
%Glc % O–antigen repeating units containing glucose
OAc O–acetylation
Rha rhamnose, Abe abequose

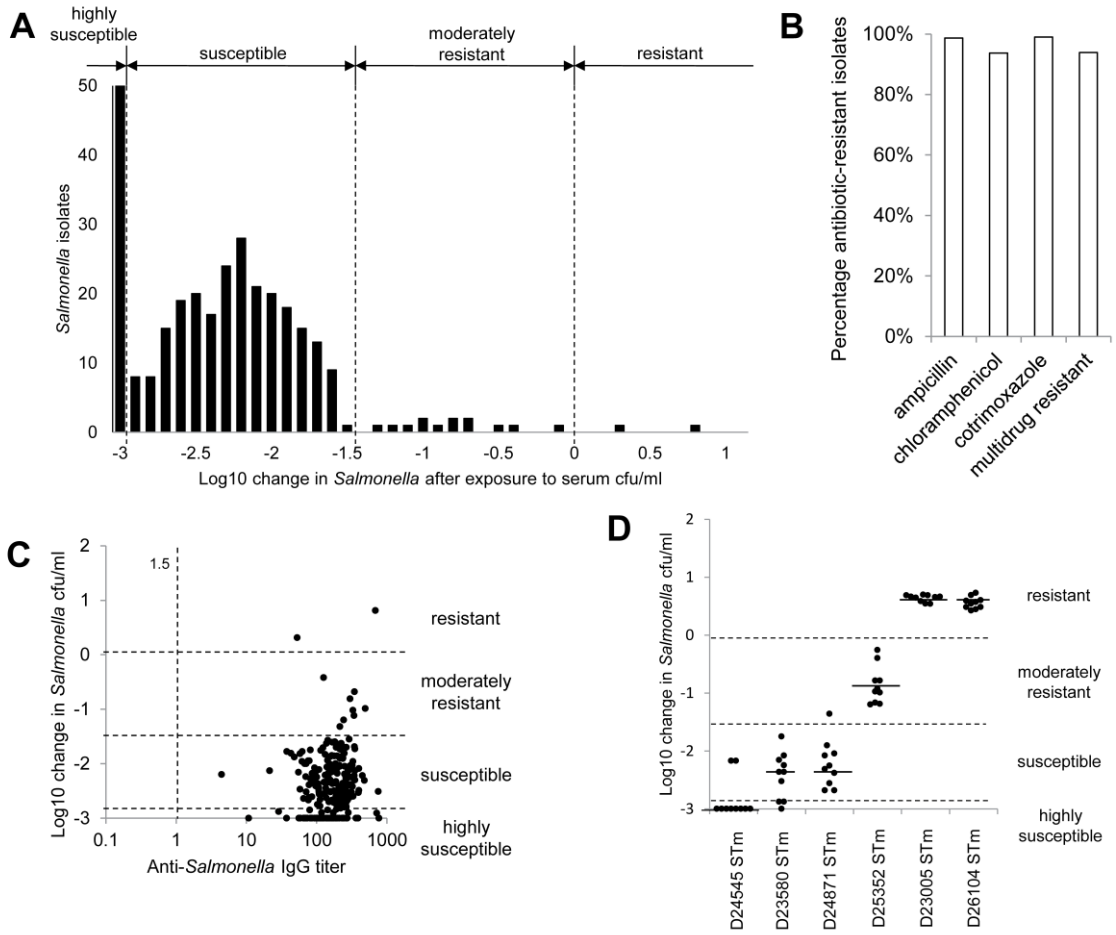


Figure 1. Antibody and antibiotic sensitivity of invasive Malawian *S.* Typhimurium isolates. **(A)** Sensitivity of 301 consecutive *S.* Typhimurium isolates to killing at 180 minutes by pooled sera from Malawian adults identifying the 'highly susceptible', 'susceptible', 'moderately resistant' and 'resistant' groups. **(B)** Percentages of invasive isolates resistant to ampicillin, chloramphenicol, cotrimoxazole or all three antibiotics (multidrug resistant). **(C)** Serum pool IgG antibody binding to each isolate compared to serum pool–induced killing. Six isolates were chosen from those in (A) to represent the range of sensitivities to antibody–induced killing. **(D)** Killing of each isolate at 180 minutes by individual sera from ten Malawian adults. Horizontal bars indicate medians. Vertical line indicates threshold of IgG deposition associated with killing of *S.* Typhimurium D23580 (11).

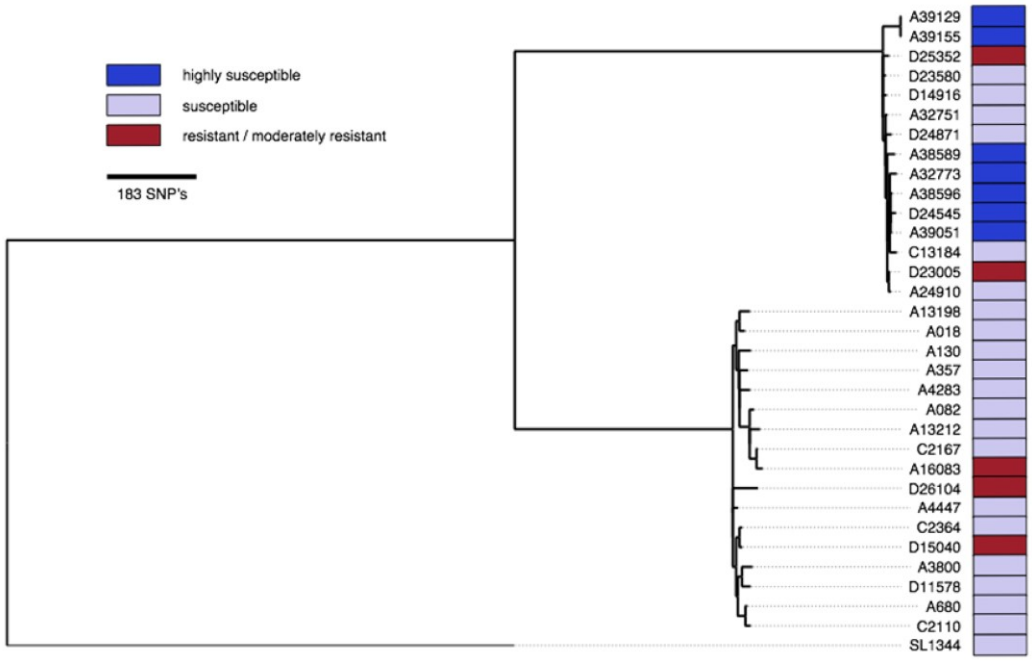


Figure 2. Phylogenetic relatedness of Malawian *S.* Typhimurium isolates. Population structure of six selected S. Typhimurium isolates, 29 other invasive Malawian S. Typhimurium isolated from 1997 to 2006 and S. Typhimurium laboratory strain SL1344 with colour–coded range of susceptibilities to antibody–induced killing.

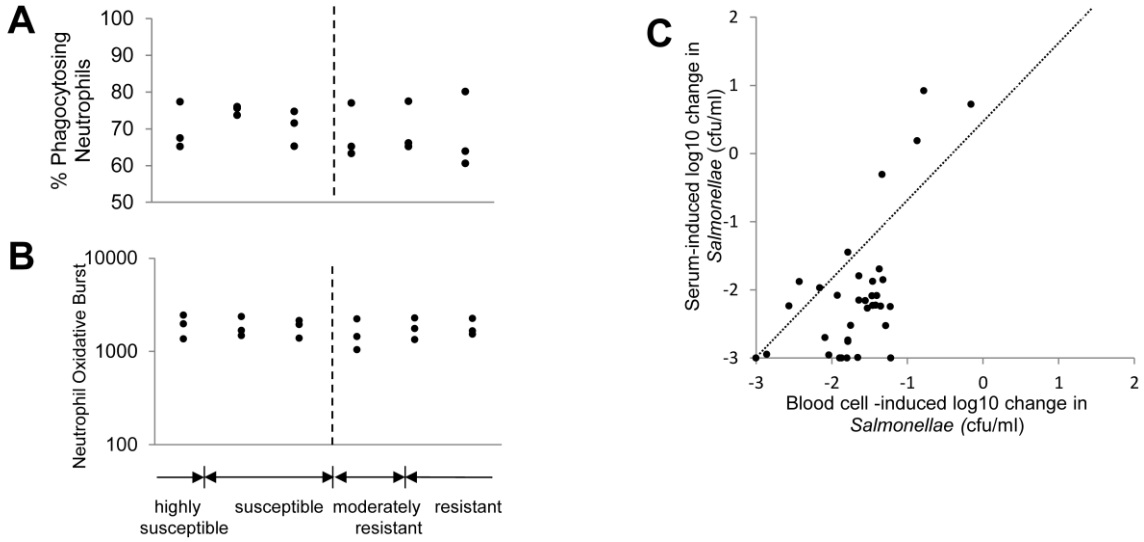
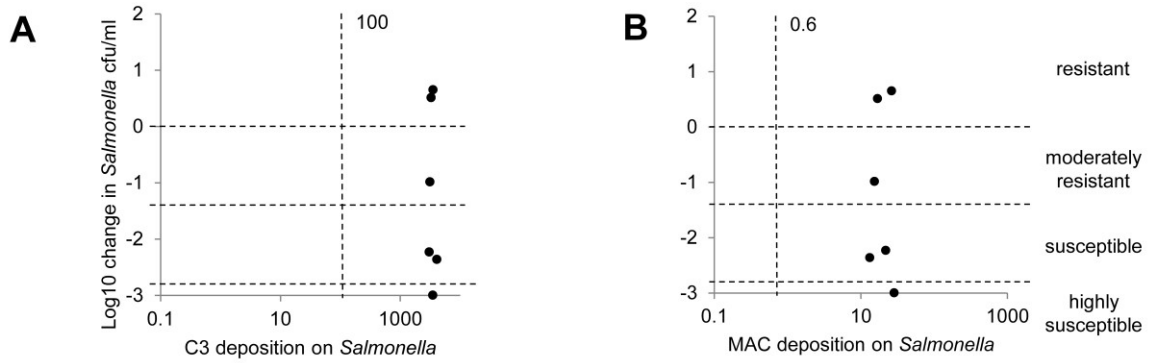
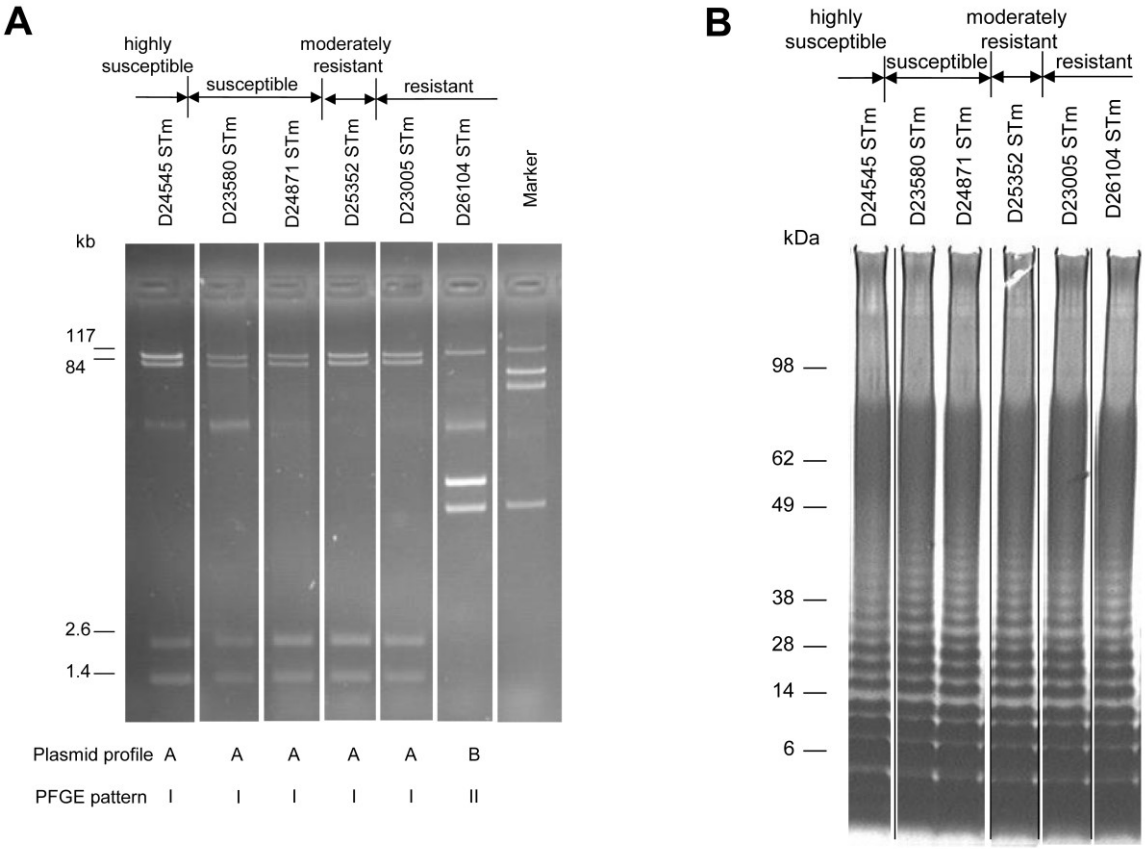


Figure 3. Phagocytosis, oxidative burst and killing of Malawian *S.* Typhimurium isolates by peripheral blood cells. **(A)** Phagocytosis and **(B)** oxidative burst by peripheral blood neutrophils of six S. Typhimurium isolates resistant or susceptible to antibody–induced killing following opsonization with the serum pool. Each vertical set of dots shows data from three experiments. **(C)** Killing at 180 minutes by washed peripheral blood cells of the six S. Typhimurium isolates and 29 other invasive Malawian S. Typhimurium isolated from 1997 to 2006, opsonized with serum pool, compared with killing by serum pool alone. Each dot represents mean of three blood cell killing experiments for one isolate. Dotted line is the line of equivalence.



Supplemental Figure 1. Serum complement deposition on invasive Malawian *S.* Typhimurium. Serum pool (A) C3 and (B) C5b–9 membrane attack complex (MAC) deposition on S. Typhimurium isolates compared to serum pool–induced killing at 180 minutes. Six isolates were those chosen from those in Figure 1D to represent the range of sensitivities to antibody–induced killing. Vertical lines indicate thresholds of complement deposition associated with killing of S. Typhimurium D23580 (11).



Supplemental Figure 2. Plasmid, pulse–field gel electrophoresis and lipopolysaccharide analysis of Malawian *S.* Typhimurium isolates. **(A)** Plasmid content of six selected S. Typhimurium isolates visualized by 0.8% agarose gel electrophoresis and pulsed–field gel electrophoresis pattern from XbaI endonuclease digests of bacterial DNA. **(B)** LPS from S. Typhimurium visualized by SDS–PAGE and silver staining. Lanes were run on the same gel but were non–contiguous.

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CHAPTER 3

The role of *sapA* and *yfgA* in susceptibility to antibody–mediated complement–dependent killing and virulence of *Salmonella enterica* Typhimurium

Running title:

Virulence of S. Typhimurium *sapA* and *yfgA* mutants

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ABSTRACT

The ST313 pathovar of *S.* Typhimurium contributes to a high burden of invasive disease among African infants and HIV–infected adults. It is characterized by genome degradation (loss of coding capacity) and has increased resistance to antibody–dependent complement–mediated killing compared with enterocolitis–causing strains of *S.* Typhimurium. Vaccination is an attractive disease–prevention strategy and leading candidates focus on the induction of bactericidal antibodies. Antibody–resistant strains arising through further gene deletion could compromise such a strategy. Exposing a saturating transposon insertion mutant library of *S.* Typhimurium to immune serum identified a repertoire of *S.* Typhimurium genes that, when interrupted, result in increased resistance to serum killing. These genes included several involved in bacterial envelope biogenesis, protein translocation and metabolism. We generated defined mutant derivatives, using *S.* Typhimurium SL1344 as host. Based on their initial levels of enhanced resistance to killing, *yfgA* and *sapA* mutants were selected for further characterization. The *S.* Typhimurium *yfgA*$^-$ strain lost the characteristic *Salmonella* rod–shape appearance, exhibited increased sensitivity to osmotic and detergent stress, lacked very long lipopolysaccharide, was unable to invade enterocytes and demonstrated decreased ability to infect mice. In contrast, the *S.* Typhimurium *sapA*$^-$ mutants had similar sensitivity to osmotic and detergent stress and lipopolysaccharide profile, and an increased ability to infect enterocytes compared with wild–type, but had no increased ability to cause in vivo infection. These findings indicate that increased resistance to antibody–dependent complement–mediated killing secondary to genetic deletion is not necessarily accompanied by increased virulence, and suggest the presence of different mechanisms of antibody resistance.

INTRODUCTION

Nontyphoidal *Salmonellae* (NTS) are a major cause of illness and death worldwide (1). While gastroenteritis is the most common clinical manifestation of the disease in the developed world, severe, often fatal disseminated disease is dominant in sub–Saharan Africa, with an estimated global burden of mortality in 2010 of 680,000 (2) and case fatality rates of up to 25% (3–5). The majority of this invasive nontyphoidal *Salmonella* (iNTS) disease is attributable to serovars Typhimurium and Enteritidis, which account for up to 95% of cases in sub–Saharan Africa (4–6). Several factors contribute to the high prevalence of iNTS disease and associated death in this region, including lack of a definitive clinical presentation, which confounds timely diagnosis (4, 5, 7), co–endemic diseases such as malaria (8, 9), and HIV (10), underdeveloped anti–*Salmonella* immunity in children (11), and multiple drug resistance (MDR) (12). MDR may have contributed to the emergence and spread of *S.* Typhimurium genotype ST313, which has been responsible for much of the epidemic iNTS disease in the region (13, 14). The difficulty diagnosing iNTS disease and widespread drug resistance among circulating strains support the need for vaccine development as an effective public health intervention (5, 15).

In iNTS–endemic regions, acquisition of *Salmonella*–specific antibodies is important for protection against bacteraemia (11, 16, 17). Waning of maternal antibody levels is followed by a peak in iNTS disease at around one year of life. Thereafter, a fall in age–related incidence is associated with production of antibody by the child's own immune system (11, 17). These antibodies effect bacterial killing through complement fixation and lysis via the classical and terminal pathways of complement (11) and opsonization of bacteria for uptake by phagocytes and oxidative burst activity (16). *S.* Typhimurium virulence and pathogenicity during systemic infection therefore, depends in part on the ability to subvert antibody–mediated killing (18). The bacterial surface and its components form an important interface through which they detect and adapt to environmental changes and host immune responses. Surface–associated mechanisms described in *S.* Typhimurium that influence antibody susceptibility include modulation of LPS length (19), and the action of proteins that impede complement activity such as Rck, PgtE, and TraT (20–22). Nevertheless, the mechanistic basis of resistance to antibody–dependent complement–mediated killing, ('serum resistance') among *Salmonella*, is poorly understood.

S. Typhimurium ST313 strains exhibit genome degradation similar to that of the human–adapted serovar, *S.* Typhi (13). This process is consistent with the possibility that this genotype is adapting from gastrointestinal towards systemic infection (23, 24), with invasive strains exhibiting decreased enteropathogenicity (25). We and others have reported higher inherent resistance to antibody–mediated killing by complement (26) and macrophages (24) compared to gastrointestinal or non–ST313 strains.

A potential threat to the successful implementation of a vaccination strategy is that further genome modification within the ST313 pathovar could result in adaptation towards enhanced resistance to antibody–mediated killing and subsequently the emergence of potential vaccine escape mutants. Acquisition of such antibody–resistance might be accelerated under the selective pressure exerted by an antibody–inducing vaccine. Nevertheless, invasive African *S.* Typhimurium ST313 isolates have mostly remained sensitive to antibody (11, 16), being killed by complement if they remain in the extracellular compartment of blood (27). We hypothesize that increased resistance to antibody and complement–mediated killing in *S.* Typhimurium impacts other functions associated with bacterial survival and virulence, so that the trade–offs between serum resistance and viability help to maintain *Salmonella* in a serum–sensitive state.

Here we report the determination of a repertoire of *S.* Typhimurium genes that, when interrupted, result in increased resistance to antibody–dependent complement–mediated killing, using a saturating transposon insertion library screen in conjunction with transposon–directed insertion site sequencing (TraDIS) (28). We subsequently investigated defined *S.* Typhimurium *yfg*A and *S.* Typhimurium *sap*A mutant derivatives in order to gain insight into their biological function and the impact of their absence on infection.

MATERIALS AND METHODS

Bacterial strains and culture. *S.* Typhimurium strain SL1344 and its derivatives were used throughout this study. SL3261 is a derivative of SL1344 harbouring a mutation in the *aroA* gene that has limited impact on susceptibility to serum killing. Bacteria were routinely cultured in Luria–Bertani (LB) broth with either 5g/l (low salt) or 10g/l (standard) NaCl, and grown aerobically at 37°C. Where appropriate, derived strains were grown in LB media supplemented with either 50μg/ml of kanamycin, 100μg/ml of trimethoprim, 30μg/ml of chloramphenicol, or 100μg/ml of ampicillin. Isogenic *phoN* mutants *S.* Typhimurium RAK113 (SL1344Δ*phoN*::*cat*) (29) and *S.* Typhimurium RAK072 (SL1344Δ*phoN*::*aph*) were used to distinguish wild–type SL1344 from test strains in competition assays by using antibiotic–resistance markers inserted into the *phoN* locus that has no impact on either virulence in mice (30) or serum susceptibility (data not shown).

Ethical approval. The study was approved by the College of Medicine Research and Ethics Committee, University of Malawi. Informed written consent was obtained from all blood donors. All animal procedures were performed in accordance with the United Kingdom Home Office Inspectorate under the Animals (Scientific Procedures) Act 1986. The Wellcome Trust Sanger Institute Ethical Review Committee granted ethical approval for these procedures.

Preparation of serum. Human blood was drawn from ten healthy HIV–uninfected Malawian adults, and allowed to clot for 2 hours at room temperature. Serum was separated from whole blood by centrifugation and stored in aliquots at −80°C until ready for use. For bactericidal assays on defined mutants, serum from a single healthy adult donor, with anti–*Salmonella* antibody and similar levels of bactericidal activity as the pooled serum, was used.

Transposon insertion library screen and TraDIS. A mini Tn5–EZ transposon insertion library containing approximately 0.9×10^6 independent insertion mutants in *S.* Typhimurium strain SL3261 (SL1344 *aroA*$^-$) described previously (31) was prepared by transferring approximately 50μl of frozen library to 10ml LB broth for overnight growth. The culture inoculum contained at least 1×10^8 cfu/ml in order to ensure that individual mutants were not lost from the library and to minimize expansion of the library by culture prior to the experiment. Log phase cultures were then prepared by inoculating 250μl of the stationary phase culture into 25ml LB broth and grown at 37°C to an OD_{600} of 0.3 with shaking. The cultures were washed twice in phosphate–buffered saline (PBS) and finally re–suspended in PBS to a concentration of 1.7×10^8 cfu/ml. Aliquots of this inoculum was

either used directly for genomic DNA preparation (input sample) or exposed to serum killing followed by culture in LB broth before preparation of genomic DNA (output sample). For serum killing, 100µl of the bacterial suspension was added to 900µl of neat pooled Malawian serum described previously, and then incubated for 3 hours at 37°C with shaking. Viable bacterial counts from serum were then determined by serial dilution and plating on LB agar at 45, 90 and 180 minutes. The remaining bacteria/serum mixture was then transferred to 25ml LB broth for expansion of the surviving bacterial mutants overnight at 37°C. Bacteria were re–suspended in 9ml PBS for DNA extraction, and 1ml of 20% SDS (w/v) and proteinase K were added to a final concentration of 200µg/ml. The suspension was mixed gently, and incubated at 37°C for 1 hour until a clear lysate formed. DNA was extracted twice using an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1 by volume) each time, followed by final extraction with chloroform. A 0.1× volume of 3M sodium acetate and 2.5 volumes ice–cold ethanol were then added to the final aqueous phase to precipitate DNA, which was then washed in 70% ethanol, dried, and resuspended in 500µl of nuclease–free water.

Sequence determination and analysis. Genomic DNA prepared from the transposon insertion library input and output samples were sheared to 300bp in size, Illumina libraries prepared and transposon insertion sites amplified by PCR as previously described (32). The amplified DNA fragment libraries were sequenced using Illumina GAII sequencer flow cells for single end reads with 54 cycles. The number of sequence reads mapping to transposon insertions was determined by parsing sequence reads containing the terminal transposon sequence. Sequence reads lacking the transposon sequence were not considered further. The transposon sequence was removed from sequence reads and the remaining sequence mapped to the reference genome *S.* Typhimurium SL1344 (FQ312003) using SSAHA. Raw sequence data is deposited in the EMBL short read archive ERR305896_1 (input sample), 5850_6 ERR305895 (output replicate 1), 6145_2 ERR305897 (output replicate 2) and 6145_3 ERR305898 (output replicate 3). Reads per insertion site were determined and a normal distribution was fitted to the $\log_2$ fold read change normalized for the differences in library read counts for each output sample dataset, as previously described (31). The distributions were all shifted to the left (in contrast to the LB passages, where the mean is at around 0) suggesting a general reduction in the number of reads observed over most genes. P values were then calculated for each gene in each replicate using this as a null distribution. To combine

information across replicates, Fisher's Method was used, which takes independent p values and combines them. This test is known to be anticonservative, so the Bonferroni correction for multiple testing was applied. Genes with >2–fold increase in sequence reads following exposure to serum and p values < 0.05 were considered biologically relevant and analysed further. Genes with fewer reads observed over the gene in any of the replicates than the average number of reads per gene in the input pool (~550) were excluded from further analysis.

Site directed mutagenesis by allelic exchange. Mutants were constructed by allelic exchange as described previously (33). PCR fragments for mutagenesis were prepared by amplification of the kanamycin resistance cassette from pKD4 using 70–mer primers (KO_forw, KO_rev, Table S1) consisting of the 50 nucleotides flanking each targeted gene (GenBank accession: NC_016810), and 20 nucleotides priming pKD4 at the 3' ends of each primer. Cycle conditions were: 95°C for 5 min followed by 30 cycles of 94°C for 1min, 60°C for 1min and 72°C for 2min. Approximately 1μg of PCR product was electroporated into SL1344 harbouring plasmid pAJD434, grown to log phase at 30°C in LB–trimethoprim broth supplemented with 20mM of L–arabinose to induce recombinase gene expression. Mutants were selected on LB–kanamycin agar. They were then cured of pAJD434 by successive replica–streaking on kanamycin and trimethoprim plates and incubation at 42°C to minimize nonspecific recombinase activity. Successful gene replacement was verified by PCR on chromosomal DNA using primers flanking the recombination site, ext_forw and ext_rev (Table S1) and sequencing.

Serum bactericidal assays on defined mutants. Log–phase cultures of each strain to be tested were prepared by diluting stationary phase cultures 1:100 in LB without antibiotics and grown for 2¼ hours. The bacteria were harvested and then washed twice in cold phosphate–buffered saline (PBS). The pellets were resuspended at an OD_{600} of 0.02 (approximately 10^7cfu/ml). 10μl of the bacterial suspension was then inoculated into 90μl of neat, freshly thawed, pre–warmed serum, and incubated for 3 hours at 37°C. Viable bacterial counts were then titrated by serial dilutions and plating on LB agar. Competition serum bactericidal assays were performed in a similar manner with 1:1 mixtures of Δ*phoN* and derived strains then grown on respective LB–antibiotic plates.

Complementation experiments. To complement the *yfgA* mutant in trans, its locus plus 274 nucleotides upstream was amplified from SL1344 chromosomal DNA by PCR using the primers *yfgA*_trans_forw and *yfgA*_trans_rev (Table S1). The gene fragment was cloned into pCR2.1 using the

TOPO TA Cloning Kit for Subcloning (Invitrogen), and then sub cloned into pWKS30. The resulting plasmid, pWKS30::*yfgA*, was transformed into SL1344Δ*yfgA* by electroporation (the resulting strain henceforth abbreviated as p*yfgA*). The *sapA* mutant could not be complemented in this way despite multiple attempts. Both mutants were therefore complemented by transduction. To achieve this, a chloramphenicol resistance cassette was inserted into the intergenic regions adjacent to each gene locus in the wild–type strain by allelic exchange as described previously, using the primers *sapA*compl_F, *sapA*compl_R, *yfgA*compl_F, *yfgA*compl_F (Table S1). Phage P22 was then used to co–transduce the marker and the wild type copy of *yfgA* and *sapA* genes into the mutant strains. Kanamycin and chloramphenicol replica plates were then used to select Km^sCm^r colonies. Presence of the *sapA* and *yfgA* genes in the selected transductants (henceforth referred to as c*sapA* and c*yfgA* respectively) was further verified by PCR using flanking primers *sapAv*F, *sapAv*R, *yfgAv*F, *yfgAv*R (Table S1).

Electron microscopy. *Salmonellae* were streaked and grown on LB agar with respective antibiotics. Single colonies were looped directly from an agar plate and resuspended in 100µl of distilled water to make a slightly turbid suspension. A 10µl drop was placed on a freshly glow–discharged carbon–coated Formvar grid and an equal volume of 2% ammonium molybdate with 0.1% trehalose added for 30 seconds before draining on filter paper and drying. Grids were imaged on a 120kV FEI Spirit Biotwin using a Tietz F4.15 CCD.

In vivo competition experiments. Groups of 5 female 4–week–old C57BL/6 mice were infected orally with 5×10^8 cfu/mouse of a 1:1 mixture of the mutant strain and an antibiotic resistance marked strain *S.* Typhimurium RAK113 (Δ*phoN*::Cm^r) or *S.* Typhimurium RAK72 (Δ*phoN*::Kan^r). The interruption of the *phoN* gene has no impact on colonization of the mouse (29, 30) but the alternative antibiotic resistance expressed by these strains and the mutant or mutant complement constructs facilitated enumeration of the strains in homogenized organs following serial dilution and culture on replica agar plates containing each antibiotic in turn. Five days post infection, a terminal bleed was performed to heparinized tubes, after which the animals were sacrificed and liver, spleen, mesenteric lymph nodes, ileum, cecum and colon recovered. Organs were homogenized and viable bacterial counts determined by serial dilution and plating on the appropriate antibiotic agar plates.

Osmotic and detergent susceptibility assays. Osmotic and detergent susceptibility assays were performed using a modification of a previously described method (34). Briefly, approx. 10^4 cfu from stationary–phase cultures were inoculated into 4ml of LB growth medium with increasing concentration of NaCl or sodium dodecyl sulphate (SDS). Inocula were determined by plating serial dilutions on LB agar. The bacteria were then grown at 37°C for 8 hours with shaking at 180rpm, after which they were titrated by serial dilutions and plating as before. Dose–response curves were obtained by plotting the $\log_{10}$ fold change from the initial to final bacterial cell density at each concentration of NaCl/SDS.

Tissue culture. To estimate the invasion of T84 cells epithelial–like cells in culture we used a gentamycin protection assay. T84 cells were seeded onto 24–well plates at 10^5/well in Ham's F–12/Dulbecco's modified Eagle's medium (DMEM) 1:1 supplemented with 2mM L–glutamine and 10% heat–inactivated foetal bovine serum (FBS) and incubated at 37°C for 24 hours. Bacteria were grown by inoculating 10µl of a stationary phase culture of each strain into 10ml of low–salt LB broth and incubated statically at 37°C for 18 hours. 500µl of washed bacteria resuspended in the cell culture media were then inoculated at an MOI of 10 followed by brief centrifugation, and then incubation for 30 min. The inoculum was removed by aspiration and replaced with F–12–DMEM medium containing 100µg/ml of gentamycin to kill non–internalized bacteria, and incubated for 90 min. The cells were then washed twice with PBS and lysed with 200µl of 0.1% Triton X–100 in PBS. Viable internalized bacteria were determined by plating serial dilutions on agar. Invasiveness was assessed by the number of viable internalized or adherent bacteria recovered as a percentage of the inoculum.

Preparation of LPS and separation by electrophoresis. *Salmonellae* were inoculated in 2ml of LB broth and cultured overnight. The bacteria were then pelleted, resuspended in saline (0.9% w/v NaCl) to an OD_{600} of 0.5, and 5ml of this suspension harvested by centrifugation. LPS was extracted using a modification of the hot phenol method previously described (35). Briefly, pellets were resuspended in 250µl of SDS–PAGE lysis buffer (2% SDS, 100mM DTT, 10% glycerol, in 50mM Tris/HCl, pH 6.8), boiled at 100°C for 15 minutes, cooled to room temperature, and then DNase and RNase added to a concentration of 100µg/ml. The samples were incubated at 37°C for 1hr, after which proteinase K was added to a final concentration of 200µg/ml, followed by incubation at 65°C overnight. LPS was then isolated using hot phenol followed by precipitation with 100% ethanol, then resuspended in deionized, autoclaved water. The samples were then separated on a

12% Bis–Tris gel (Invitrogen), and stained using the SilverQuest Silver Staining Kit (Invitrogen) according to the manufacturer's instructions.

RESULTS

Identification of *S.* Typhimurium transposon insertion mutants with enhanced survival in serum. To determine genes modulating antibody and complement–dependent serum killing in *S.* Typhimurium, a saturated transposon insertion mutant library (approximately 9×10^5 independent mutants) was subjected to the bactericidal activity of pooled serum from healthy Malawian adults. Approximately 1×10^9 cfu of the log–phase (input) library was used to ensure that each insertion mutant in the library was represented around 1,000 times in the input mutant pool. After three hours of incubation with human serum, the viable count decreased to ~3.5% of the initial viable counts (Fig. 1A). The surviving mutants (output library) were cultured overnight in the absence of serum to permit extraction of genomic DNA from surviving, but not killed mutants. Genomic DNA was also prepared from the input library *Salmonella* in order to compare the relative number of insertions at each site in the genome by transposon directed insertion site sequencing (TraDIS).

A total of 46 genes were significantly overrepresented in the output pool with a $\log_2$ read ratio of 2 or more from comparisons of output compared to input mutant libraries (Table 1). The biological functions of this subset included genes involved in protein/peptide translocation (*sapA, sapB, sapC, sapF, tatA, tatB, tatC, clpX*) and metabolism, including transferases (*ybeA, cbiE, rfaY, ksgA, metB, mdoH, pfkA, mreD, metL*), and pentose phosphate pathway enzymes (*deoB, zwf, pfkA*). Twenty–three (50%) encoded either membrane protein constituents of the bacterial envelope or proteins involved in synthesis of the bacterial envelope, including peptidoglycan biosynthetic genes (*murF, murB*, penicillin binding protein 2 (PBP2)/*mrdA*) and four of the six known cell shape–determining genes in Gram–negative bacilli (*mreC, mreD, mrdA,* and *yfgA/rodZ*).

To independently verify the impact on antibody–dependent complement–mediated killing of mutation in genes identified in the mutant library screen, we selected eight genes for site–specific deletion in *S.* Typhimurium SL1344 by allelic exchange with an antibiotic resistance gene. Increased representation of particular mutants in the output pool could be due to gene inactivation secondary to transposon insertion leading to increased resistance to antibody–dependent complement–mediated killing (more likely to be relevant where genes encoded membrane components), or

enhanced bacterial growth (more likely to be relevant where genes are primarily involved in metabolism).

First, we selected twenty genes whose transposon–insertion mutants were over–represented following exposure to serum indicated by the highest ratio of output reads compared with input reads (>3.5–fold). As the objective of the study was to understand the impact of mutations on resistance to antibody–dependent complement–mediated lysis, rather than on bacterial growth, we chose to focus our attention on genes encoding membrane components. For some genes, it proved not to be possible to generate a viable definitive mutant, which could be due to an inherent lack of viability of such mutants. This led to the eight genes selected for generation of definitive mutants. From these eight, *yfgA* and *sapA* mutants were selected for further study based on their enhanced survival in serum compared with that of the other definitive mutants.

We replaced each of eight genes (Δ*mdoG*, Δ*mrdA*, Δ*mreD*, Δ*murB*, Δ*osmY*, Δ*sapA*, Δ*yfgA* and Δ*SL1344_0630*) with an antibiotic resistance gene by allelic exchange in *S.* Typhimurium SL1344, and tested serum susceptibility of these derivatives individually. Six of the mutants were less sensitive to antibody–mediated complement–mediated killing than the parent strain, while *mrdA* and *SL1344_0630* definitive null mutants were found to be more susceptible than the wild–type strain in contrast to the screening data (Fig 1B). Deletion of two genes in particular, *sapA* and *yfgA*, resulted in a greater than 10–fold decrease in susceptibility to killing by serum.

As these genes had been identified in a screen containing a mixture of mutants, we tested the strains by a second approach using a mixed inoculum assay with *S.* Typhimurium SL1344 containing a kanamycin or chloramphenicol resistance gene at the *phoN* locus for selection. We determined whether their phenotypes could be complemented by providing the wild type gene on a plasmid or reconstituting the gene on the bacterial chromosome. The number of viable bacteria with *sapA* or *yfgA* mutations was greater than the comparator strain after 1 hour of co–incubation in serum ($p=0.017$ and 0.039 respectively), and had approximately 6–7–fold more viable counts after 3 hours (Fig. 1C). Restoration of the *sapA* gene onto the chromosome resulted in a partial return of antibody sensitivity and introduction of *yfgA* on a plasmid resulted in an increase in susceptibility to serum relative to the wild type ($p<0.01$), possibly due to the impact of increased *yfgA* copy number relative to wild type (Fig. 1C).

Mutation of *yfgA* and *sapA* likely contribute to decreased susceptibility to antibody–dependent complement–mediated killing by distinct mechanisms. Loss of functional YfgA and SapA proteins resulted in a similar decrease in susceptibility to antibody– and complement–mediated serum killing. However, the functions of these proteins are distinct. YfgA is a structural protein that plays a role in maintenance of cell shape, while SapA is a component of a peptide transport complex. In order to gain insight into the mechanisms by which the loss of these proteins contribute to antibody susceptibility, we investigated whether increased resistance to serum bactericidal activity was related to increased tolerance to other lytic agents acting non–specifically on the bacterial membrane.

To this end, we tested the susceptibility of *sapA* and *yfgA* mutants to detergent and osmotic stress. Wild type and mutant derivatives were grown in media containing dilutions of either NaCl or SDS and then change in viable counts was determined after 8 hours of incubation. The *yfgA* mutant was more susceptible to both 1M NaCl (p=0.003) and 1% SDS (p=0.004) than the wild–type equivalent (Fig. 2), while the *sapA* mutant had comparable levels of susceptibility to NaCl and SDS lytic activity to wild–type SL1344, suggesting that the loss of these proteins, leading to decreased susceptibility to lysis by the membrane attack complex, is likely to be due to distinct mechanisms.

Further insight into the basis of this difference in mechanism came from the observation that the Δ*yfgA* derivative exhibited profound alterations in cell shape that is likely to be the result of underlying structural weakness of the cell envelope. Compared to wild–type SL1344, the long axis was markedly absent from the *yfgA* mutant, resulting in spherical bacteria (Fig. 3). Complementation of *yfgA* on a plasmid partially restored cell shape.

Δ*yfgA* but not Δ*sapA* is associated with alterations in very long chain LPS. To assess the impact of the absence of the two periplasmic proteins on the gross composition of lipopolysaccharide expression on the outer leaflet of the bacterial envelope, LPS from stationary phase cultures grown in standard LB broth was extracted from equivalent quantities of each of the derivatives studied, and separated by SDS–PAGE. Long–chain LPS was present in all the derivatives (Fig. 4). However, a high molecular weight band corresponding to very long LPS was appreciably less intense in the *yfgA* mutant than either wild type or its two complemented counterparts, despite similar proportions of short and long O–antigens, suggesting the absence of or a substantial reduction in production of very long chain LPS by this strain (Fig. 4). Furthermore, given that LPS was extracted from equivalent quantities of each bacterial strain, it also appears that the *yfgA*$^{-}$ mutant had reduced LPS expression on the cell

surface in comparison to the wild type. Deletion of *sapA* did not have an obvious effect on LPS expression.

Decreased susceptibility to antibody–dependent complement–mediated killing of Δ*sapA* and Δ*yfgA* derivatives is associated with altered interaction with host cells, but no increase of in vivo virulence. Since deletion of *sapA* and *yfgA* in S. Typhimurium resulted in enhanced survival in serum, we addressed the question as to how this impacts on the interaction of the pathogen with host enterocytes in culture and ability to colonize the host, using a mouse model of *Salmonella* infection. We first determined the relative ability of Δ*sapA* and Δ*yfgA* derivatives to invade T84 epithelial cells in culture. The Δ*yfgA* mutant derivative invaded T84 cells significantly less ($p<0.01$) than the SL1344 wild type parent, while the Δ*sapA* mutant derivative exhibited increased invasion ($p<0.01$) (Fig. 5). We then determined the ability of Δ*sapA* and Δ*yfgA* mutant derivatives to compete with wild–type SL1344 for colonization of genetically–susceptible mice in mixed inoculum experiments. A 1:1 ratio of mutant and wild type were inoculated into groups of five mice by the gastrointestinal route and five days later the derivatives were enumerated in organ homogenates. The Δ*sapA* mutant derivative was recovered from the colon at more than 10–fold lower levels than SL1344 ($p=0.04$), but at similar levels from the ileum, liver, spleen, blood and mesenteric lymph nodes (Fig.6). In comparison, the *yfgA* mutant was not recovered from any site in the mice five days post infection. Complementation of the gene knock out by replacement of the wild type copy on the chromosome resulted in near or complete return to wild type levels of colonization (Fig. 6).

DISCUSSION

The current epidemic of iNTS disease across sub–Saharan Africa is significantly associated with the S. Typhimurium genotype ST313. S. Typhimurium ST313 strains are characterized by genome degradation and pseudogene formation, with similarities to that observed for the human–restricted serovars S. Typhi and S. Paratyphi A (13), leading to speculation that ST313 is currently in an evolutionary bottleneck which may lead to host adaptation (13). Increasing antibiotic resistance among ST313 and the inability to make a clinical diagnosis have hampered antibiotic–based strategies to manage the epidemic of iNTS disease, leaving vaccination as an attractive public health intervention once licensed vaccines against iNTS disease become available.

Many current vaccine strategies are based on glycoconjugates (15) and are designed to induce protective antibody responses. It is therefore important to understand whether the propensity of *S.* Typhimurium ST313 to deletion and/or loss of function of individual genes will lead to isolates with increased resistance to antibody–dependent complement–mediated killing that could escape vaccine–mediated killing and undermine future vaccine campaigns. To gauge the potential public health threat posed by such mutants, it is also important to ascertain their virulence in vivo.

By screening a saturated transposon mutant library, we identified mutants with reduced susceptibility to killing by antibody. Many of these insertions were in genes encoding membrane proteins, or proteins involved in the synthesis of cell envelope components or determining cell shape, such as *yfgA*. Other mutations were in genes involved in protein translocation, such as *sapA*, and metabolism. In order to better understand the potential of these mutants to cause disease by escaping vaccine–induced and naturally–acquired immunity to *Salmonella*, we selected *S.* Typhimurium *sapA* and *yfgA* mutant derivatives for further investigation, including assaying their effects on virulence. Despite a similar impact on susceptibility to serum killing, inactivation of these genes had contrasting impact on virulence and susceptibility to non–specific cell envelope stress. Deletion of *sapA* resulted in a marked decrease in colonization of the colon of mice but had little impact on colonization of systemic sites. This mutation had little impact on tolerance to osmotic and detergent stress. The *sapA*$^{-}$ mutant had a similar LPS composition to the wild–type strain, but was hyper–invasive for epithelial cells, consistent with previous reports (36). In contrast, virulence was substantially diminished in the *yfgA*$^{-}$ mutant, demonstrated by the inability of the strain to establish infection in vivo. This was accompanied by diminished ability to invade epithelial cells, reduced membrane stress tolerance, and altered LPS composition.

The *sapA* gene is the first of the 5–cistron *sap* operon, which encodes ABC family proteins involved in peptide transport. *SapA* is hypothesized to confer resistance to antimicrobial peptides by shuttling them away from the membrane into the cell, where they are degraded by cytoplasmic proteases (37, 38). Its expression in response to antimicrobial peptides is under control of the *phoPQ* two–component system (39). A primary role of *yfgA* (*rodZ*) together with other bacterial cytoskeletal proteins including the actin homolog MreB is to confer cell morphology in rod–shaped bacteria (40, 41).

The lack of increased virulence in vivo or in vitro despite increased viability in serum in the *sapA* and *yfgA* mutants corroborates pleiotropic effects from studies of other mutants defective in genes encoding proteins associated with the cell envelope or synthesis of cell envelope components. Deletion of osmoregulated periplasmic glucan (OPG) synthesis genes, *mdoG* and *mdoH*, for instance, results in strains with a mucoid phenotype, resistant to endogenously expressed phage lytic activity, but renders the strains more susceptible to bile and osmotic stress, and diminished virulence in vivo (42–44). Insertion mutants in the *mdoG* and *mdoH* genes were also overrepresented in the output library following exposure to serum, and the *mdoG* mutant was significantly less susceptible to killing relative to wild–type SL1344.

Regarding virulence in vivo, *sap* operon mutants of Gram–negative bacteria are known to have diminished capacity for colonization in animal models of infection. In *H. ducreyi* for example, *sapBC* mutants are avirulent, while the *sapA* mutant is partially attenuated (45). The *H. influenzae sapA* mutant also has diminished ability to infect rabbits compared to the parent strain (46). In *S.* Typhimurium, attenuated transposon mutants with insertions mapped to the *sap* locus first identified the contribution of these genes to virulence in–vivo (47). To our knowledge, however, there is no prior description of the specific involvement of the *sapA* gene to in vivo survival in a defined null mutant of *S.* Typhimurium. Absence of *sapA* was associated with increased invasion of epithelial cells in culture, a phenotype also described in the *H. influenzae sapA* mutant (36).

Substantial reduction in the ability of the *yfgA* mutant to invade epithelial cells and/or survive osmotic and detergent stress may be an explanation for its inability to colonize mice. Besides altered morphogenesis, absence of other bacterial cytoskeletal proteins is associated with decreased SPI–1 T3SS protein expression but without loss of the ability to cause systemic infection (48). This may explain the loss of epithelial invasiveness in the *yfgA* mutant. Moreover, the reduction of very long O–antigen in this mutant might suggest a role for YfgA in the trimodal expression of LPS O–antigen on the bacterial surface. It is worth noting, therefore, that despite loss of this form of O–antigen, the *yfgA* mutant was still more serum resistant than its wild–type counterpart, especially in view of the defined role of very long O–antigen in conferring resistance to complement (19). While very long O–antigen may be redundant in the presence of long O–antigen chains during complement–mediated killing in *S.* Typhimurium, (49), its transient, inducible expression may confer a fitness advantage, to both complement killing and elements of non–specific immunity such as bile

(50, 51). Therefore, while long O–antigen may be sufficient for resistance to antibody and complement in the *yfgA* mutant, the role of very long O–antigen may be required for overall virulence or fitness.

In conclusion, using TraDIS technology, we have demonstrated that deletion of single genes from the genome of *S.* Typhimurium can confer increased resistance to in vitro killing by antibody. However, using *sapA* and *yfgA* deficient strains as examples, we have shown that their deletion does not necessarily translate to increased virulence in vivo. Hence, even as invasive *S.* Typhimurium isolates appear to be adapting to decreased gastrointestinal colonization in favour of systemic infection, emergence and establishment of highly antibody resistant strains may be countered by associated fitness costs.

ACKNOWLEDGEMENTS

We wish to thank Derek J. Pickard, Leanne Kane and Katherine Harcourt at the Wellcome Trust Sanger Institute for their kind assistance with the experiments.

Table 1. Significantly over–represented *S.* Typhimurium genes with transposon insertions detected by transposon–directed insertion site sequencing (TraDIS) following exposure of a *S.* Typhimurium SL3261 (SL1344 aroA$^-$) saturating transposon insertion library to immune Malawian adult serum for 180 minutes. All genes with a $\log_2$ read ratio >2 for output reads compared to input reads are included.

Systematic ID*	Gene name	No. of Reads		$\log_2$ Read Ratio	p value	Description
		Input	Output			
SL1344_1088	*mdoH*	1661	1825516	10.018	5.2E−22	glucans biosynthesis glucosyltransferase H
SL1344_1087	*mdoG*	1017	1092859	9.934	5.30E−22	glucans biosynthesis protein G
SL1344_4050	*metL*	2101	255244	6.858	6.02E−12	bifunctional aspartokinase II/homoserine dehydrogenase
SL1344_3345	*mreC*	139	14158	5.899	7.69E−11	rod shape–determining protein MreC
SL1344_0718	*sucA*	92	10103	5.732	1.63E−10	2−oxoglutarate dehydrogenase E1 component
SL1344_3433	*cap*	1123	54257	5.474	8.81E−09	septum formation protein Maf
SL1344_0124	*murF*	10	4709	5.450	9.98E−16	UDP−N−acetylmuramoylalanyl−D−glutamyl−2,6−diaminopimelate−D−alan alanyl ligase
SL1344_0628	*mrdA*	39	5599	5.358	1.24E−12	penicillin−binding protein 2
SL1344_1820	*zwf*	219	12961	5.356	2.86E−08	glucose 6−phosphate dehydrogenase
SL1344_4496	*deoB*	216	10604	5.082	5.07E−08	phosphopentomutase
SL1344_3954	*glnA*	678	23924	4.949	5.19E−07	glutamine synthetase
SL1344_3344	*mreD*	83	4583	4.678	6.92E−09	rod shape−determining protein
SL1344_1622	*sapA*	100	4811	4.618	1.79E−07	peptide transport periplasmic protein
SL1344_2486	*yfgA*	187	6936	4.616	7.08E−07	cytoskeleton protein RodZ
SL1344_0630	–	113	4968	4.572	8.55E−08	ribosome–associated protein
SL1344_4489	*osmY*	389	9390	4.279	1.69E−06	hyperosmotically inducible periplasmic protein
SL1344_0665	*nagA*	113	3794	4.192	7.88E−07	N−acetylglucosamine−6−phosphate deacetylase
SL1344_0629	*ybeA*	111	2881	3.820	1.45E−06	(pseudouridine1915−N3)−methyltransferase

SL1344_4081	*murB*	99	2347	3.620	1.55E−06	UDP−N−acetylenolpyruvoylglucosamine reductase
SL1344_3894	*cyaA*	5791	67552	3.522	4.62E−04	adenylate cyclase
SL1344_3376	*sapG*	270	3361	3.226	6.53E−04	trk system potassium uptake protein
SL1344_1474	–	125	1871	3.131	1.61E−71	putative multidrug efflux protein
SL1344_3939	*trkH*	1713	15780	3.131	6.45E−03	trk system potassium uptake protein TrkH
SL1344_1623	*sapB*	18	909	3.096	6.80E−70	dipeptide transport system permease protein
SL1344_2209	–	86	1198	2.803	5.59E−57	tail fiber assembly protein
SL1344_1684	–	385	3105	2.724	9.59E−54	putative regulatory protein
SL1344_3929	*tatC*	1223	8324	2.671	1.35E−51	sec−independent protein translocase protein
SL1344_4049	*metB*	1768	10496	2.504	4.40E−02	cystathionine gamma−synthase
SL1344_0448	–	131	1206	2.499	5.01E−45	hypothetical protein
SL1344_1442	*dcp*	372	2505	2.464	9.45E−44	dipeptidyl carboxypeptidase II
SL1344_0443	*clpX*	509	3227	2.450	3.23E−43	ATP−dependent clp protease ATP−binding subunit
SL1344_4011	*pfkA*	268	1910	2.449	3.64E−03	6−phosphofructokinase
SL1344_2007	*cbiE*	118	1041	2.388	1.34E−03	precorrin−6Y C5,15−methyltransferase
SL1344_1693	*narK*	160	1229	2.354	8.11E−40	nitrite extrusion protein; MFS transporter
SL1344_3682	*rfaY*	495	2892	2.330	5.29E−39	lipopolysaccharide core biosynthesis protein
SL1344_3280	–	147	1118	2.302	4.84E−38	hypothetical protein
SL1344_2377	*nupC*	204	1303	2.206	7.05E−35	nucleoside permease NupC
SL1344_3927	*tatA*	408	2225	2.194	1.73E−34	sec−independent protein translocase protein
SL1344_3726	*slsA*	1288	6222	2.187	2.89E−34	hypothetical protein
SL1344_0091	*ksgA*	187	1158	2.132	1.63E−32	dimethyladenosine transferase (adenine1518−N6/adenine1519−N6)−dimethyltransferase
SL1344_3928	*tatB*	1013	4552	2.063	2.06E−30	sec−independent protein translocase protein
SL1344_0678	*ybfF*	117	793	2.041	9.67E−30	putative esterase/lipase
SL1344_1624	*sapC*	47	501	2.032	1.84E−29	peptide transport system permease
SL1344_4424	–	4353	18008	2.024	3.12E−29	type II restriction enzyme
SL1344_1236	–	49	502	2.014	5.87E−29	putative MutT family protein
SL1344_1626	*sapF*	32	430	2.005	1.08E−28	peptide transport system ATP−binding protein

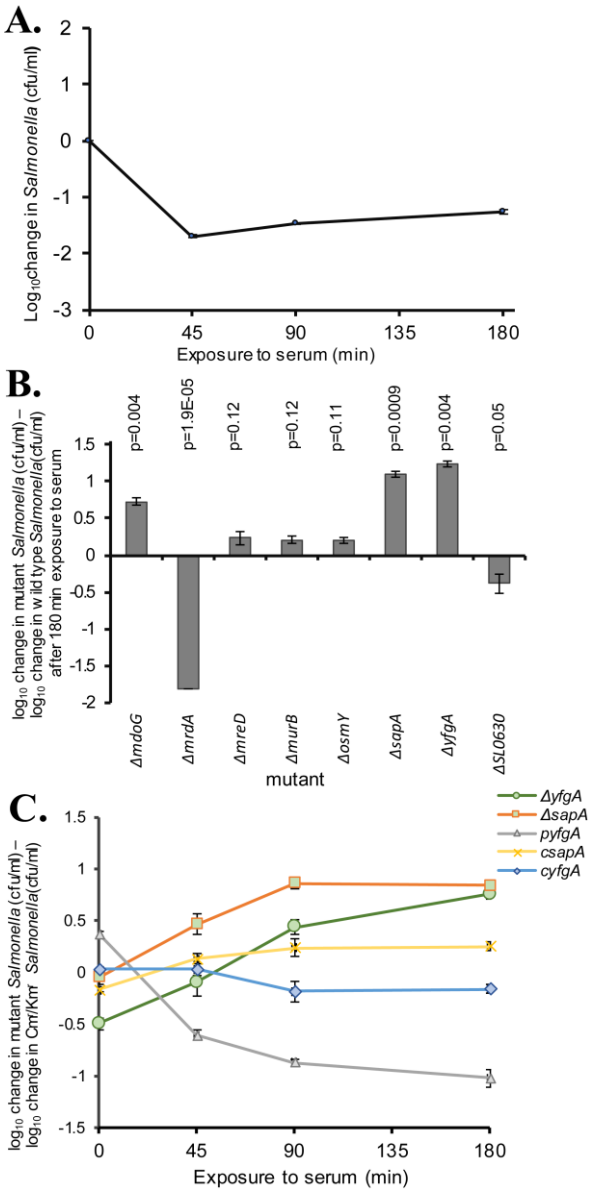

Fig. 1. *S.* Typhimurium SL1344 mutants with single gene deletions exhibiting enhanced resistance to antibody–dependent complement–mediated killing. All data are from serum bactericidal assays with exposure to immune Malawian adult serum at 37°C for 180 minutes. **A**. Killing of a S. Typhimurium SL3261 (SL1344 aroA$^-$) saturating transposon insertion library with starting concentration 10^8cfu/ml. Negative values indicate killing. Significantly over–represented S. Typhimurium genes with transposon insertions in the output pool compared with the input pool are shown in Table 1. **B**. Resistance to killing of eight mutant S. Typhimurium SL1344 strains with definitive single gene deletions selected from Table 1. Bars represent mean $\log_{10}$ change in mutant *Salmonella* less $\log_{10}$ change in wild type S. Typhimurium SL1344 after 180 min exposure to serum. Positive values indicate resistance to killing. Starting bacterial concentrations were 10^6cfu/ml. All means are from three single independent experiments. Error bars: SEM. **C**. Resistance to killing of S. Typhimurium SL1344 *sapA*$^-$ and *yfgA*$^-$ mutants and corresponding complemented strains in a mixed inoculum serum bactericidal assays with a S. Typhimurium SL1344 strain with intact *yfgA* and *sapA* genes and either Cm^r or Km^r antibiotic–resistance marker inserted at the *phoN* locus. Strain designations: 'Δ*sapA*': SL1344Δ*sapA*::*aph*; 'Δ*yfgA*': SL1344Δ*yfgA*::*aph*; 'pyfgA': SL1344ΔyfgA::*aph* pWKS30::*yfgA*; 'csapA' & 'cyfgA': cis–complemented strains from Δ*sapA* and Δ*yfgA* mutants (SL1344::*cat*). Strains were mixed at a 1:1 ratio with starting bacterial concentrations of 10^6cfu/ml and exposed to immune serum for 180 minutes. Positive values indicate resistance to killing. All means are from three single independent experiments. Error bars: SEM.

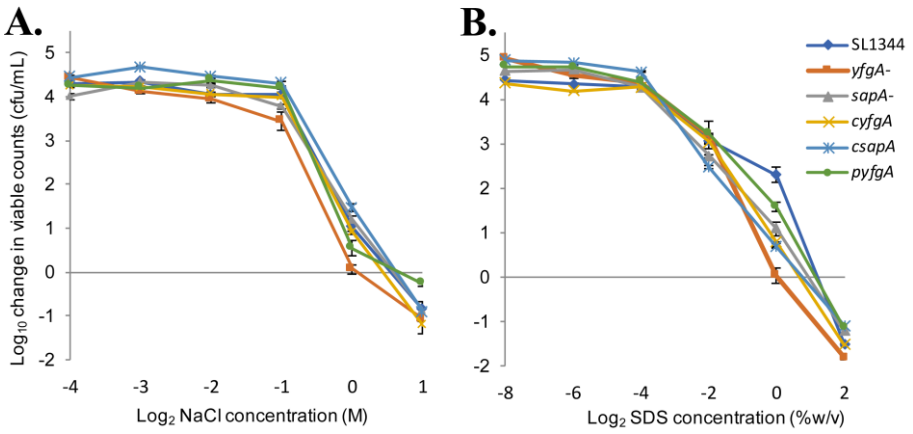

Fig. 2. Susceptibility of *S.* Typhimurium SL1344 *yfgA* and *sapA* mutants to osmotic and detergent stress. Log_{10} change in viable bacterial counts of *S.* Typhimurium SL1344 *yfgA*$^-$ and *sapA*$^-$ definitive mutants, complemented strains and wild type bacteria following incubation in LB broth containing varying concentrations of either **A.** NaCl, or **B.** SDS, at 37°C for eight hours. Growth of the *yfgA*$^-$ mutant, but not the *sapA*$^-$ mutant, was significantly reduced compared to wild type *S.* Typhimurium (p=0.03 in NaCl and 0.004 in SDS). The concentrations of NaCl and SDS needed to prevent net growth of *S.* Typhimurium in LB broth were significantly lower for the *yfgA*$^-$ mutant, but not the *sapA*$^-$ mutant, compared with wild type (1.02 and 1.55 M NaCl, p=0.04; 1.1% and 2.8%, p=0.005). Starting bacterial concentrations were 2.5 x 10^3 cfu/ml. Strain designations: *sapA*$^-$: SL1344Δ*sapA*::*aph*; *yfgA*$^-$: SL1344Δ*yfgA*::*aph*; pyfgA: SL1344Δ*yfgA*::*aph* pWKS30::*yfgA*; c*sapA* & c*yfgA* cis–complemented strains from Δ*sapA* and Δ*yfgA* mutants (SL1344::*cat*). Data represent means of two independent experiments. Error bars: SEM.

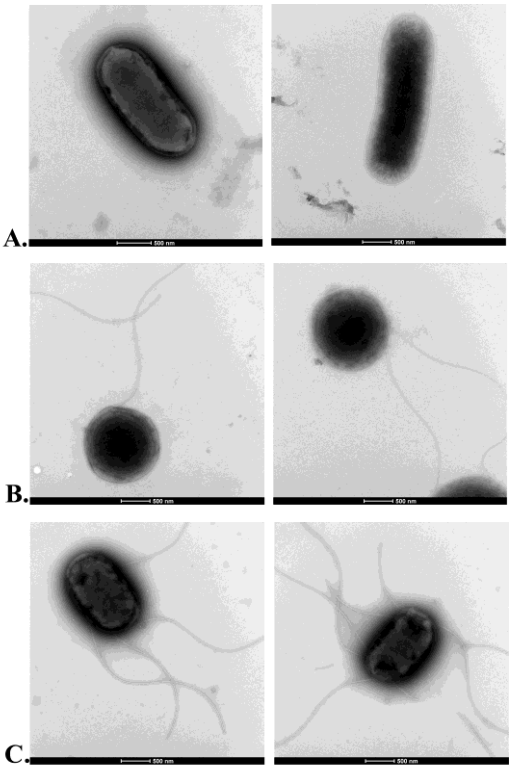

Fig. 3. Morphological change in *S.* Typhimurium SL1344 following deletion of *yfgA*. Representative transmission electron micrographs of negative–stained *S.* Typhimurium SL1344 strains: **A.** wild–type, **B.** *yfgA* mutant demonstrating loss of rod cell shape, and **C.** complemented *yfgA* mutant, p*yfgA* (SL1344 Δ*yfgA*–pWKS30::*yfgA*).
Scale bar: 500nm.

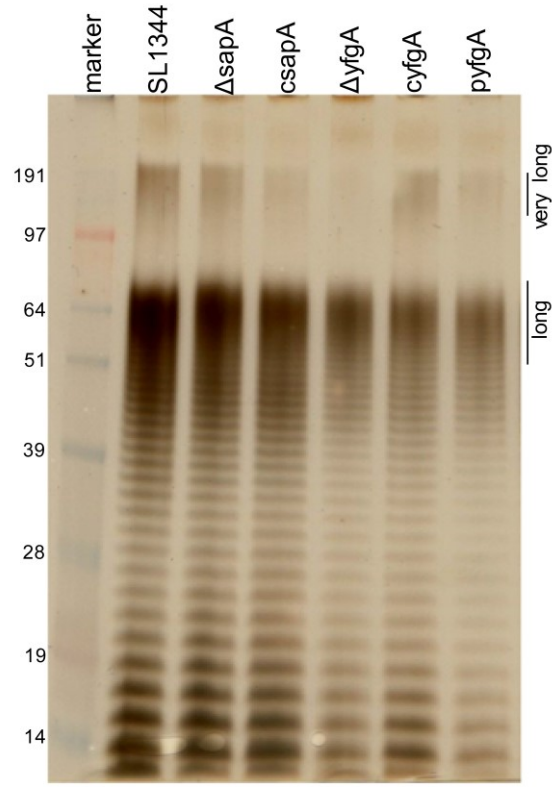

Fig. 4. Lipopolysaccharide content of *S.* Typhimurium SL1344 wild type, *sapA* and *yfgA* mutants, and complemented strains Lipopolysaccharide was extracted from overnight cultures containing equivalent numbers of bacteria from each strain using hot phenol, separated by PAGE on a 12% Bis–Tris gel and visualized by silver staining. Long and very long populations are indicated. Strain designations: Δ*sap*A: SL1344Δ*sapA*::*aph*; Δ*yfg*A: SL1344Δ*yfgA*::*aph*; pyfgA: SL1344Δ*yfgA*::*aph* pWKS30::*yfg*A; c*sap*A & c*yfg*A: cis–complemented strains from Δ*sap*A and Δ*yfg*A mutants (SL1344::*cat*). Molecular weights of the standard in kDa are indicated.

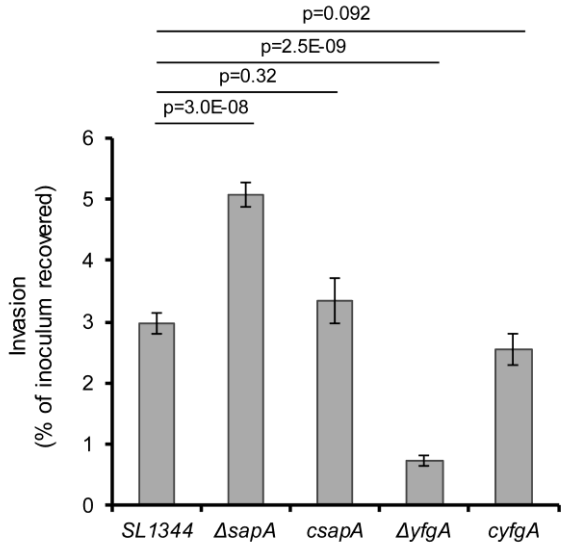

Fig. 5. Invasion of enterocytes by *S.* Typhimurium SL1344 wild type, *sapA* and *yfgA* mutants, and complemented strains Means and standard errors of viable internalized bacteria as percentage of the starting inoculum from 6 wells per strain recovered after co–incubation with 10^5/well colon–derived T84 cells for 2 hours at a MOI of 10. Two independent experiments were performed in triplicate. P–values are from t–test comparisons of means of the 4 derived strains to SL1344.

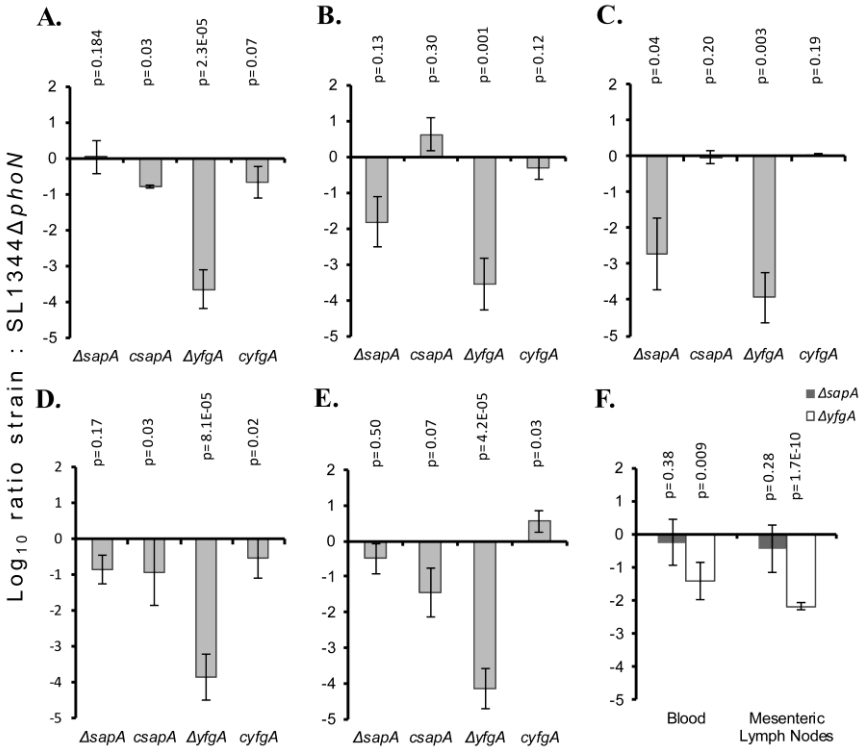

Fig. 6. Mixed mouse in vivo infections with *S.* Typhimurium SL1344 wild–type, *yfgA* and *sapA* mutants, and complemented strains. Mice were infected by the oral route with 5×10^8 cfu of a 1:1 mixture of each mutant or complemented mutant strain and an isogenic *phoN* mutant of *S.* Typhimurium SL1344 carrying an antibiotic resistance marker. Bars represent $\log_{10}$ ratios of viable cfu (per gram of organ or ml of blood) recovered from **A.** ileum, **B.** cecum, **C.** colon, **D.** liver, and **E.** spleen after 5 days. Viable bacterial counts from **F.** blood and mesenteric lymph nodes were determined from a separate experiment, where the complemented strains were not tested. P values are from t–test comparisons of each strain against SL1344Δ*phoN*. Error bars: SEM

Table S1. Primers used in this study

Oligo name	Sequence 5'–3'
*murB*_KO_forw	CCAACACAGATACGGTAAACTATTGCCGATTGAGTATCAGGAAAGCAGCCGTGTAGGCTGGAGCTGCTT
*murB*_KO_rev	CCGTCGGCAAGTAGTGAGATCAGCGTCAGGGGAACGGTAGTATCTTTCATCATATGAATATCCTCCTTA
*murB*_ext_forw	TAACCACAAGCCTTCCCAAC
*murB*_ext_rev	GCTCGCCTGAGTGAAACTCT
*osmY*_KO_forw	TTGCCTGAGCTCAAATTACGAGCAAACTATACAGGACAAAATCGATGACTGTGTAGGCTGGAGCTGCTT
*osmY*_KO_rev	GGTGCACATTACGCCTCCCGACAAACGTCGGGAGGACGAATTACGACGAACATATGAATATCCTCCTTA
*osmY*_ext_forw	TGCCTGAGCTCAAATTACGA
*osmY*_ext_rev	GGAGGACGAATTACGACGAA
*mreD*_KO_forw	CATCGCCTTCGGGCGCGACCACGCCGCCTGCGCGTGCGCCGGGAGGGTAAGTGTAGGCTGGAGCTGCTT
mreD_KO_rev	CTGGCGACGCGGGGAACCGGAAGCAAGATACAGAGTTGTCATATCGACCTCATATGAATATCCTCCTTA
*mreD*_ext_forw	GGGATTACTCAGCCATCTGC
*mreD*_ext_rev	GGAACCGGAAGCAAGATACA
*mrdA*_KO_forw	ACCCTTATCACCGTGAGTGATCGTGAAGTCTTGAGAAGATTAAGCAGCGGGTGTAGGCTGGAGCTGCTT
*mrdA*_KO_rev	GATATGAATTTTATCCCAGAAGGTTTTTTTGTTCGGATTATCCGTCATGACATATGAATATCCTCCTTA
*mrdA*_ext_forw	CACCGTGAGTGATCGTGAAG
*mrdA*_ext_rev	CAGTAACGCCAGCAGAATGA
*SL1344_0630*_KO_forw	TTGGCCTTTTTTCTATCGTTATAACGCAATTATTCACCCAGGGGGAAAACGTGTAGGCTGGAGCTGCTT
*SL1344_0630*_KO_rev	ACCCAGTCGGGCATCTTCGTGCCGACAGCGACAAGTTGCAGCTTCACGCACATATGAATATCCTCCTTA
*SL1344_0630*_ext_forw	AACGCAATTATTCACCCAGG
*SL1344_0630*_ext_rev	CGGAAAACGACGCAGATATT
*yfgA*_KO_forw	GGCTCGTCGGCGCCTGAATCCTAATTTACACGTACCTGTAGCTGTAGCGAGTGTAGGCTGGAGCTGCTT
*yfgA*_KO_rev	TTGAATCGGAGCCTGGTTATGCATGAAAAACCTCCCGCGTTACCCGTCTGCATATGAATATCCTCCTTA
*yfgA*_ext_forw	CTAACCTGATGTTTCGCGGT
*yfgA*_ext_rev	GTTGAATCGGAGCCTGGTTA

*sapA*_KO_forw	GGCCGAAGTGCGATACACTTTGCAAATTGAACTTCAAAAACTTAACTATTGTGTAGGCTGGAGCTGCTT
*sapA*_KO_rev	GAAGAAGAGCGTCACCAGCAACAGCAATAACCGACGCAGGGTGAAGATAACATATGAATATCCTCCTTA
*sapA*_ext_forw	GCCAGTTATCCACCGACATT
*sapA*_ext_rev	AGGAAGAAGAGCGTCACCAG
*mdoG*_KO_forw	TAAGCACACAAAGGGGGAAGTGCTTACTTATTATGAAACATAAACGACAAGTGTAGGCTGGAGCTGCTT
*mdoG*_KO_rev	TCACGTTCAGAAAGCAGCAATGCGTCAATATACTCAGTTGTTTTATTCATCATATGAATATCCTCCTTA
*mdoG*_ext_forw	TGGATCGGATCGATATAAGCA
*mdoG*_ext_rev	CTCACGTTCAGAAAGCAGCA
*sapA*compl_F	TTATCTTCACCCTGCGTCGGTTATTGCTGTTGCTGGGTGTAGGCTGGAGCTGCTTC
*sapA*compl_R	AGGCTAAAGCCGATAAAGGTCAGGAAGAAGAGCGTCATGGGAATTAGCCATGGTCC
*yfgA*compl_F	CAAGTACAACGCCAGACATTATTCAACTGACATTGCGTGTAGGCTGGAGCTGCTTC
*yfgA*compl_R	TAGCAGTTGCGCCACGGGGCATTCATTATACCGTGGATGGGAATTAGCCATGGTCC
*sapAv*F	TATCGATCCTTTTCAACGCC
*sapAv*R	GAAATCCAGGCGTCAATGAT
*yfgAv*F	GGGGCATTCATTATACCGTG
*yfgAv*R	GATAAGCTTGAACGCTTCGG
yfgA_trans_forw	GGGGCATTCATTATACCGTG
yfgA_trans_rev	GATAAGCTTGAACGCTTCGG

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CHAPTER 4

Rapid transcriptional responses to serum exposure are associated with sensitivity and resistance to antibody–mediated complement killing in invasive *Salmonella* Typhimurium ST313

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Abstract

Background: *Salmonella* Typhimurium ST313 exhibits signatures of adaptation to invasive human infection, including higher resistance to humoral immune responses than gastrointestinal isolates. Full resistance to antibody–mediated complement killing (serum resistance) among nontyphoidal *Salmonellae* is uncommon, but selection of highly resistant strains could compromise vaccine–induced antibody immunity. Here, we address the hypothesis that serum resistance is due to a distinct genotype or transcriptome response in S. Typhimurium ST313.

Methods: Six *S.* Typhimurium ST313 bloodstream isolates, three of which were antibody resistant, were studied. Genomic content (single nucleotide polymorphisms and larger chromosomal modifications) of the strains was determined by Illumina and PACBIO sequencing, and functionally characterized using RNA–seq, transposon directed insertion site sequencing (TraDIS), targeted gene deletion and transfer of selected point mutations in an attempt to identify features associated with serum resistance.

Results: Sequence polymorphisms in genes from strains with atypical serum susceptibility when transferred from strains that were highly resistant or susceptible to a strain that exhibited intermediate susceptibility did not significantly alter serum killing phenotype. No larger chromosomal modifications typified serum resistance or susceptibility. Genes required for resistance to serum identified by TraDIS and RNA–seq included those involved in exopolysaccharide synthesis, iron scavenging and metabolism. Most of the down–regulated genes were those associated with membrane proteins. Resistant and susceptible strains had distinct transcriptional responses to serum, particularly related to genes responsible for polysaccharide biosynthesis. There was higher upregulation of *wca* locus genes, involved in the biosynthesis of colanic acid exopolysaccharide, in susceptible strains and increased expression of *fepE*, a regulator of very long–chain lipopolysaccharide in resistant strains.

Conclusion: Clinical isolates of *S.* Typhimurium ST313 exhibit distinct antibody susceptibility phenotypes that may be associated with differences in gene expression on exposure to serum.

Introduction

Invasive nontyphoidal *Salmonella* (iNTS) infections are estimated to cause ~3.4 million illnesses and over 680,000 deaths annually (1), with a majority of these cases occurring in sub–Saharan Africa (1, 2). Predisposing factors include co–morbidities such as malaria (3–6), HIV co–infection (7–9), young age (10, 11), and malnutrition (12–14). A clonal genotype of *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) of sequence type 313 (ST313) (15), dominates iNTS in sub–Saharan Africa and this clone frequently encodes multiple drug resistance (16–21). These factors likely contribute to the relatively high prevalence of invasive disease in the region compared to that observed with nontyphoidal *Salmonella* in other parts of the world, where gastroenteritis dominates.

A prominent signature in the genomes *S.* Typhimurium ST313 isolates is genetic degradation, associated with gene deletion and the accumulation of pseudogenes, resembling that observed in host–restricted *Salmonella* serovars such as *S.* Typhi, the causative agent of typhoid fever (15). Nevertheless, the ST313 found in Africa are monophyletic, falling into two highly conserved clades known as clades I and II (15). Phenotypically, ST313 isolates of both clades broadly exhibit decreased enteropathogenicity (16, 22), hyper–dissemination from the intestine (23), hyper–resistance to phagocytic killing (24) and complement (20, 25), and altered multicellular behaviour (17).

The predilection for invasive disease exhibited by ST313 underscores an important role for an effective humoral response to NTS during extra–intestinal infection (11, 26). Antibodies mediate opsonisation and killing by phagocytic cells (26), and direct bacterial killing by complement during inter–macrophage spread (11). Vaccines eliciting these responses, therefore, are a potentially promising means of protecting individuals from developing iNTS disease.

Multiple, and possibly redundant mechanisms for evading antibody responses have been described for *Salmonella*. These include survival in phagocytes associated with virulence factors such as *Salmonella* Pathognicity Island (SPI–2) (27), and the production of proteins that actively degrade or inhibit complement proteins such as PgtE (28, 29), Rck (30), PagC (31). and TraT (32). Recently, a SNP was identified in the promoter of *pgtE* resulting in high expression of the PgtE virulence factor in Lineage II African ST313 *S.* Typhimurium. PgtE increases degradation of factor B component of human complement, likely contributing to serum resistance of the ST313 pathovar of *S.* Typhimurium (20).

Lipopolysaccharide (LPS) O–antigen length and composition can also play a key role in bacterial survival during bloodstream infection, by shielding bacteria against direct complement attack and killing by blood phagocytes (33–35). Subunit vaccines that can induce antibodies capable of directing complement–mediated killing are under development for the prevention of iNTS disease in sub–Saharan Africa (36–40). The presence of variants that evade antibody–mediated killing conferred by a vaccine could undermine this strategy. Here we address the hypothesis that variation in genotype or transcriptional response to serum of clinical isolates of iNTS correlates with serum susceptibility.

Materials and Methods

Bacteria and culture conditions. Six *S.* Typhimurium from a series of 329 iNTS isolates from bacteraemic children admitted to Queen Elizabeth Central Hospital, Blantyre, Malawi with defined susceptibility to complement–mediated bactericidal activity were used in this study. Bacteria were routinely maintained in normal (10 g/l) or low salt (5 g/l) Luria–Bertani broth and grown aerobically at 37°C. Where appropriate, derivative mutant strains were grown in LB media supplemented with 100 μg/ml of hygromycin, or 50 μg/ml of kanamycin.

Investigation of the stability of the serum susceptibility phenotype. Suspensions of two isolates, D23005 (serum resistant) and D24545 (highly serum sensitive) were spread on Luria–Bertani (LB) agar plates at a density of approximately 500 cfu and grown overnight. 100 colonies of each isolate were then grown to log phase in LB broth and exposed to serum for three hours. Serum susceptibility phenotype was then determined using serum bactericidal assays (described below).

Identification of single nucleotide polymorphisms. Sequence read alignment and detection of single nucleotide polymorphisms (SNP's) were performed using paired–end Illumina sequence data mapped to the reference genomes *S.* Typhimurium D23580 and SL1344 with SMALT (http://sourceforge.net/projects/smalt/files/smalt_manual.pdf). SNPs were identified using samtools mpileup and filtered with a minimum mapping quality of 30 and quality ratio cut–off of 0.75.

Transfer of point mutations by transduction. Nonsynonymous polymorphisms were transferred into *S.* Typhimurium D23580 by co–transduction with a selectable marker. A kanamycin resistance cassette was inserted into intergenic regions within 1kb of each selected point mutation in the donor strains by homologous recombination as described previously (41), using primers designed to avoid disrupting gene

promoters (Table S1). Phage P22 lysates of the resulting derivative strains were then used to infect D23580, using the kanamycin marker to select for transductants. Colonies were screened for the transfer of the desired allele using specific PCR oligonucleotide primers (Table S1) and confirmed by sequencing.

Serum bactericidal assays. Normal human serum was obtained from the blood of a healthy adult donor. Clotting was performed at room temperature within two hours of the blood draw. Serum was then separated from the clotted fraction by centrifugation and stored in aliquots at –80°C until use. Test *S.* Typhimurium strains were grown to mid–log phase by inoculating 100–fold dilutions of overnight cultures into fresh LB broth and grown aerobically for 135 minutes at 37°C. The bacteria were then washed twice with phosphate–buffered saline (PBS), and approximately 1×10^6 cfu/ml were incubated with serum for 3 hours at 37°C. Serum susceptibility was determined by the change in numbers of viable bacteria after 3 hours of serum exposure, using serial dilutions of the serum–bacteria mixtures plated on LB agar.

Transposon mutant library construction and screening. A derivative of the transposon EZ–Tn5 (Epicentre) was phosphorylated with polynucleotide kinase (New England BioLabs Inc.), and incubated with EZ–Tn5 transposases (Epicentre) at 37°C for 1 hour to prepare the transposome, which was stored at –20°C until ready for use. D23580 electrocompetent cells were mixed with 0.5 µL of transposomes and transformed by electroporation in a 0.2 cm cuvette (Bio–Rad Laboratories) using parameters (2.5 kV, 25 F and 200 Ω) in Gene Pulser Xcell Electroporation System (Bio–Rad Laboratories). Cells were immediately re–suspended in 1 mL of SOC medium (Invitrogen) and incubated at 37°C for 2 hr before being spread on LB agar supplemented with kanamycin (10 µg/mL). The D23580 library consisted of 6×10^5 mutants. Stationary phase cultures were then prepared by 1/200 dilution of mutant library in LB broth, vortexed briefly, and incubated aerobically at 37°C overnight with shaking. Log phase cultures (input library) were then prepared by 1/100 dilution of the overnight culture in LB broth and 2.5 hours of incubation at 37°C (without venting), on a rocker plate at 20 rev/sec. Viable counts of the input library were determined by serial dilution in PBS and plating of a sample of the neat input culture. An aliquot of the input library was harvested by centrifugation at 6000 rpm for 5 minutes. The supernatant was decanted, and the pellet washed three times in PBS (pH 7.4), then resuspended in PBS to a final concentration of 1×10^8/ml bacteria. Serum bactericidal assays were performed as described above but using pooled serum from 10 healthy Malawian adults. After 180 minutes, the entire serum–bacteria

suspension was transferred to 25 ml LB broth, vortexed and incubated overnight aerobically 37°C, to enable the surviving library to outgrow killed components. Viable counts were determined from the output library, and the remaining output culture pelleted at 6000 rpm for 20 minutes at 4°C, the supernatant removed, and frozen at –80°C until ready for use.

Transposon directed insertion site sequencing (TraDIS). Genomic DNA (gDNA) of the initial (input) and selected (output) mutant pools was extracted using Qiagen Genomic–tip 100/G (Qiagen, Hiden, Germany). Approximately 2µg of gDNA was fragmented to insert size of ~300 bp using covaris. The fragmented DNA was end repaired, A–tailed and adapter–ligated using Illumina DNA fragment library preparation kit (New England BioLabs Inc.) according to the manufacturer's instructions. The libraries were then enriched by 10–20 PCR cycles using a transposon–specific forward primer and a reverse primer that included Illumina flow cell binding sites (42). The enriched libraries were purified using the Agen court AMPure XP beads (Beckman Coulter) and quantified on an Agilent DNA1000 chip (Agilent Technologies) as per the manufacturer's instructions. The libraries were sequenced on a HiSeq2000 Illumina platform as single–end reads of 100bp. The reads were processed using the Bio::TraDIS toolkit (43). Briefly, reads were filtered for 10 bases matching the expected transposon tag sequence. Filtered reads were then mapped against D23580 reference genome (GenBank accession number FN424405) using SMALT version 0.7.2, and transposon insertion sites and insertion indices (obtained by dividing the number of unique insertion sites by the gene length) were determined for every gene. Genes with read counts of less than 20 in all conditions were excluded from the analysis. Log_2 fold changes and significance of changes in mutant abundance before (input; the basic mutant pool) and after selection (output; biological replicates of mutant pool screened in the presence of bactericidal serum) were calculated using edgeR after normalization using TMM method (44).

Targeted gene deletion in D23580. Deletion of genes in *S.* Typhimurium D23580 was performed by recombinase–mediated allelic exchange as described previously (41). PCR fragments were made by amplifying the kanamycin resistance gene from plasmid pKD4, with each primer consisting of 50–mer homology arms flanking each targeted gene such that the entire open reading frame was deleted, and 20 nucleotides priming pKD4 at the 3'ends (primers in Table S1). A second round of PCR using gel–purified products from the first reactions as templates was performed to eliminate pKD4 from the material used for electroporation. Recombinase–proficient D23580 bacteria harbouring the pSIM18 helper plasmid

were transformed using approximately 1 μg of PCR product. Transformants were then selected on LB plates with kanamycin, and the gene replacement verified by screening colonies using PCR from extracted genomic DNA and primers specified in Table S1. Culture of transformants at 42°C (non–permissive temperature for pSIM18 replication), and isolation of kanamycin resistant and hygromycin sensitive colonies by replica plating was used to cure and verify loss of pSIM18.

PacBio sequencing and assembly. To prepare high molecular weight genomic DNA, log–phase cultures of each strain were prepared in 10 ml of LB broth as described above. Each culture was then centrifuged and the pellets resuspended in 2 ml of 25% (w/v) sucrose in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). 5 mg of lysozyme (Roche) was then added to each suspension, and incubated for 45 minutes at 37°C. 2 mg of proteinase K (Roche), 300 μg of RNase A (Roche), EDTA (pH 8.0) at a final concentration of 0.02 M, and 250 μl of 10% Sarkosyl NL30 (BDH/Fisher) were added to the suspensions, then left on ice for 2 hours. Lysates were placed on a 50°C block overnight, then reconstituted to 5 ml using TE buffer. Samples were mixed with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) in 15 ml phase–lock gel tubes (Eppendorf), centrifuged for 15 minutes at 4000rpm, and the aqueous phases transferred to a fresh tube, and repeated three times. The aqueous phase was mixed with 2.5 volumes of 100% ethanol and incubated at –20°C for 1 hour to precipitate the DNA. The DNA was separated from the ethanol by centrifugation, washed with 70% ethanol, resuspended in TE buffer, and stored at –80°C until use.

PacBio sequencing was performed on a PacBio RS sequencer. Sequence reads were assembled using HGAP v3 (45) of the SMRT–Analysis software v2.3.0 (46). Sequence coverage for picking the minimum fragment length for assembly was set to 30, and the approximate genome size was set to 3 Mbp. The assembly was circularized using the pre–assembled reads in Circlator v1.1.3 (47). The circularized assembly was then improved using the PacBio RS_Resequencing protocol and Quiver v1 of the SMRT analysis software v2.3.0 (46).

RNA sequence analysis. Log–phase (OD_{600} 0.2) cultures of each strain were prepared in 10 ml of LB broth as described previously. Bacteria were resuspended to an OD_{600} of 2.0. 100 μl of this suspension was then inoculated into 900 μl of LB and 900 μl of normal human serum (to a final concentration of approximately10^8 cfu/ml) in 2ml microcentrifuge tubes. The tubes were incubated in a horizontal position with shaking at 60rpm at 37°C for 10 minutes, after which the bacteria were pelleted by

centrifuging at 13000 rpm for 2 minutes, and the media/serum discarded. The pellets were resuspended in 1ml of ice–cold PBS. RNA was immediately stabilized as described previously (48), by adding 400µl (20%v/v) of a cold mixture of 100% ethanol and phenol saturated with 0.1 M citrate buffer at pH 4.3 (Sigma) (19:1 v/v) to each sample and incubating on ice for 30 minutes. Extractions were performed using the FastRNA Pro Blue Kit (MP Biomedicals) according to the manufacturer's protocol. Genomic DNA was removed from the samples using Turbo DNase (Ambion) with 4% v/v of RNAsecure RNase Inactivation Reagent (Ambion) added to each sample during DNase treatment to prevent RNA degradation. RNA was then purified using phenol–chloroform, with final elution in DEPC–treated water. Quality and concentration were checked by using a 2% agarose gel, and the Agilent 2100 Bioanalyzer RNA Nano protocol. Samples were then stored at –80°C until use. Extractions were performed in three biological replicates. RNA–seq was performed using an Illumina HiSeq 2000 sequencer, yielding 9–23 million reads per library from 100 cycles of paired–end sequencing (Sequencing and mapping statistics are in Table S2). Sequence reads from each sample were mapped against the S. Typhimurium D23580 genome (Genbank accession number: FN424405). The reference was indexed, and the reads aligned using default parameters, with read trimming quality threshold set to 15 (q=15) and maximum insert size adjusted per library as the maximum requested fragment size of the sequencing library, implemented using BWA v0.7.12 (49). Expression values were computed from the read alignments to the coding sequence to generate the number of reads mapping (50, 51), including only reads with a mapping quality score of 10. Output spreadsheet files with features previously described (52) were obtained for each sample. Total read counts for each gene in every sample were used, with zero values in the data thresholded to 1. Principal Component Analysis (PCA) plots of normalized counts were used to identify samples that did not cluster with their replicates, and one sample (run accession ERR731338) was excluded from subsequent analysis. Data normalization, filtering, and calculation of fold changes were performed using DESeq2 (53), and heatmaps and cluster dendrograms created using the *heatmap.2* function of the *gplots* package, all implemented in R v.3.1.3.

Sequencing data accession number. The RNA–seq and PacBio data generated in this study were submitted to the European Nucleotide Archive, available under study accession number ERP005455.

Ethical approval. Ethical approval was from the College of Medicine Research and Ethics Committee, University of Malawi. Peripheral blood samples were obtained following consent from each donor.

Results

***S.* Typhimurium isolates exhibit a stably inherited serum susceptibility phenotype.** We have noted that even phylogenetically highly related ST313, that vary by only a few SNPs, can exhibit reproducible differences in their resistance to serum killing. Thus, in order to investigate this further we selected five isolates of lineage II (D23580, D24545, D23005, D25352, D24871) and two (A130 (a reference isolate), D26104) of lineage I with different serum susceptibilities for detailed genotypic or phenotypic analysis. Two of these isolates, D23580 and D24871, were moderately susceptible to killing in normal serum., one, D24545 was highly susceptible, while the other three had net growth after three hours of incubation in the same immune serum (Figure 1 A and B). To investigate if serum susceptibility was a stably inherited phenotype during culture, we performed serum killing assays on 100 independent colonies each of a serum resistant (D23005) and susceptible (D24545) isolate. No obvious differences in phenotype for either isolate was observed (Figure S1), therefore high frequency changes were considered unlikely to account for the observed variation in serum sensitivity.

Complete genome analysis of the iNTS isolates with different serum susceptibility. The genetic basis for variation in serum susceptibility between ST313 isolates is unknown. Therefore, to facilitate detailed genetic comparisons, we determined the complete genome sequence of each isolate using a combination of Illumina–generated short reads and PacBio long–read sequences (54). No major alterations in chromosome arrangement were apparent in any isolate using D23580 as the reference (Figs S2A and 2B). For example, there were no large chromosomal inversions and no major deletions involving multiple or single genes. The *traT* and *rck* genes, two known serum resistance genes, were both intact and had 100% sequence identity in all the six isolates. Similarly, no difference, other than single SNPs were found in the 88kb p14–95A plasmid between D23580 and other isolates was observed (Figure S2C). Differences between ST313 clade I and isolates from clade II reflected lineage–specific genome evolution reported previously (55) (Figure 1) (Figure S2). We therefore concluded that gene flux and major genome rearrangements do not obviously correlate with serum susceptibility phenotype.

To investigate whether nucleotide substitutions (SNPs) or small indels impact the degree of susceptibility to serum killing, SNPs in gene coding and promoter regions were analysed in five lineage II isolates with D23580 as the reference (Figure 1B). A total of 52 SNPs were identified (Table S3),

confirming the high genetic similarity. Thirteen of these SNPs resulted in predicted nonsynonymous amino acid substitutions that were unique to either the highly sensitive (D24545) or one of two resistant isolates (D25352 and D23005) (Table 1).

We tested whether these individual nonsynonymous SNPs accounted for the altered serum susceptibility phenotype by introducing them into D23580. Three of the SNPs (in *acrB*, *uxuR*, and *pepP*) were specific to D24545 that was highly susceptible to serum killing and three were present in resistant isolates D25352 or D23005 (*uxuB*, *ispD*, and *STMMW_29841*). All of the recombinant D23580 derivatives exhibited similar sensitivity to the wild type D23580 at all sampled time points, indicating that these polymorphisms had no detectable impact on serum susceptibility (Figure 2).

For functional analysis of the fifty–two SNPs occurring in clade II isolates, we compared RNA–seq and TraDIS data corresponding to the regions where these SNPs occur. No significant changes, either in the viability of the corresponding transposon mutants or on the relative levels of transcription were observed (Table S3). D26104, a lineage I isolate, had significantly diminished expression of *pgtE* when compared to reference isolate D23580, consistent with lacking the promoter mutation known to increase serum resistance in lineage II isolates (20) (Table S8). Despite this, D26104 is a highly serum resistant isolate, suggesting an alternative mechanism of resistance in this isolate that is unlikely to be associated with the *pgtE* gene.

Identification of genes required for full serum resistance of *S.* Typhimurium D23580. By a complementary approach, we identified genes required for serum resistant ST313 using a whole genome functional screen with a saturating Tn5 transposon insertion library in D23580. The number of Tn5 transposon insertion mutants in each gene before and after exposure to serum for 180 minutes was determined by transposon directed insertion site sequencing (TraDIS). We classified genes as 'serum resistance determinants' if their disruption resulted in decreased viability in serum and therefore under–representation at the final timepoint. Under–representation was determined by a reduction in mapped read counts (relative to the library prior to serum exposure) of at least 2–fold and with an adjusted p–value of <0.05 or less. Similarly, we defined genes as 'serum susceptibility determinants' if their disruption resulted in increased viability in serum and therefore over–representation at the final timepoint. We identified 555 serum resistance determinants and 82 serum susceptibility determinants (Complete results in Table S4).

To validate the transposon insertion library screen, we constructed defined mutations in D23580 by independently replacing twenty of the serum–resistance determinants with the *aph* gene conferring kanamycin resistance, by allelic exchange. The deleted genes were from a broad range of functional categories. Nine of the mutants tested (*entD*, *osmC*, *uspF*, *wzzB*, *arnD*, *pmrD*, *wecA*, *cpxP* and *yjbE*) had significantly increased susceptibility to serum compared to wild–type D23580 (Figure 3). The inherent differences in the assays (for example competitive vs single populations) and serum sources used may partly explain this.

Gene ontology (GO) terms associated with proteins encoded by serum–resistance determinants (Table S5) identified significant enrichment of membrane components, particularly genes responsible for membrane and extracellular polysaccharides such as lipopolysaccharide (LPS) and enterobacterial common antigen (ECA) synthesis (33 genes) (Figure 4). Also, genes involved in iron transport, iron ion binding, or iron–sulfur cluster assembly binding were significantly represented (30 genes total), consistent with the iron–limiting conditions of serum contributing to diminished bacterial viability (Figure 4, Table S5).

Distinct transcriptional response is associated with serum susceptibility. To investigate whether variation in gene expression was associated with serum sensitivity in isolates with differential degrees of sensitivity, we analysed changes in transcription from bacteria exposed to normal human serum for 10 minutes relative to standard laboratory conditions (mid log culture in LB). This identified 170 genes as differentially expressed in all six isolates, 88 of which were up–regulated and 82 down–regulated (Tables S6, S7). A significant proportion (49%) of the up–regulated genes included ones involved in iron scavenging and iron–associated metabolism, including *fhu, ent, fep, suf, iro, exb, feo,* and the *sit* operon (Table S6). The second most abundant group (12.5%) consisted of genes involved in colanic acid biosynthesis, including the *rcsA* regulator (Table S6). A majority of the down–regulated genes (55%) were associated with the bacterial envelope (Table S7). Notably, *S.* Typhimurium serum resistance genes *rck*, *traT*, and *pgtE* (20), were not significantly up–regulated in our experiments (Table S8).

We also identified transcriptional patterns that distinguished the isolates by serum susceptibility phenotype. Unsupervised clustering of the six *S.* Typhimurium strains based on the magnitude of change in expression for all chromosomal genes revealed a pattern that approximately grouped the

isolates by relative serum sensitivity (Figure 5A). The transcriptional response to serum killing of D26104 and D23005 that had the greatest resistance to serum killing were most similar to one another, D25352, D24871 and D23580 that were moderately resistant or sensitive clustered, whilst the extremely susceptible strain D24545 had a distinct profile that was nonetheless most closely related to other susceptible strains. Since both colanic acid and LPS polysaccharides are known to be associated with serum resistance in pathogenic Enterobacteria (34, 35, 56) we investigated the transcriptional profile genes involved in their biosynthesis in the six strains (Figure 5B and C). A modest level of clustering of resistant and susceptible strains was also observed for genes involved in colanic acid biosynthesis and to a greater degree LPS biosynthesis. Notably, despite significant induction of a majority of the *wca* locus genes in all the strains (Table S6), elevation of transcription of these genes was higher in serum susceptible than serum resistant strains (Figure 5B).

We also identified genes that exhibited a significant difference in mean expression greater than 2–fold in the three resistant strains compared to the three susceptible strains, ($p<0.05$). A total of 45 genes had a ratio of 2 or more from comparison of means of either group, most of which are associated with putative or uncharacterized proteins (Figure 5D). Genes more highly expressed in response to serum in sensitive compared with resistant strains included *yrb*C, *yiaD*, which is a putative outer membrane (*ompA*) family protein, *yibD*, which is expressed in response to phosphate starvation, *yggE*, an oxidative response protein, heat shock protein *hslJ*, homeostasis protein *cut*C, lysozyme inhibitor *mli*C, flagellar biosynthetic protein *fliR*, metabolic proteins *eut*S, *btuR*, *galU* and *oda*A, *cysZ* transporter, *yjb*G, which increases resistance to bacitracin, and *yaiW*, a putative surface–exposed outer membrane lipoprotein. Twelve genes were more highly up–regulated in response to serum in resistant compared with sensitive strains, and included *glpB*, porin protein *ompD*, *hilD*, a transcriptional regulator for multiple virulence genes, and *bssR*, which is induced in biofilms.

Notably, two LPS O–antigen genes, *wzzB* and *fepE* exhibited distinct responses to serum in resistant and susceptible strains. While the O–antigen polymerase *wzzB* was on average more highly up–regulated in serum–susceptible strains relative to serum–resistant ones, *fepE*, which modulates expression of very long O–antigen chains (34), was on average 2–fold more highly expressed in serum–resistant strains than susceptible strains (Figure 5D).

Discussion

S. Typhimurium ST313 that are highly resistant to serum killing have been identified raising the possibility that these may be the basis of escape mutants capable of evading vaccine–mediated antibody and complement killing. To better understand this problem, we investigated the molecular basis of resistance to antibody–mediated complement killing among iNTS isolates. First, we analysed the genomic content of six bloodstream isolates with differing levels of resistance, to identify SNPs or larger modifications that may have an impact on serum resistance. By this means, we were unable to find a basis for the differences in resistance.

TraDIS and transcriptomic profiling analyses revealed the repertoire of genes involved in survival and growth in serum in these S. Typhimurium ST313 isolates. Two major functional groups of differentially selected or expressed genes following serum exposure were identified: genes involved in nutrient scavenging, mainly the uptake and assimilation of iron, and genes involved in the synthesis of bacterial envelope components, particularly of extracellular polysaccharides. Several genes significantly up–regulated on exposure to serum, including *ent*, *iro*, *sit*, *feo* operons, *tonB*, *exbB* and *exbD* were essential for normal levels of viability in serum. For example, deletion of the *entD* gene in D23580 resulted in 40–fold lower viability than wild–type following 3 hours of exposure (Figure 3). Genes classically associated with serum resistance in S. Typhimurium, such as *rck*, *traT*, *pgtE*, however, were not significantly up–regulated at the time point investigated (Table S6).

The bacterial membrane and its associated components are important for viability in serum. Whilst LPS genes were not significantly up–regulated during serum exposure, we did observe differences in expression patterns of LPS genes between the serum resistant and susceptible strains. In particular, the expression of O–antigen polymerase genes *wzzB* and the very long O–chain determinant *fepE*, were significantly different among these strains, with an average two–fold higher expression of *fepE* detectable within 10 minutes in resistant compared with susceptible strains as determined by RNAseq. Differences in the induction of these two genes resulting in preferential polymerization of LPS into trimodal (including very–long) rather than bimodal (with long) O–chain lengths confers resistance to antibody–dependent complement–mediated serum bactericidal activity in S. Typhimurium (34).

Increased expression of *wca* locus genes, responsible for the synthesis of colanic acid exopolysaccharide was observed. Twenty genes comprise this operon in S. Typhimurium (57), ten of which were differentially expressed in all six strains, along with *rcsA*, which regulates their expression. Rcs–associated up–regulation of colanic acid genes in response to serum has been observed in pathogenic *E. coli* (56, 58), and is also induced by exposure to antimicrobial peptides in S. Typhimurium (59). Expression of colanic acid is thought to mediate serum resistance either through formation of a barrier against lysis by the membrane attack complex (56) or preventing complement activation by limiting access to LPS (57). Although we found that colanic acid biosynthetic genes increased expression on exposure to serum, induction was greatest in susceptible strains. Furthermore, Tn5 insertions in colanic acid biosynthetic genes were not counter–selected in the TraDIS screen, and a null *wcaB* mutant was not more sensitive to antibody killing than the wild–type strain.

The relative lower induction of colanic acid biosynthetic gene in resistant strains may be linked to the higher increase in *fepE* expression in these strains. FepE is a positive regulator of very long LPS O–antigen chain associated with enhanced serum resistance (60). In S. Typhi, *fepE* is a pseudo gene (61), and very long LPS O–antigen chain interferes with the expression of the Vi exopolysaccharide capsule (61). Lower expression of colanic acid biosynthesis genes may therefore facilitate the elaboration of very long LPS O–antigen chain and enhance serum resistance.

The large–scale genome–wide approaches used in this study have provided a robust platform for investigating the full complement of genes necessary for survival of S. Typhimurium in serum. As with most high–throughput assay systems, some caveats apply. For instance, with mutant libraries, it is not possible to distinguish between mutations associated with an overall decrease in bacterial viability or fitness over ones specifically necessary for complement resistance during selection in serum. Additionally, since the environmental signals ex vivo or in vitro are not identical to those in vivo, the information from the assays may not fully recapitulate the in vivo response to antibody–dependent complement killing. However, complementary results from the multiple approaches used in this study, mitigate these limitations to an extent.

We have defined gene functional classes and specific genes associated with serum resistance in six S. Typhimurium bloodstream isolates. Investigation of the transcriptional response of resistant and

sensitive isolates highlighted putative factors responsible for the diverse serum sensitivity phenotypes exhibited by isolates of S. Typhimurium ST313. While susceptibility to antibody was not associated with an identifiable genotype, distinct transcriptional responses were evident in resistant and sensitive isolates, in particularly transcriptional response of genes associated with surface polysaccharides. It is hoped that this study will provide a basis for further research into modes of escape from natural vaccine–induced immunity among invasive *Salmonella* strains.

Author contributions

Conceptualization: EMO, EJK, CLM, GD, RAK, CAM; Formal Analysis: EMO, EJK, MAEG, LB, RAK; Funding Acquisition: GD, RAK, CAM; Investigation: EMO, CLM, MAEG, JNH, DJP, RAK; Methodology: EJK, GD, RAK, CAM; Software: LB, RAK; Supervision: GD, RAK, CAM; Validation: EMO, EJK, RAK, CAM; Original Draft Preparation: EMO, EJK, RAK, CAM; Review & Editing: All authors.

Competing Interests

Edna Ondari and Calman MacLennan are former employees of Novartis Vaccines Institute for Global Health (under the ownership of GlaxoSmithKline at the time of data analysis and writing). Calman MacLennan has been the recipient of a Clinical Research Fellowship from GlaxoSmithKline.

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Table 1. Polymorphisms occurring in clade II strains with atypical serum susceptibility. Only SNPs occurring in gene–coding regions and unique to each strain/phenotype are included. SNPs from *acrB*, *pepP*, *ispD*, *uxuB*, *uxuR*, and *STMMW_29841* genes were transferred to D23580 and tested for their impact on resistance or sensitivity to serum killing by normal human serum.

Position in D23580	Systematic Gene ID	Gene name	Description	D23580 base	SNP base	D24545 (highly sensitive)	D23005 (resistant)	D25352 (resistant)	AA CHANGE
570516	STMMW_05451	*acrB*	acriflavin resistance protein B	C	T	T	.	.	G796S
1200631	STMMW_11271	*agp*	glucose–1–phosphatase precursor, secreted	T	C	C	.	.	L322P
1264174	STMMW_11931	*rne*	ribonuclease E	G	T	T	.	.	R1062S
4277479	STMMW_40091	_	alcohol dehydrogenase	T	C	C	.	.	K78E
4390204	STMMW_41061	*rpoB*	DNA–directed RNA polymerase, beta–subunit	G	A	A	.	.	D516N
4783703	STMMW_44531	*uxuR*	uxu operon transcriptional regulator	G	A	A	.	.	W221STOP
3211805	STMMW_30181	*pepP*	proline aminopeptidase II	C	A	A	.	.	E79D
3064062	STMMW_28931	*ispD*	2–C–methyl–D–erythritol4–phosphate cytidylyltransferase	G	T	.	T	.	R128W
3175291	STMMW_29841	_	probable amino acid transport protein	T	G	.	G	.	S255L
1947840	STMMW_18471	_	putative membrane protein	T	A	.	.	A	D181E
1875804	STMMW_17771	_	conserved hypothetical protein	G	A	.	.	A	R349W
1966357	STMMW_18691	*pykA*	pyruvate kinase A	G	A	.	.	A	G124S
3289745	STMMW_30961	*uxuB*	D–mannonate oxidoreductase	A	C	.	.	C	N361T

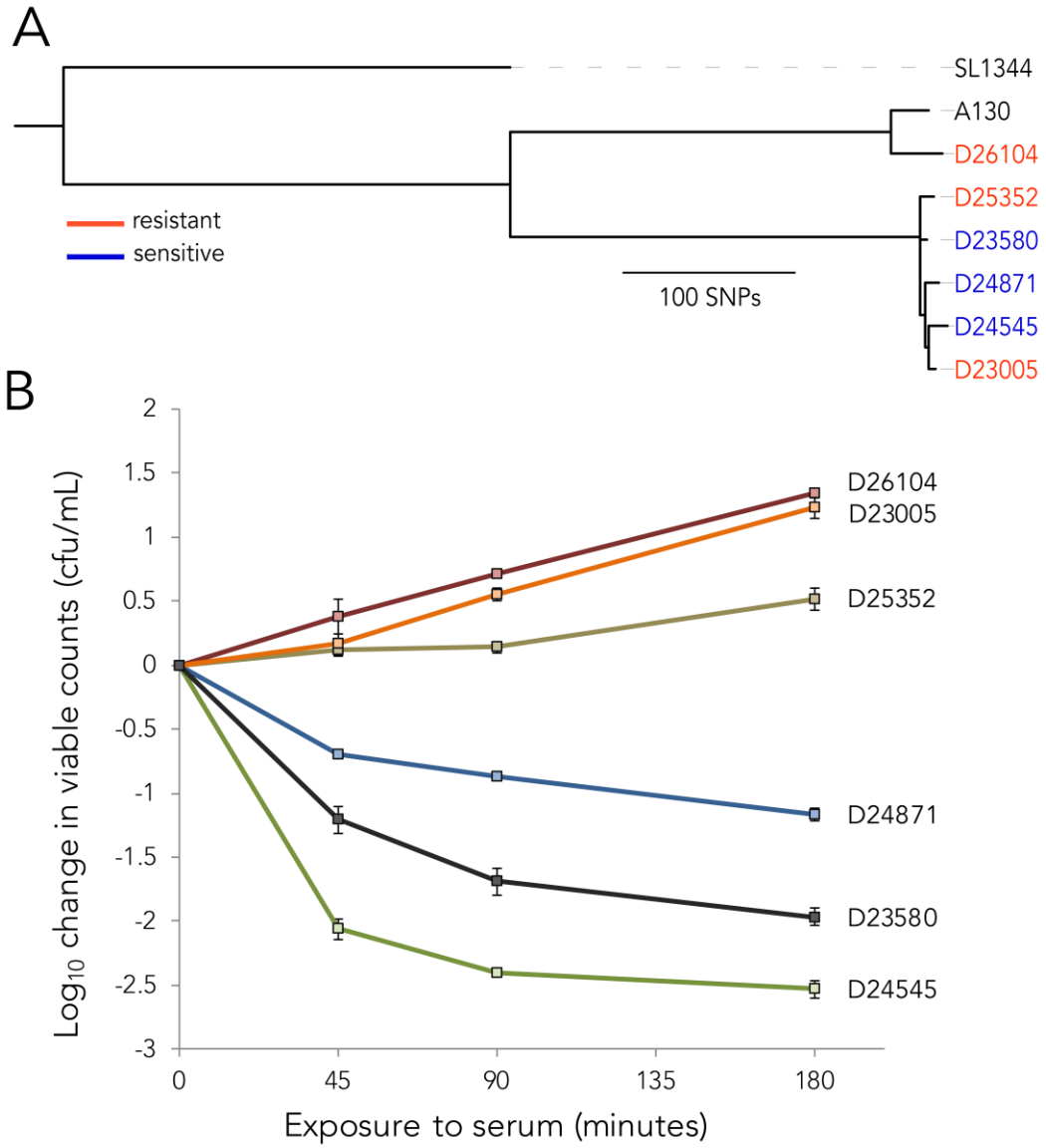

Figure 1. Characteristics of the six NTS isolates. A. Phylogenetic tree representing the relationships between the six NTS isolates studied. **B**. Fold change in bacterial counts following 3 hours of exposure to immune serum from a healthy adult donor. Each isolate was incubated at 37°C for 180 minutes with sampling at 45, 90 and 180 minutes. Data represent mean +/– standard error of two independent experiments, performed in triplicate.

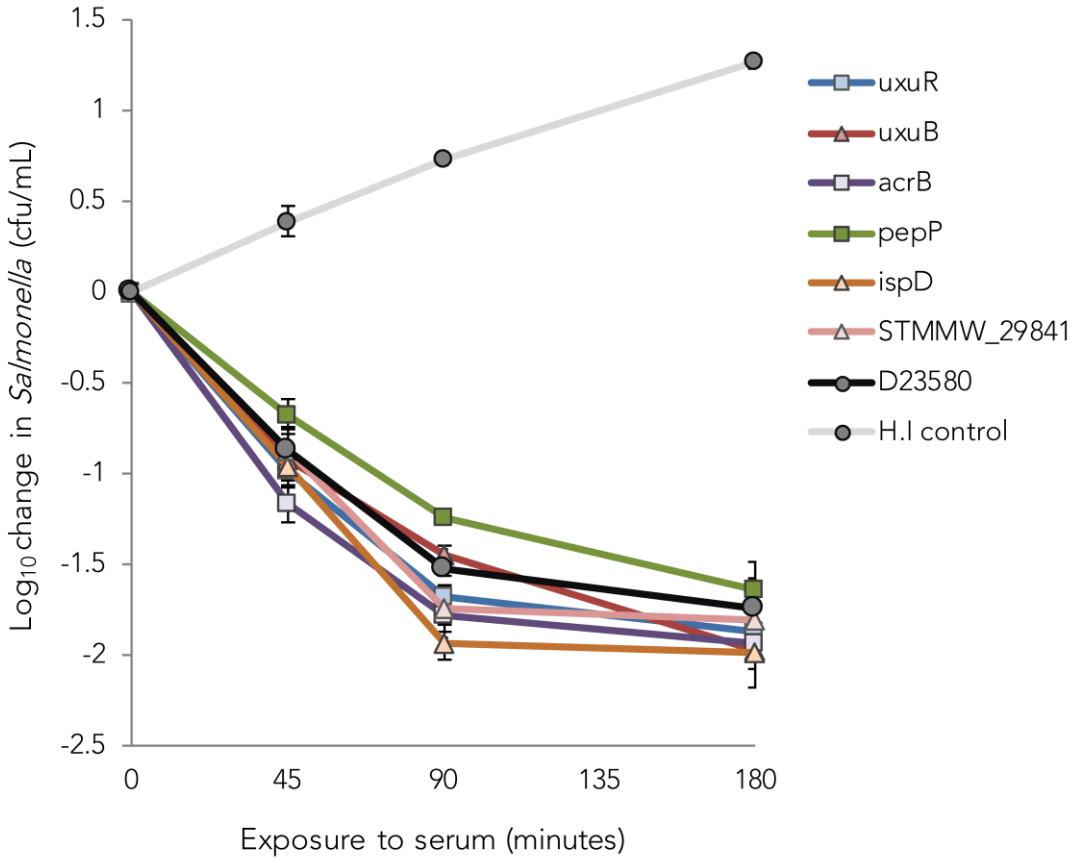

Figure 2. Relative serum sensitivity of D23580–derived strains with nonsynonymous polymorphisms transferred from serum resistant (D23005 and D25352) and susceptible (D24545) *S.* Typhimurium strains. Each strain was exposed to immune human serum at 37°C for 180 minutes with sampling at 45, 90 and 180 minutes. Squares represent strains with alleles from D24545, triangles from the resistant strains. Data represent means of two independent experiments, performed in triplicate. Error bars: Standard Error. HI control: Heat–inactivated control (Wild–type D23580 exposed to heat–inactivated serum).

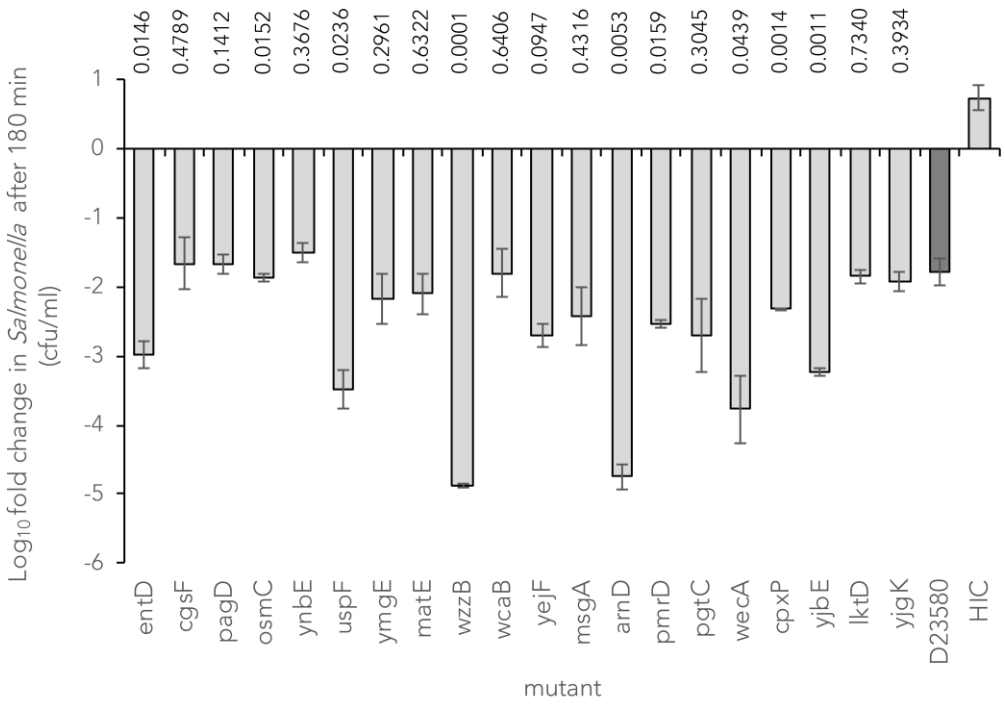

Figure 3. Susceptibility of D2580 mutants to the bactericidal activity of normal human serum relative to the wild–type strain. Bars show the mean $\log_{10}$ fold change +/– standard error of the mean in viable counts of twenty S. Typhimurium D23580 mutants following exposure to serum for 180 minutes. HIC: Heat–inactivated control (Wild–type D23580 exposed to heat–inactivated serum). P values above the bars represent t–test probabilities of pairwise comparisons of fold changes between each mutant strain and the wild–type strain.

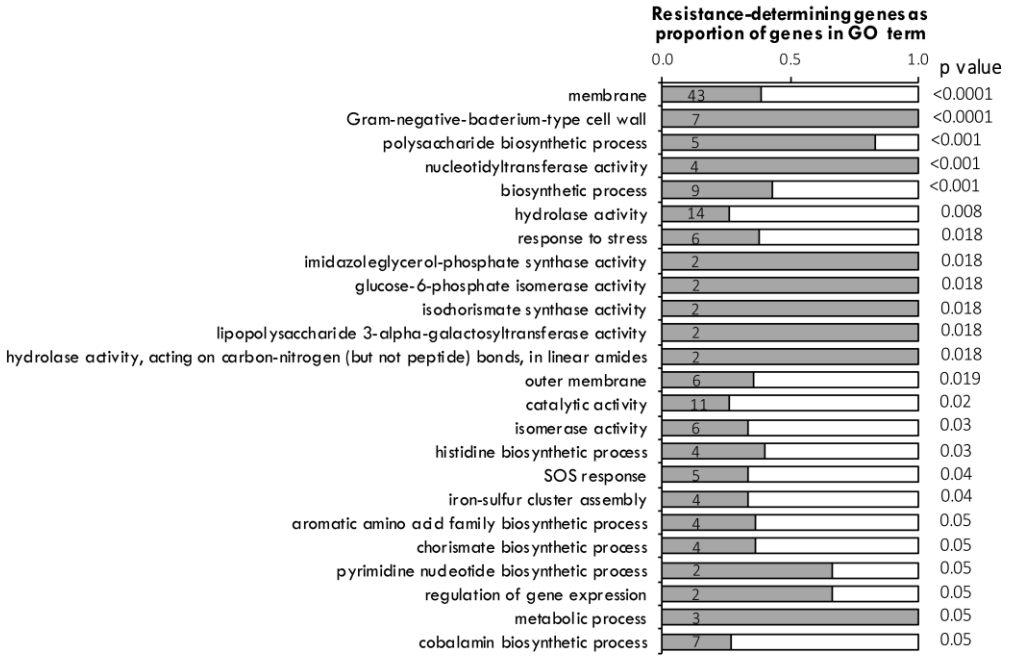

Figure 4. Significantly enriched gene ontology (GO) terms for 555 serum resistance–determining genes in D23580. Genes included in the analysis were those whose corresponding mutants had a fold change of 2^{-2} or lower from comparisons of input and output mutant libraries identified by TraDIS. P values are hypergeometric probabilities calculated using the *phyper* function in R. Comparisons were made using the number of genes associated with each term in the gene set versus the total number of genes associated with each term in the genome, using S. Typhimurium LT2 GO annotation (http://www.uniprot.org/proteomes/UP000001014). Numbers within the bars represent the total number of genes in the essential gene set associated with each term.

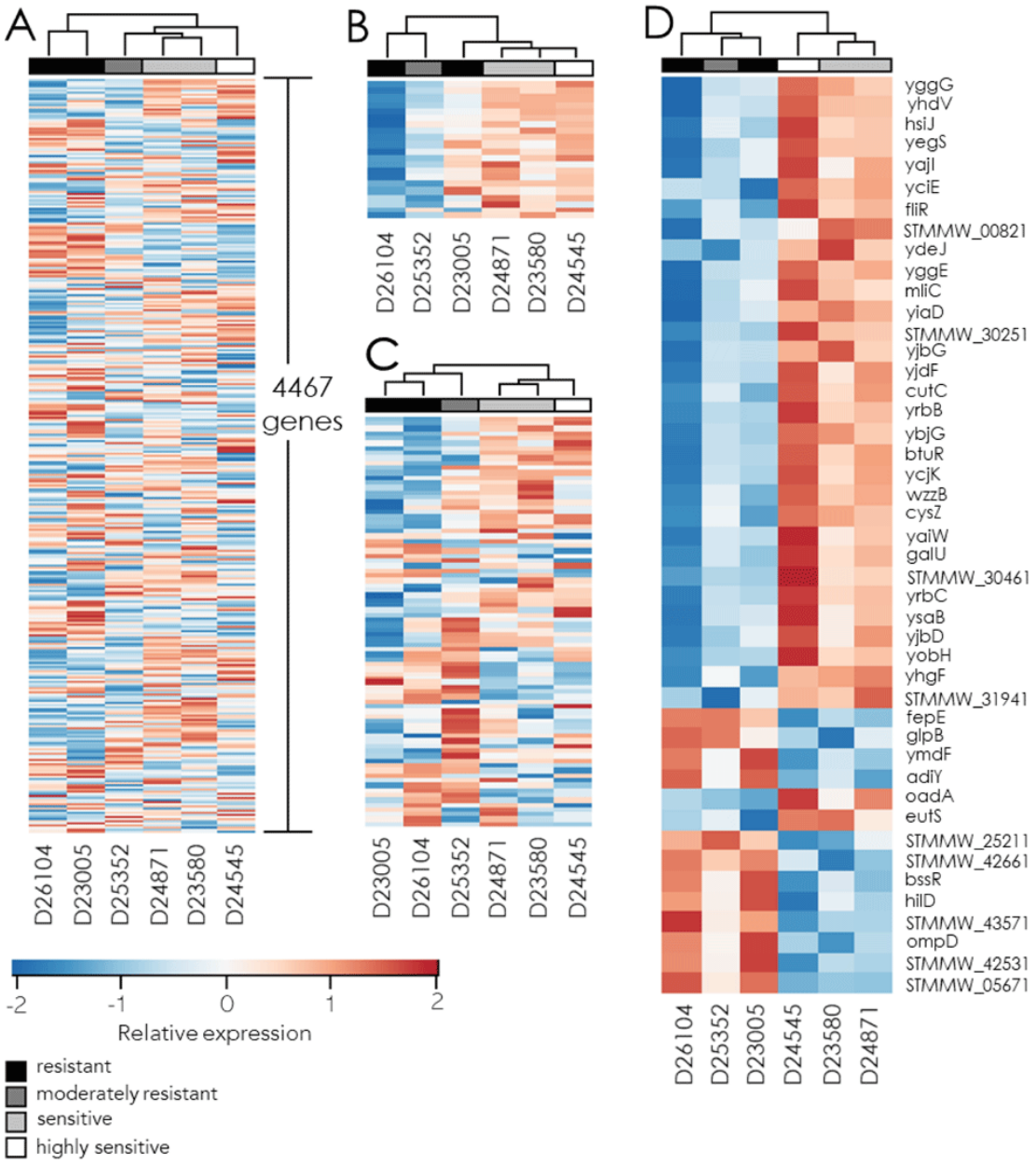

Figure 5. Relative gene expression in six invasive *S.* Typhimurium strains in response to serum exposure. Heat maps indicate $\log_2$ fold changes in transcript abundance following a 10–minute growth in serum compared to log–phase cultures in LB. Patterns of transcriptional responses are clustered and their relationship indicated by dendrograms. The intensities of each cell colour represent the deviation (Z–score) of each strain from the average fold change (centered to zero, represented with white on the heatmap) per gene for higher (red) or lower (blue), for all chromosomal genes (A), genes in the *wca* (colanic acid biosynthesis) locus (B), LPS biosynthesis genes (C), or genes satisfying a t–test cut–off of ≤0.05 and a ratio of ≥2 (D).

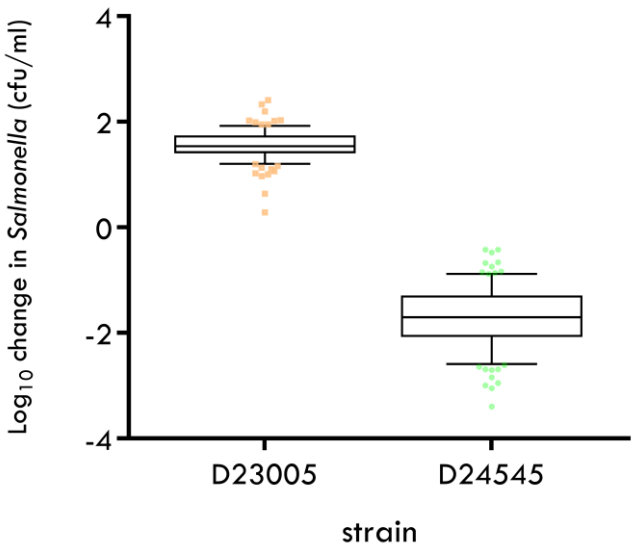

Figure S1. Susceptibility of 100 clones of *S.* Typhimurium ST313 strains, D23005 (serum resistant) and D24545 (serum susceptible) following exposure to normal human serum for 3 hours.

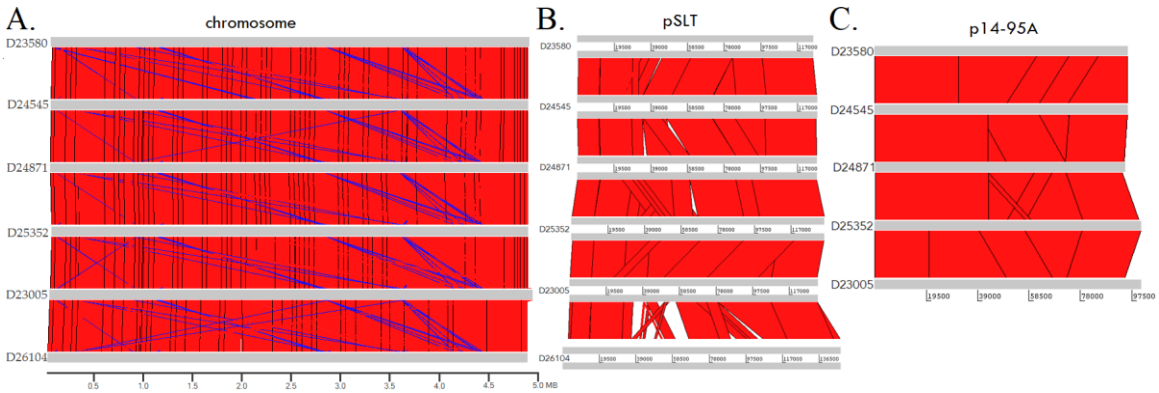

Figure S2. Comparisons of chromosomes and major plasmids in six *S.* Typhimurium strains displaying different antibody sensitivities. Blocks represent pairwise blastn comparisons of **A.** chromosomes **B.** pSLT virulence plasmid and **C.** *S.* Typhimurium p14–95A from clade II strains. *p14–95A was absent from D26104. Comparisons were made using the Artemis Comparison Tool (ACT).

Table S1: Primers used in this study

Insertion of kanamycin cassette for phage transduction		
Gene with SNP	**Left primer sequence (5′-3′)**	**Right primer sequence (5′-3′)**
uxuR	ATCTGTTTGAGTCATGGCCGCTGGACAACGTTGA TGCCTGAGTGTAGGCTGGAGCTGCTT	TTATACCTGGAATAGCGTGGGCAGACCCAGTCCACGT TTTACTTCCTGTAGCCATATGAATATCCTCCTTA
uxuB	GGCTATCCAGCGCGCTTTCTTTAGCAAATAACCT TCTGTGTAGGCTGGAGCTGCTT	CTATTGCATTGAGTAGGGGAAATCGCATGACGCGTCG CGCCATGCGCATATGAATATCCTCCTTA
acrB	TTTGCTCACTGTTGATAAGGCCGCGCAAGCGGCC TTTTTTACGCGTGTAGGCTGGAGCTGCTT	GGATTATACCTTTTATTAATATGGCAAAAAATAATCA ATCTAACAATAAGCATATGAATATCCTCCTTA
pepP	ACGAAATGGAGGCCATGTCATGACTCAGCAGGAA TACCAACGGTGTAGGCTGGAGCTGCTT	CTCCGGCGCGGCAAAGATCAGCGCGGCGCTGCCGGGC TGCATTCATATGAATATCCTCCTTA
ispD	GGTAGACGGGTTATCACTGCGCCTCCGGTCATAA TTCAAACCGTGTGTAGGCTGGAGCTGCTT	GATGTTACGTATACCCGAGTAAGGAGAACATAATCTC ATTGTTGGCATATGAATATCCTCCTTA
STMMW_29841	TGATGTTTTTCGGCTAAAGTAAAAGATAGCCGGG AGTGTAGGCTGGAGCTGCTT	GTGCTGGATTCTTATATAAAATAGCCGGGAGTTATAC ATATGAATATCCTCCTTA
Verification of SNPs transferred to D23580		
uxuR_allele1	CGCGCAGCGAAGCTGGCGATGTG	
uxuR_allele2	CGCGCAGCGAAGCTGGCGATGTA	
uxuR_seq	ATCGCTGAGAGGCTCAATGT	
uxuR_rev	ACCTGCCTGCAAGAACTGAT	
uxuB_allele1	TGTTGATTGCGCGTTTCACTAA	
uxuB_allele2	TGTTGATTGCGCGTTTCACTAC	
uxuB_seq	TGTGAAGTGAACCTGATGCC	
uxuB_rev	ACTTAGCCAGCGCTTCTACC	
acrB_allele1	CTGGTACGTTCGTGGTAGCGATA	
acrB_allele2	CTGGTACGTTCGTGGTAGCGATG	
acrB_seq	ACAAGGTTGAAGCGATTACC	
acrB_rev	CCATTAAGTCTTTGGCGAAT	
pepP_allele1	CGAATATGCGTATGCCGACGAG	
pepP_allele2	CGAATATGCGTATGCCGACGAT	
pepP_seq	CCATGTCATGACTCAGCAGG	
pepP_rev	AACCTGCGTCGATAAGCACT	
ispD_allele1	CTGGCTATCAGCGAAAACAGCC	
ispD_allele2	CTGGCTATCAGCGAAAACAGCA	
ispD_seq	ATGGCAGCCACTTTATTGGA	
ispD_rev	ATGCTTTCTCCTGGTGGATG	
STMMW_29841*_seq	AACATACTGGCCGCATAAGG	
STMMW_29841_rev	CATAGAGATGGCATTCGGGT	
***verified only by sequencing, without genotyping PCR**		
Targeted gene deletion in D23580		
gene name	**Left primer sequence (5′-3′)**	**Right primer sequence (5′-3′)**
entD	AGGGATCTCATTTATCGGGGTATTGCGCTAAGTATAGTGTA GGCTGGAGCTGCTT	**TCCCTCCCTCATTCGGGGAGGGAATTGGCAAAAACCATATGAAT ATCCTCCTTA**
csgF	AGCGCGGCATGATTTATATCCTTTAGTGACTGTAGCGTGTA GGCTGGAGCTGCTT	ACATGATTTTTATCCGGAGGCTGTCATGCGTGTTACATATGAAT ATCCTCCTTA
pagD	AAAGTAGTCGTTCGGCACCATTGTAGATAAATAACAGTGTA GGCTGGAGCTGCTT	AACTTCACAAGTCATTATATATAACAGGAGGTGCTCATATGAAT ATCCTCCTTA
osmC	CCCGATCAACCCATCGTTACCGGGCTGGAGAGGTTAGTGTA GGCTGGAGCTGCTT	TCTATACTTTATTTTTTGAGCCAACAGGAGAGCAACATATGAAT ATCCTCCTTA
ynbE	AGCACCCATAGTATTAAATACTTTTTCATTATGGCCGTGTA GGCTGGAGCTGCTT	TAAATAATGAAAATGAGTATTGCCATGCTGTCAGCCATATGAAT ATCCTCCTTA
uspF	TAGGGAGTAGAATTTCAGTGGATACTTAAACAGGAGGTGTA GGCTGGAGCTGCTT	ATTTGAGCCCGCACTCTCTTATGCGGGCTCGGGTACATATGAAT ATCCTCCTTA
ymgE	TGCTGACCGGGTCAACGCTTACACGTTCTGCAGCGGGTGTA GGCTGGAGCTGCTT	TACTTAAAACGTTTTTACTGGAAAGAGGGTTCGCACATATGAAT ATCCTCCTTA
matE	TCACCGTTTCCTGATGATTTTCACAGCAAAAAGCGCGTGTA GGCTGGAGCTGCTT	AGCTTTGCAGCCTTACGATTTCTCGCTCATTATTGCATATGAAT ATCCTCCTTA
wzzB	TACCTGTCGTAGCCGACCACCATCCGGCAAAGAAGCGTGTA GGCTGGAGCTGCTT	GTCTCCAGCTTCATCCTTTTTTTAGTTAGGGTATCCATATGAAT ATCCTCCTTA
wcaB	GTGCTGACCTTCACCCGTGCTTTTTCTCCCACCACCGTGTA GGCTGGAGCTGCTT	GATGCTGGAAGACTTACGCGCGAATAGCTGGAGCTCATATGAAT ATCCTCCTTA
yejF	GATGACATCTCCATTGCTTGCGATTGAAAATTTATCGTGTA GGCTGGAGCTGCTT	GCGTCAGCTTAACGCGAGTAGCTGACGCGTATAGGCATATGAAT ATCCTCCTTA

msgA	CGGATCTTCCCCGATGCTGATGTTCGGGTTAAACCGGTGTA GGCTGGAGCTGCTT	GCGATCAAAACGTAACTATGGCGGATATTGAAAAACATATGAAT ATCCTCCTTA
arnD	CGAAAGTCGGTTTACGCATTGACGTTGATACCTTGCGTGTA GGCTGGAGCTGCTT	TCATGAGGCGCTCGCTGCCTGTTGACACCCCAGCCCATATGAAT ATCCTCCTTA
pmrD	TGGCTTGCGCGTCAACCGCTGCCATTCGTCGCAGGAGTGTA GGCTGGAGCTGCTT	ACGTGTTATGAACAATCAGCGTGAAACGGGGGCGCCATATGAAT ATCCTCCTTA
pgtC	TTCAGCTCTTCCAGTTTGTGAATCGCCAGCCGCTGAGTGTA GGCTGGAGCTGCTT	CCAGAGTGGGCGCTTTTACGCTATGGCTGGCGGCACATATGAAT ATCCTCCTTA
wecA	TTTAAGGAAATGATATATGCTGAGAGCACATACACTGTGTA GGCTGGAGCTGCTT	GTATCCTCATTTACTTGGTTAAGTTTGGCCGGTTCCATATGAAT ATCCTCCTTA
cpxP	GTTGAATCGCGACAGAAAAGATTTTGGGAGCAAGCGGTGTA GGCTGGAGCTGCTT	GACAGGGATGGTGTCTATGGCAAGGAAAACAGGGTCATATGAAT ATCCTCCTTA
yjbE	TTGGGTAAATCTCCATTCATTCAATGAAGGGAAGTTGTGTA GGCTGGAGCTGCTT	ACCGGAGTGTGGTTATGGTTAAACGTACCGATACTCATATGAAT ATCCTCCTTA
lktD	TGAAAGTGAGTTAAATGTAATGAAATGAATAGAAGAGTGTA GGCTGGAGCTGCTT	ATATTATATTCACTCTCAAGGTGTATCTAATCGTTCATATGAAT ATCCTCCTTA
yjgK	AGAAAAATAAAACGCTGTTTTAACTAAGGGGATGCGGTGTA GGCTGGAGCTGCTT	ATCGCAACGTGCAGGCGCCGCCATCCGGCACAAAACATATGAAT ATCCTCCTTA

Verification of D23580 null mutants

Oligo name	Left primer sequence (5′-3′)	Right primer sequence (5′-3′)
km_int_rev		GAAGCCCAACCTTTCATAGA
entD_v	GCGTATAAGGTTCAGAAACG	
csgF_v	TAAGCATCCGCTCAACA	
pagD_v	AAGTAGTCGTTCGGCACC	
osmC_v	TATATTCATCAGCCCGATCA	
ynbE_v	AGCGTCAGCACCCATAGTA	
uspF_v	AGGGAGTAGAATTTCAGTGGA	
ymgE_v	GGTCAACGCTTACACGTTC	
matE_v	GCTTTATTCCACACTTCGG	
wzzB_v	GATCGACAATCTTCGTAAGC	
wcaB_v	GAATATTCATGTGCTGACCTTC	
yejF_v	TCCTGCTAAGGCGGTATAAG	
msgA_v	GGAGAATTGACTAAGAGGGTTC	
arnD_v	GCATTGACGTTGATACCTTG	
pmrD_v	CAGATCATGATGGCTTGC	
pgtC_v	CAGCTCTTCCAGTTTGTGAA	
wecA_v	GATATATGCTGAGAGCACATACACT	
cpxP_v	CGTAAACTGTCTCTCGTTGAAT	
yjbE_v	GACATAATCCGCCTGCTT	
lktD_v	GCATATGTAAGCATGATCGAC	
yjgK_v	CGATGAGAGTAAGGGTAGTATGG	

Table S2: Mapping statistics

Strain	Replicate	Condition	ENA Run Accession no.	Number of reads mapped	Mapped %	Paired %	Mean Insert Size	Depth of Coverage	Depth of Coverage SD
D23005	1	LB	ERR731309	13361556	97.4	95.7	219	123.09	230.79
D23005	2	LB	ERR731315	20019172	97.2	95.3	213	158.16	264.52
D23005	3	LB	ERR731321	12744506	97.3	95.6	207	118.07	228.59
D23580	1	LB	ERR731310	13256050	96	93.8	212	118.2	228.15
D23580	2	LB	ERR731316	17367808	96.7	94.7	207	139.22	247.94
D23580	3	LB	ERR731322	12658964	96.6	94.9	201	108.75	219
D24545	1	LB	ERR731326	15511002	95.7	90.8	191	142.7	242.65
D24545	2	LB	ERR731332	16528940	95.8	92.9	193	136.94	244.33
D24545	3	LB	ERR731338	16951864	96.3	94	198	146.25	255.59
D24871	1	LB	ERR731327	15953754	96.3	91.9	197	142.48	242.06
D24871	2	LB	ERR731333	16062318	96.1	93.6	196	135.41	242.14
D24871	3	LB	ERR731339	16002634	95.8	92.5	196	136.99	246.91
D25352	1	LB	ERR731328	23107586	95.9	91.7	196	169.28	271.37
D25352	2	LB	ERR731334	14916950	96.4	92.6	195	126.72	236.73
D25352	3	LB	ERR731340	16862840	95.8	91.9	195	141.32	252.84
D26104	1	LB	ERR731308	14367902	97.1	94.5	217	117.24	224.83
D26104	2	LB	ERR731314	13668920	97.7	95.7	207	122.73	231.86
D26104	3	LB	ERR731320	9310172	97.6	96	206	86.8	195.71
D23005	1	serum	ERR731312	12129916	96.6	94.3	198	123.92	228.37
D23005	2	serum	ERR731318	21591460	96.5	93.7	187	190.25	283.97
D23005	3	serum	ERR731324	13549348	96.4	94.1	192	96.48	200.27
D23580	1	serum	ERR731313	17042480	96	94	201	156.77	261.52
D23580	2	serum	ERR731319	15455926	96.2	94.2	194	147.55	249.19
D23580	3	serum	ERR731325	19226200	95.4	93.9	195	133.74	236.1
D24545	1	serum	ERR731329	15236920	95.2	91	186	149.17	244.51
D24545	2	serum	ERR731335	16081260	95.7	91.2	183	150.19	253.91
D24545	3	serum	ERR731341	9308772	93.1	89.4	208	64.63	112.09
D24871	1	serum	ERR731330	14640364	95	91.1	190	145.29	242.47
D24871	2	serum	ERR731336	15998200	95.1	89.5	184	155.66	256.59
D24871	3	serum	ERR731342	10535672	93.3	89.7	213	77.39	116.76
D25352	1	serum	ERR731331	15763416	94.8	90.8	191	152.56	249.75
D25352	2	serum	ERR731337	15913162	95.4	91	190	148.49	248.41
D25352	3	serum	ERR731343	15304748	95.1	92.7	191	116.29	185.2
D26104	1	serum	ERR731311	16318278	96.8	94.8	204	152.13	250.14
D26104	2	serum	ERR731317	16266924	97.4	95.6	209	154.63	254.21
D26104	3	serum	ERR731323	12456810	97.1	95	200	103.28	207.43

Table S3: Comprehensive analysis of SNP's in lineage II *S.* Typhimurium isolates

Position in D23580	Gene ID	Gene name	Description	D23580 base	SNP base	resistant		susceptible		AA CHANGE**	Log_2 Fold difference vs D23580 (LB)				Log_2 Fold difference vs D23580 (SERUM)				Log_2 Fold regulation in serum					TraDIS log_2 Read Ratio	TraDIS adjusted p value
						D23005	D25352	D24545	D24871		resistant		susceptible		resistant		susceptible		resistant		susceptible				
											D23005	D25352	D24545	D24871	D23005	D25352	D24545	D24871	D23005	D25352	D24545	D24871	D23580		
62717	STMMW_00541	citA	sensor kinase citA	A	C	C	.	C	.	L – V	-0.0596	-0.1165	0.0839	-0.1556	-0.1734	0.2267	0.2846	0.3350	0.0671	-0.2416	-0.6052	-0.3256	-0.4792	0.8401	1.0000
75492	intergenic	_	_	T	C	C		C	C	_	0.7111	-0.0696	-0.7542	0.1608					4.3514	4.3282	3.8057	4.9138	4.8518		
*	STMMW_00661	pseudogene	_	.	.	.	.	.	.	_	_	_	_	_	_	_	_	_	_	_	_	_	_		
271609	STMMW_02371	dnaE	DNA polymerase III, alpha chain	A	G	G	G	G	G	NC	-0.2422	0.0968	0.0101	0.1873	-0.1729	-0.1093	-0.1462	-0.0632	-0.4941	0.0188	-0.1636	0.0934	0.0685	_	_
570516	STMMW_05451	acrB	acriflavin resistance protein B	C	T	.	.	T	.	G – S	0.2451	0.0861	-0.1629	0.1059	-0.2006	-0.3441	-0.2310	-0.2323	-0.1879	-0.1447	0.1221	0.2290	0.3618	-1.4440	0.9840
954102	STMMW_08961	moeB	molybdopterin biosynthesis protein	C	T	.	.	T	.	NC	-0.2329	-0.0893	-0.2506	0.0204	-0.3871	-0.3923	-0.2529	-0.2280	-0.0757	0.2406	0.5357	0.6263	0.5598	2.8770	0.6241
1086992	intergenic	_	_	T	C	.	.	.	C	_	0.0000	-0.0895	0.0717	0.5860					0.3762	0.1604	-0.1599	-0.1335	-0.7373	_	_
*	STMMW_10101	ompF	outer membrane protein F	.	.	.	.	.	.	_	-1.4074	-0.0444	0.2000	0.5608	0.2485	0.0061	-1.4372	0.1849	0.7786	0.3410	0.3512	0.0601	-0.3024	0.9126	1.0000
1200631	STMMW_11271	agp	glucose-1-phosphatase precursor (G1Pase), secreted	T	C	.	.	C	.	L – P	-1.1223	-0.0022	0.4288	0.1013	-0.3224	-0.0506	-0.3510	-0.0337	-0.4400	-1.3478	-1.4705	-1.1304	-1.3508	-0.6826	1.0000
1219536	STMMW_11421		glucose-1-phosphatase precursor (G1Pase), secreted	G	T	T	.	T	T	V – F	-1.1403	-0.0752	0.0485	0.0184	-0.0983	-0.2428	-0.2604	-0.2062	0.2628	-0.0792	-0.0723	-0.1160	-0.2944	1.7667	1.0000
1264174	STMMW_11931	rne	ribonuclease E	G	T	.	.	T	.	R – S	0.3148	0.0693	0.1719	0.0720	-0.0627	0.0223	-0.1092	-0.0041	0.1502	0.6427	0.5624	0.8423	0.7016	5.3482	0.1074
1450047	STMMW_13761	sufB	conserved hypothetical protein	C	T	T	T	T	T	P – S	0.3396	0.0278	-0.0029	-0.0391	-0.0647	-0.3732	-1.0072	-0.4768	4.2983	3.9260	3.5813	3.9103	4.5249	3.9714	0.3831
1558953	intergenic	_	_	C	.	.	.	.	T	_	-0.0272	-0.1912	-0.2893	-0.3062					0.3576	0.5047	0.3493	0.2931	0.1149		
*	STMMW_14851	asr	putative secreted stress response protein	.	.	.	.	.	.	_	-0.3209	-0.1880	0.2214	-0.2761	-0.0695	0.0415	0.3548	-0.3445	-0.4725	-0.7575	-0.7624	-1.1791	-1.0133	-2.6481	0.3824
1639987	STMMW_15611	scfA	NAD-linked malic enzyme	A	G	.	.	G	.	NC	0.1943	0.0152	0.2618	-0.1654	-0.0053	-0.2384	-0.6257	-0.4788	-0.0769	-0.4405	-0.2588	-0.2094	0.0569	1.9300	1.0000
1763888	STMMW_16731		putative oxidoreductase	A	T	.	.	.	T	K–N	0.2925	0.2671	-0.1503	-0.0196	0.3850	0.1319	0.1336	0.0196	0.6503	0.4066	0.5759	0.3085	0.2258	-5.3240	0.0312
1875804	STMMW_17771		conserved hypothetical (phage) protein	G	A	.	A	.	.	R – W	-0.0551	0.0275	-0.3770	0.0638	0.2419	0.2958	-0.2091	0.0046	0.5447	0.3607	-0.3448	0.0314	-0.1724	0.5743	1.0000
1947840	STMMW_18471		putative membrane protein	T	A	.	A	.	.	D – E	0.0296	-0.0888	0.0467	-0.3630	0.5695	0.5696	0.2611	0.1767	-0.0048	0.0224	-0.3001	-0.4188	-0.1658	0.7504	1.0000
1966357	STMMW_18691	pykA	pyruvate kinase A	G	A	.	A	.	.	G – S	-0.2614	0.1612	0.3879	-0.1639	-0.0280	0.3340	0.1055	0.0778	-0.5032	-0.6062	-0.3341	-0.2277	-0.5475	3.6552	0.2871
2077197	STMMW_19932		phage protein	C	T	T	.	.	.	_	0.8772	0.2100	-0.0997	0.1228	0.5329	0.2943	-0.0137	-0.0670	0.0176	0.1287	0.0218	-0.0594	-0.2761	2.6094	1.0000
2083222	STMMW_20021		phage portal protein	G	T	T	.	.	.	_	0.9883	0.3267	-0.5282	-0.2105	0.7135	0.2566	-0.3844	-0.1314	-0.4499	-0.8161	0.2417	-0.3217	-0.6925	2.1850	0.6835
2151824	STMMW_20821	pduQ	putative propanol dehydrogenase	G	A	.	A	.	.	NC	0.7944	0.2554	-0.0458	-0.0327	0.0879	0.1960	0.6498	-0.0102	0.7473	0.3800	1.7870	1.4372	0.3958	-0.5009	1.0000
2351365	STMMW_22571	yejK	Nucleoid-associated protein YejK	C	A	.	.	.	A	S–I	0.2598	0.2704	-0.1891	-0.1598	0.6529	0.1013	0.0539	-0.1993	-0.5185	-0.0200	-0.3304	-0.1624	-0.1387	-2.6494	0.5192
2389768	STMMW_22921	ompC	outer membrane protein C	G	A	.	A	.	.	NC	0.1790	0.1692	0.1370	0.1111	1.0918	0.1468	0.7688	-0.1156	-1.1297	-2.8510	-1.9947	-3.1169	-2.8391	2.2225	1.0000
2791066	intergenic	_	_	A	C	.	C	.	.	_	0.4681	-0.2755	0.3291	-0.0369					0.4098	-0.2095	-0.0547	-0.1258	-0.5771	_	_
*	STMMW_26411		putative prophage protein	.	.	.	.	.	.	_														-6.2689	0.0163
3064062	STMMW_28931	ispD	2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase	G	T	T	.	.	.	R – W	0.4287	0.0403	-0.2616	0.1767	0.0926	0.0311	-0.2773	0.1397	-0.2707	0.3490	0.0476	0.3540	0.3743	-0.4384	1.0000
3089285	intergenic	_	_	T	G	.	.	G	.	_	-0.1260	-0.4463	0.7805	-0.2952					0.5814	1.2135	-0.6163	-0.8360	0.6831	_	_
*	STMMW_29141	ygcF	hypothetical protein	.	.	.	.	.	.	_	0.2222	-0.0710	0.1810	0.1030	0.3363	-0.0517	0.1933	-0.0409	0.0410	0.0514	-0.4086	-0.3703	0.0481	-1.0159	1.0000
3175291	STMMW_29841		probable amino acid transport protein	T	G	G	.	.	.	S – L	0.3337	-0.1516	-0.4882	-0.0226	0.6540	0.6270	0.1750	0.4231	-1.1516	0.2137	-1.0213	-0.6092	-0.7149	0.0517	1.0000
3183733	intergenic	_	_	G	T	T	.	T	T	_	-0.0610	-0.0487	0.0240	0.0685	0.6540	0.6270	0.1750	0.4231	0.6996	0.7207	0.0355	0.9127	-0.3842	_	_
*	STMMW_29911	stdA	fimbrial protein StdA	.	.	.	.	.	.	_	-0.1192	-0.0490	-0.2478	0.0123	0.6771	0.2120	0.5722	0.2448	-0.2351	-0.8867	-0.4176	-0.3114	-0.6330	0.3696	1.0000
3211805	STMMW_30181	pepP	proline aminopeptidase II	C	A	.	.	A	.	E – D	0.2535	0.0241	-0.1499	0.1412	-0.0524	-0.1735	-0.2147	-0.2120	0.3646	0.1623	0.1776	0.2601	0.3942	2.0749	0.9869
3272126	STMMW_30821		arylsulfatase	G	A	A	.	A	.	R – C	0.3355	0.0417	-0.2812	-0.0047	0.4412	0.1446	0.2287	0.2243	0.4100	0.1187	-0.0467	-0.0213	-0.2137	-0.2376	1.0000
3289745	STMMW_30961	uxuB	D-mannonate oxidoreductase	A	C	.	C	.	.	N – T	-1.3643	-0.2234	3.1051	-0.2693	-0.0198	-0.0648	4.0791	0.3974	0.0717	-0.2616	0.4000	0.3359	-0.4127	-1.1379	1.0000
3341146	STMMW_31641	ygiM/htrG	outer membrane channel protein/SH3 domain protein	G	A	.	A	.	.	NC	-0.1466	0.0143	-0.1617	0.0733	-0.5291	-0.4104	-0.0137	0.1565	1.4284	2.3009	3.2757	3.0461	2.5618	1.2415	1.0000
3438567	STMMW_32471	garK	glycerate kinase	T	C	.	C	.	.	NC	-0.4351	0.0506	0.0055	0.1175	0.1915	-0.0652	0.1536	-0.2070	-0.2081	-2.3387	-2.7092	-3.3681	-2.7930	1.0674	1.0000
3571190	intergenic	intergenic	_	C	T	.	.	T	.	_	0.6048	0.1259	0.7149	-0.3043					-0.4306	-0.5890	-0.3488	-0.3661	-0.5195	_	_
*	STMMW_33741	yhdH	oxidoreductase	.	.	.	.	.	.	_	0.0889	-0.1158	-0.0156	-0.0345	-0.1338	0.0730	0.2752	0.2767	-0.0326	-0.1007	0.4921	0.3163	-0.0651	-1.1172	1.0000
3890627	STMMW_36671		hypothetical protein	T	C	.	.	.	C	_	-0.3791	0.0500	0.3574	-0.0813	-0.5751	-0.1768	-0.1846	-0.0912	-0.1665	0.0823	0.1066	0.3132	0.3686	0.6612	1.0000
3935113	STMMW_37051	rfaY	lipopolysaccharide core biosynthesis protein	C	T	.	.	.	T	V–I	-0.1729	-0.0203	0.1956	0.3465	_	_	_	_	-1.2999	-0.5084	-0.5489	-1.2481	-1.0723	4.9223	0.0135
3944659	STMMW_37151	mutM	formamidopyrimidine-DNA glycosylase	C	T	.	.	.	T	NC	0.1177	-0.0870	0.0764	-0.0967	-0.0151	-0.1803	-0.9431	-0.2928	-0.8293	-1.0935	-0.7635	-0.2160	-0.4932	-0.8785	1.0000
3965328	STMMW_37361	yicE	putative purine permease	A	G	G	G	G	G	NC	0.3983	-0.1413	-0.4725	0.0560	0.0837	0.0649	0.2361	0.1046	0.5869	0.5522	0.5471	0.4219	0.5942	0.6831	1.0000
4131525	intergenic	_	_	G	T	T	.	T	T	_	0.0587	-0.1574	0.4579	-0.1405					0.8018	0.4989	-0.0831	0.3641	0.1571	_	_
*	STMMW_38751	yifB	putative competence protein (chelatase)	.	.	.	.	.	.	_	-0.1807	-0.1047	-0.1759	0.1787	-0.2375	-0.1162	0.0371	0.0720	0.8880	0.6607	0.5722	0.6062	0.6815	-0.6936	1.0000
4254152	STMMW_39841	yihR	aldose-1-epimerase	C	T	.	.	.	T	G–D	-0.0359	-0.1253	-0.2566	-0.2719	-0.3894	-0.0905	0.1333	-0.0236	0.3856	0.1684	0.5225	0.5149	0.2695	0.1322	1.0000
4277479	STMMW_40091		alcohol dehydrogenase	T	C	.	.	C	.	K – E	-0.6723	0.0809	0.5365	-0.0878	-1.0793	-0.1649	0.2076	0.0547	0.2492	-0.2347	-0.1528	-0.0792	0.1386	2.0055	0.6240
4390204	STMMW_41061	rpoB	DNA-directed RNA polymerase, beta-subunit	G	A	.	.	A	.	D – N	0.3065	0.0898	-0.4581	0.0938	0.0605	-0.3139	-0.0183	-0.2457	-0.5416	-0.4098	0.0442	-0.1511	-0.0165	0.7850	1.0000
4563175	STMMW_42471	adi	arginine decarboxylase	C	T	T	.	.	.	NC	0.0029	0.0272	-0.2118	-0.0429	-0.0562	-0.0090	-0.0393	0.0778	0.0881	-0.5809	-0.0158	-0.9032	-0.1787	0.5242	1.0000
4758579	intergenic	_	_	C	T	T	.	.	.	_	0.4810	-0.0514	0.1373	-0.1813					0.2042	0.0245	-0.3482	-0.3422	-0.1582	_	_
*	STMMW_44351		putative restriction endonuclease	.	.	.	.	.	.	_	-0.9200	-0.0405	0.4140	0.1420	-0.2553	-0.1513	-0.1035	-0.1614	0.3831	-0.0821	-0.1317	-0.1023	-0.0293	0.8795	1.0000
4783703	STMMW_44531	uxuR	uxu operon transcriptional regulator	G	A	.	.	A	.	W – STOP	-0.0238	-0.0988	1.5331	-0.2952	-0.0676	0.0055	1.9754	0.0335	0.0766	-0.4971	-0.0316	-0.3157	-0.4815	-1.4251	1.0000

*Gene downstream of intergenic region with SNP **NC: no amino acid change

Tables S4 – S7 are accessible on OSF: https://doi.org/10.17605/OSF.IO/TM5WN

Table S8: Expression of classical serum resistance genes in *S.* Typhimurium

Expression of serum resistance-associated genes *rck*, *traT*, and *pgtE* during serum exposure. Values are log_2 fold changes of read counts of each gene from serum-exposed bacteria relative to LB controls.

locus_tag	gene name	**D23580**	**D24545**	**D24871**	**D25352**	**D26104**	**D23005**
SLT-BT0011	*rck*	-0.3482	0.1431	0.098	-0.2599	-0.5446	-0.157
SLT-BT1041	*traT*	-0.2	-0.1862	-0.0236	-0.0357	0.4077	0.4728
STMMW_24161	*pgtE*	-1.4733	-1.7662	-1.6223	-1.3241	-1.2014	0.9584

Expression of *pgtE* in each isolate compared to D23580. Values are log_2 fold differences of read counts of *pgtE* in each strain compared to D23580 (from strains exposed to LB).

		D24545	**D24871**	**D25352**	**D26104**	**D23005**
STMMW_24161	*pgtE*	-0.40228	-0.26942	0.024125	-3.84386	-0.00068

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CHAPTER 5. OVERALL DISCUSSION, CONCLUSIONS, OUTLOOK

This chapter is a summary of the main findings of the thesis project, how they relate to other studies on vaccinology, *S.* Typhimurium and pathogen biology, and potential future steps.

5.1. Increased complement resistance can occur independently of overall virulence in *S.* Typhimurium

Early studies of human *Salmonella* infections demonstrated that systemic disease is more likely to result from infections with serum resistant than susceptible strains (Roantree and Rantz, 1960), positing that resistant strains are inherently more pathogenic or virulent. It also now appears evident that African *S.* Typhimurium ST313 strains are more invasive (Carden *et al.*, 2017), thus more likely to cause fatal, systemic disease than gastrointestinal isolates over which they now predominate in sub– Saharan Africa. The emergence of *S.* Typhimurium ST313 has largely been driven by multiple drug resistance, leading to the hypothesis that drug resistance and virulence have emerged together in this pathovar (Msefula, 2009). Selection that may occur due to immunity or novel pressure such as vaccines, therefore, would potentially co–select for strains that are not only resistant to bactericidal antibodies, but also more virulent overall.

Most of the *S.* Typhimurium ST313 isolates causing invasive disease in humans characterised thus far are susceptible to antibody–mediated killing (Chapter 2), (Onsare *et al.*, 2015), a possible consequence of circulating in susceptible (either immune compromised or naïve) individuals. The fraction that is fully resistant thus becomes an important focus of this study. Genome sequence variation, either due to polymorphisms, genome degradation or incorporation of phage elements is a significant factor in *S.* Typhimurium ST313 pathogenesis.

The effect of genome alterations that increase resistance to killing by antibody and complement on *Salmonella* virulence and fitness was demonstrated experimentally by identifying and characterising mutants with increased viability under selection in immune human serum from saturating mutant libraries. SL3261, an attenuated *aroA*$^-$ derivative of *S.* Typhimurium SL1344 (Hoiseth and Stocker, 1981) was used as the parent strain for library construction. SL3261, like most invasive ST313 strains, is complement–susceptible. A comparison of this experiment with one derived from the invasive African ST313 strain D23580 also identified a congruence of 49 mutants with enhanced complement

resistance (Appendix I). This set–up also allowed comparisons of isogenic strains, thus controlling for effects of multiple natural variations within different clinical isolates. Two of these genes, *sapA*, and *yfgA*, common to both the SL3261 and D23580 screens, were disrupted in S. Typhimurium strain SL1344. In vivo and in vitro virulence, specifically the ability to invade colon–derived epithelial cells, establish murine infection (both systemic and gastrointestinal), and resist osmotic lysis and detergent activity were then assayed in the resulting definitive mutants, and then each phenotype compared to that observed in the wild–type strain. For both mutants, these traits were either similar to or substantially lower than those of their parent strain, suggesting that increased resistance to killing by antibody and complement in S. Typhimurium occurred without an overall increase in virulence. Similarly, complement resistance did not appear to be advantageous among invasive *Salmonella* ST313 strains when exposed to agents acting on the bacterial membrane such as high NaCl or SDS concentrations (Appendix II) nor an increase in case fatality when compared to susceptible strains (Chapter 2) (Msefula, 2009). The emergence of the ST313 clade II (related to D23580) which includes complement resistant strains, was not associated with increased virulence, nor was virulence restricted to resistant strains (Msefula, 2009; Okoro *et al.*, 2012).

The lack of association between phenotypes that may confer a selective advantage without enhancing bacterial virulence is consistent with reports from studies of other S. Typhimurium strains and pathogenic Enterobacteria. S. Typhimurium strains better able to survive in blood, for instance, were not more invasive in human epithelial cells than isogenic, attenuated ones (Herrero-Fresno *et al.*, 2014a). Likewise, conjugation of an avirulent *Escherichia coli* strain with a plasmid carrying antibiotic, complement resistance and putative virulence genes conferred complement resistance, but not virulence (Johnson *et al.*, 2002). Indeed, despite diminished SPI–1 T3SS protein expression and loss of epithelial cell invasiveness in S. Typhimurium lacking cytoskeletal proteins (such as *yfgA*), the ability to cause systemic murine infection was not lost (Bulmer *et al.*, 2012). Similarly, acquisition of multiple drug resistance in clinical isolates of S. Typhimurium DT104 did not confer enhanced virulence when intramacrophage survival, invasion of epithelial cells, susceptibility to reactive oxygen and nitrogen compounds, and lethal murine infection were compared to a drug–susceptible strain (Allen *et al.*, 2001). This phenomenon is also particularly evident with acquired colistin resistance in *A. baumanii*, which impairs virulence in clinically relevant strains (López-Rojas *et al.*, 2013; Pournaras

et al., 2014; Rolain *et al.*, 2011), postulated to be an explanation for their limited transmission and dissemination in vivo (López-Rojas *et al.*, 2011).

Therefore, genome modifications or selection of traits that increase bacterial fitness or adaptation to invasive disease, such as that which seems to be occurring in ST313 strains, may be limited to the extent of the benefit to the bacteria imposed by a single selective pressure, or a few essential virulence factors, such as has been seen with drug resistance. This phenomenon is partly evident in the high prevalence of novel virulence genes restricted to ST313 strains such as *std–td313* (Herrero-Fresno *et al.*, 2014b), or phage–encoded *gtr*C (Kintz *et al.*, 2015) in S. Typhimurium, which do not seem to be confined to complement–resistant strains, nor affect viability in serum when disrupted. These novel virulence mechanisms could, therefore, be involved in other pathogenic traits of ST313, but do not seem to make a considerable contribution to complement resistance.

5.2. Genotypic variation is likely not associated with complement resistance among *S.* Typhimurium ST313 strains

Most virulence factors are acquired and disseminated within pathovars by horizontal transfer of mobile genetic elements and, less frequently, adaptive or compensatory polymorphisms or mutations (Beceiro *et al.*, 2013; Kelly *et al.*, 2009). Changes in ST313 genomes that affect components of the bacterial envelope have the potential to influence their responses to immune defences, for instance, the presence of the prophage gene *gtr*C, which modulates LPS O–antigen length and resistance to phage infection (Kintz *et al.*, 2015). As earlier alluded to, such non–conserved, horizontally acquired genes outside the O–antigen cluster often contribute to antigenic diversity through the addition of sugar residues that modify O–polysaccharide structure, potentially affecting O–antigen–specific immune responses (Liu *et al.*, 2014), and by extension, antibody–dependent complement–mediated killing. The impact of genetic variation specific to ST313 strains on susceptibility to antibody–dependent complement–mediated serum bactericidal activity, however, is largely unknown. Since protection from iNTS disease depends on eliciting specific antibodies, the presence of selectable genetic elements that confer an advantage during the transient extracellular exposure to antibodies might counteract vaccine immunity.

First, it was hypothesized that resistance to complement killing, like to multiple antibiotics among iNTS strains, is the result of identifiable genetic events, such as polymorphisms or gene flux, that lead to the expansion of invasive *S.* Typhimurium populations, therefore capable of causing a dominance of strains resistant to antibody immunity. This hypothesis was tested by examining the phylogenetic relatedness among 32 invasive African strains isolated between 1997 to 2006, with varying degrees of susceptibility to antibody and complement.

These strains fell into the two major ST313 lineages identified previously (Okoro *et al.*, 2012), associated with strain A130, and the more recent D23580, representing the majority of invasive strains isolated from Africans since 2002 (Msefula, 2009; Okoro *et al.*, 2012). Fully resistant strains (ones that had a net growth when exposed to immune human serum in vitro) were represented in both lineages, with three resistant strains in the A130–like clade I and two in clade II (Chapter 2). Antibody–resistant strains did not form a distinct cluster, and two of these isolates (D23005, and D25352) were interspersed among highly susceptible strains in the phylogenetic tree (hence more closely related to highly susceptible strains than other resistant ones). In contrast, there was considerable clustering of isolates related to the more recent D23580–associated clade, particularly of highly sensitive strains, distinguished from other clade II isolates by three SNP's (Appendix III, Chapter 2), Chapter 4. Genomic features (SNP's, insertions, deletions, inversions, repetitive sequences, or novel mobile genetic elements) of a representative subset of six strains, including fully resistant ones, were analysed more extensively. None of these features, however, were found exclusively among complement–resistant strains (Chapter 4).

Since there was no common genomic feature among all resistant strains, the impact of individual single nucleotide polymorphisms/mutations occurring in the invasive strains were investigated. A total of 52 SNP's occurring in gene–coding regions or closely upstream (putative promoter) regions in 5 strains closely related to D23580 were identified. The impact of each polymorphism was then estimated by correlation with changes in transcript levels of each corresponding gene during serum exposure by RNA–seq, or change in viability of D23580 mutants of each gene following selection in fresh serum. Additionally, a subset of SNP's predicted in–silico to have an impact on protein function were transferred individually to D23580 by phage transduction, and the impact on complement sensitivity tested in serum bactericidal assays. No association between SNP's and changes in gene

expression or alterations in viability in immune serum that suggested a direct effect on susceptibility to killing by antibody and complement were found among invasive ST313 strains.

These results, therefore, suggest that, in addition to resistance not occurring in these strains by emerging from a common resistant isolate, complement resistance in these invasive S. Typhimurium strains was not linked to acquiring a common mobile genetic element conferring complement resistance. These observations also suggest the absence of natural antibody immunity acting as a selective influence on S. Typhimurium population structure among invasive African strains. Since S. Typhimurium among African populations largely infects and persists in individuals with inadequate antibody immunity, the high prevalence of complement–susceptible strains could have a fitness advantage among these strains, with the occasional occurrence of resistant clones deleterious to transmission due to high associated mortality or unsustainability of the resistant phenotype.

5.3. Identification of bacterial factors that enable *S.* Typhimurium strains to survive complement–mediated killing acting via antibody

Identifying the important determinants of antibody immunity is an important consideration for the design of antibody–inducing vaccines for invasive *Salmonellae*. These immune responses are a function of several genetic, epigenetic and growth characteristics (Taylor, 1983). It is therefore unlikely that antibody resistance (or indeed other virulence mechanisms) act in isolation (Dobrindt, 2005), and full expression of these traits depend on combinations of factors, which may either synergise, or regulate a major resistance factor, such as O–antigen or other membrane structures.

Most of the S. Typhimurium strains causing invasive disease that have been studied so far are genotypically homogenous (Msefula *et al.*, 2012), and among ST313 strains, this study did not find a common genetic determinant of complement resistance (Chapter 2, Chapter 4). The significant genetic relatedness of the fully resistant and highly susceptible strains in this study, therefore, suggests that resistance does not solely depend on the presence or absence of resistance–associated genetic features or genes, and was thus hypothesized to be due to variation in levels of expression between isolates with either resistant or susceptible phenotypes, as is the case with virulence traits determined by multiple factors (Pitout, 2012).

Responses of resistant and susceptible isolates to antibody–dependent complement–mediated bactericidal activity were thus analysed by measuring changes in gene expression levels during in vitro incubation in normal, immune human serum by RNA–seq. Similar studies on *Salmonella* and other Gram–negative invasive bacterial pathogens have yielded various results, partly influenced by variations in experimental conditions. To overcome the practical challenge posed by incubation in active human serum on the quality and stability of bacterial RNA for transcriptome analyses, many studies use dilute serum, enabling longer incubation times (Huja *et al.*, 2014; Miajlovic *et al.*, 2014). In–vitro assays on iNTS strains, however, demonstrate that an exposure time of 10 minutes is sufficient for antibody–dependent complement–mediated killing and uptake by macrophages to occur detectably (Siggins *et al.*, 2014), hence longer extracellular exposure times in blood or serum are less likely to recapitulate this initial response. The experiments thus analysed changes in transcription following a 10–minute exposure to neat, active human serum. To distinguish between innate humoral immunity and adjustment to nutrient scarcity in serum, we also compared differentially expressed genes between resistant and susceptible ST313 strains, identifying factors contributing specifically to resistance to antibody and complement–mediated killing.

First, a common set of differentially expressed genes from serum–incubated bacteria relative to standard laboratory conditions (growth in LB) were identified in six ST313 strains (three susceptible, three resistant), to identify factors necessary for their survival of in serum (Chapter 4, Appendix IV). The fold changes were then contrasted between the resistant and susceptible isolates, so as to isolate putative determinants of complement resistance. Additionally, the full complement of genes necessary for serum survival of *S.* Typhimurium strain D23580 was described by identifying genes and gene functions significantly diminished in transposon mutants selected in immune serum. Two major gene functions were identified; iron scavenging or metabolism, and membrane polysaccharide synthesis.

Most of the up–regulated genes (47%) were associated with iron assimilation and iron–associated metabolism in the iNTS strains studied. There was also a significant loss of viability in corresponding serum–exposed mutants (Chapter 4). Assimilation of iron is an essential component of growth of extraintestinal pathogenic bacteria in serum or blood, where up to 80% of the genes (regulated by the RpoS/Fur stress response system), are associated with iron scavenging and metabolism (Huja *et*

al., 2014; Pettersen *et al.*, 2016; Huja *et al.*, 2015). Growth factors such as iron and zinc are critical for the establishment of invasive disease in bacteria, and their sequestration from serum is an important aspect of the host's response to invasive bacterial infection (Hood and Skaar, 2010; Skaar, 2010). This is particularly evident in individuals with severe malaria, where risk of severe bacteraemia is highest during peak parasite haemolytic activity as a result of increased availability of free iron (Takem *et al.*, 2014).

There was also significant up–regulation of genes (12.5% of the total up–regulated genes) in the *wca* cluster in all the strains, which are responsible for the synthesis of colanic acid (CA) polysaccharide (Chapter 4). While CA is important for environmental survival, its role in survival within the human host, particularly in evading host responses is less adequately studied. This study identifies the potential role of this polysaccharide in the growth of invasive S. Typhimurium in immune serum. The *wca* operon is disrupted in S. Typhi (Nuccio and Bäumler, 2014), a possible consequence of restriction to a human, rather than an environmental reservoir, or possibly redundancy of the colanic acid polysaccharide due to the presence of the Vi capsule. This gene cluster was also significantly up–regulated in extraintestinal pathogenic *E. coli* (ExPEC) during exposure to serum, and has been postulated to enhance evasion of complement bactericidal activity by shielding the bacterial membrane (Miajlovic *et al.*, 2014). Another possible mode of colanic acid modulating immunity is masking O–antigen and other pathogen–associated molecular patterns (Li *et al.*, 2017a), repressing complement activation and other components of the immune system. This effect has been demonstrated by the enhanced immunogenicity observed in a colanic acid–deficient S. Typhimurium vaccine strain (Wang *et al.*, 2013), an effect similar to that of loss of the Vi capsule in S. Typhi (Crawford *et al.*, 2013).

Most of the downregulated genes (55%) were those associated with synthesis of components of the envelope (Chapter 4). Significant downregulation of *phoPQ* and several *phoPQ*–regulated genes, in particular, was observed among all the S. Typhimurium strains during exposure to immune serum, including fully resistant strains. Under these conditions, transcriptional profiles of virulence–associated genes (specifically SPI genes) did not show correlation with complement resistance either (genes did not cluster by sensitivity phenotype), despite LPS and CA genes being associated with complement resistance phenotype (Chapter 4). This suggests restriction of *phoPQ*–mediated

virulence factors to other phases of infection, such as the intramacrophage environment. Similarly, *phoP* and *pmrB* mutants of S. Typhimurium (defective in *phoPQ* and *pmrAB* two–component system activity respectively), had negligible to no impairment of fitness when exposed to antimicrobial peptides, pH and oxidative stress, serum and bile, and during murine infection (Lofton *et al.*, 2015).

There was no strong evidence found for a direct contribution of classical serum resistance genes such as *rck*, *pgtE*, or *traT*, to evasion of complement lytic activity in the iNTS strains in this study, as they were all present and intact in all the strains regardless of their sensitivity to complement, and were not differentially expressed during serum exposure, nor did their absence in strain D23580 affect viability in serum (Chapter 4). The *mli*C gene (membrane–bound lysozyme inhibitor of C–type lysozyme), however, was significantly up–regulated in all the six iNTS strains. Transcription of *mli*C like the *wca* operon, is controlled by Rcs, a major regulator for stress response genes (Miajlovic *et al.*, 2014). Although its role in S. Typhimurium virulence is not well established, expression of *mli*C increased significantly in pathogenic *E. coli* exposed to serum (Miajlovic *et al.*, 2014), and absence of the gene substantially diminished in vitro resistance to complement killing (Vanderkelen *et al.*, 2012). Lytic activity of complement is a function of lysozyme acting on murein in the bacterial envelope, thus *mli*C and other inhibitors of lysozyme have a well–described role in contributing to resistance to complement bactericidal activity (Li *et al.*, 2015a; Vanderkelen *et al.*, 2012). Whereas SL3261 or D23580 TraDIS screens were not able to adequately pinpoint changes in *mli*C mutants during serum exposure, perhaps more precise or targeted experiments could be used to explore the contribution of *mli*C and other lysozyme inhibitors to S. Typhimurium survival in the bloodstream, and to invasive disease.

To isolate the effect of gene regulation affecting complement resistance, comparisons of log fold changes in mRNA levels following serum incubation of the iNTS isolates were performed. This identified a global transcriptional pattern distinguishing resistant and susceptible strains. Genes associated with lipopolysaccharide biosynthesis and colanic acid (*wca* cluster genes) in particular, clustered by complement sensitivity phenotype. Unsurprisingly, LPS mutants also had significantly reduced viability in serum (Chapter 4).

Two O–polysaccharide–associated genes were of particular interest; *fepE*, which determines expression of very long O–antigen chains, was two–fold more highly up–regulated in resistant strains,

while mRNA levels of *wzzB*, associated with O–antigen chains averaging between 16 and 35 repeating units, were upregulated 2.4 times higher in susceptible strains. Differential expression of very long O–antigen on the bacterial surfaces is associated with a more effective blockade of complement deposition on the bacterial membrane and insertion of the membrane attack complex than long O–antigen (Murray *et al.*, 2005), and subsequently a higher level of resistance. Transcriptional adaptation of *S.* Typhimurium to environmental changes begins as early as 4 minutes (Rolfe *et al.*, 2012). Since the rate of bactericidal activity is highest within the first 45 minutes of exposure to serum in vitro, increase transcription of *fepE* in resistant strains within 10 minutes of incubation in serum suggests more rapid O–antigen adaptation, and may explain the differences in the responses of the two groups of strains to the serum bactericidal activity.

The dominance of serum–susceptible over resistant isolates circulating in Africa implies a cost to fitness associated with VL–Oag expression. Acquisition of some traits in *S.* Typhimurium is cost–free, for example, an antimicrobial peptide–resistant strain defective for the LPS core biosynthesis gene *waaY* did not have diminished ability to survive stressors such as bile, serum, low pH, oxidative stress, or antimicrobial peptides (Lofton *et al.*, 2015). Adaptation to bloodstream or systemic infection may, therefore, imply that strains with longer O–antigen would be under intense selection pressure causing a dominance of complement–resistant strains, but there is no evidence of this occurring among ST313 isolates. Complement–resistant iNTS strains are not known to cause higher fatality than sensitive ones, which might otherwise be a potential threat to fitness that limits transmission (Msefula, 2009). Very long–chain O–antigen synthesis, however, is genetically and metabolically costly, thus wzz_{fepE} is dispensable during much of an *S.* Typhimurium infection (Murray *et al.*, 2005). This might be a disadvantage to resistant strains, especially since immune selection in predominantly susceptible hosts seems to be virtually absent, and that other factors, such as the rate at which *S.* Typhimurium ST313 enter, shelter and survive within macrophages seem to offset sensitivity to complement lysis (Ramachandran *et al.*, 2015; Siggins *et al.*, 2014). It is evident, from studies of other bacteria expressing O–antigen, that LPS–mediated resistance is not cost–free, and can be disadvantageous for fitness in the absence of selective pressure (Durante-Mangoni *et al.*, 2015; Vila-Farres *et al.*, 2015; Crawford *et al.*, 2013). Significant down–regulation of *wzz*–regulatory genes *phoPQ* and *pmrD* in serum (Chapter 4), also suggests a specific pathway for O–antigen length modification

that is independent of *phoPQ*, which is predominantly associated with intramacrophage survival in the bloodstream.

Upregulation of colanic acid genes was also markedly higher in susceptible strains (Chapter 4, Appendix IV). Since disruption of the *wca* operon in S. Typhimurium D23580 (either in multiple transposon mutants or in a defined *wcaB* null mutant) resulted in a negligible change in susceptibility to serum bactericidal activity, colanic acid expression seems to have a nonessential but synergistic role in protecting the bacteria from immune attack, and that up–regulation of *wca* gene expression (and increased CA synthesis) is perhaps a less costly means of survival of S. Typhimurium in serum, and that an optimal level of O–antigen expression in the presence of surface polysaccharides is attributable to the differences in susceptibility (Crawford *et al.*, 2013). The significant up–regulation of this gene cluster in antibody–susceptible and highly susceptible strains may also be indicative of an inherent inability of susceptible strains to increase LPS production, or perhaps an unknown membrane defect. *E. coli* strains with structural defects in lipopolysaccharide, for instance, had increased colanic acid production (Ren *et al.*, 2016).

The hypothesis that *wca* operon transcription is increased in compensation for a membrane defect is also supported by an increase in resistance to serum bactericidal activity when S. Typhimurium genes encoding proteins responsible for the synthesis of other membrane polysaccharides are deleted. Two mutants deficient in membrane–derived oligosaccharide/MDO (also called osmoregulated periplasmic glucan/OPG) synthesis, SL1344Δ*mdoG*::*aph*, SL1344Δ*mdoH*::*aph*, for instance, were more resistant to killing in normal human serum than the isogenic wild–type strain (Chapter 3). S. Typhimurium *mdo* knockout strains have increased colanic acid synthesis (Ebel *et al.*, 1997), and also increased resistance to endogenously expressed phage lytic activity (Höltje *et al.*, 1988), although the presence of the *mdoGH* locus is necessary for S. Typhimurium virulence in vivo (Bhagwat *et al.*, 2009). This increase in colanic acid synthesis is characteristic of a common extracytoplasmic stress response in bacteria (Rowley *et al.*, 2006), mediated by sigma factor *rpoS*.

Similarly, disruption of several genes responsible for the synthesis of components of the bacterial envelope enhanced resistance to serum bactericidal activity (Chapter 3). This suggests a contribution, at least in part, of a proper conformation of the bacterial envelope in facilitating complement–

mediated lysis. *mdoGH* mutants, for instance, have been shown to have a wider periplasmic space that interferes with phage lytic activity (Höltje *et al.*, 1988), and could possibly also have mitigated the extent of damage induced by disrupting insertion of the membrane attack complex in the *mdoGH* mutants in this study (Chapter 3). Increased colanic acid synthesis could also mask important epitopes on the surface, and therefore decrease complement activation despite similar levels of available antibody.

Natural variation in serum susceptibility in the S. Typhimurium ST313 strains studied herein, however, does not seem to be the direct result of presence or absence of genes affecting complement resistance, but rather differences in the relative expression of different components of the bacterial envelope, more specifically of membrane polysaccharides. Given their significant antigenic nature, changes in expression of surface polysaccharides can vary considerably, with impacts on clinical outcome. Unencapsulated strains of S. Typhi, *N. meningitidis,* and S. *pneumoniae*, for example, are known cause invasive disease, and in some instances associated with complement resistance, such as with *H. influenzae* b (Williams *et al.*, 2001). Given that surface polysaccharides are not static features of the bacterial envelope, the impact of host environments on gene regulation affecting evasion of host immunity might have implications for vaccines and vaccine–induced immunity.

To analyse the effect of phase variation causing nonuniform bacterial populations and potentially facilitating immune evasion, serum bactericidal assays on 100 clones of a single generation of two closely related strains D23005 (serum resistant) and D24545 (serum–susceptible) were performed (Chapter 4). This did not reveal both resistant or sensitive isolates of the same strain, although some heterogeneity in complement susceptibility phenotype was observed. However, no direct effect of a change in DNA sequence was found to be associated with complement resistance in S. Typhimurium ST313 isolates despite differences in transcription between resistant and susceptible strains being detected within 10 minutes of serum exposure. This observation suggests a yet unidentified epigenetic mechanism regulating complement resistance in these strains, possibly not associated with phase variation. Indeed, traits in ST313, such as *gtr*–mediated control of O–antigen length and phage resistance, are determined epigenetically via phase variation (Kintz *et al.*, 2015). Phase variation has also been shown to affect *gtr*–dependent O–antigen glucosylation, associated with decreased antibody recognition, complement binding and subsequently enhanced serum survival in S. Typhi (Kintz *et al.*,

2017) and, importantly, was demonstrated under infection–relevant conditions, which perhaps might reveal associations in S. Typhimurium 313 less likely to be replicated during growth in standard laboratory conditions.

5.4. Conclusions

An effective vaccine is an important prospect for iNTS disease control in sub–Saharan Africa, particularly under the current clinical circumstances. Current strategies for vaccination target major surface components, chiefly the LPS O–antigen. O–antigens are uniquely central to both protective immune responses and complement resistance, hence subject to selective pressure and antigenic variation. It has previously been demonstrated, however, that modifications to the sugar composition of O–antigen in invasive S. Typhimurium isolates have a negligible effect on complement susceptibility or immunogenicity, and that most strains are susceptible to the bactericidal activity of immune serum (Onsare *et al.*, 2015), (Chapter 2).

This study identifies a putative role for O–antigen chain length in mediating resistance in invasive S. Typhimurium strains by increased *fepE* expression, but the potential metabolic cost associated with complement resistance could present a challenge to bacterial fitness among community isolates, and without an appreciable benefit to overall virulence. Additionally, complement resistant isolates are genetically distinct, and do not share any genetic loci explicitly associated with the resistance trait. These observations, therefore, support the advancement of O–antigen–based antibody–inducing vaccines for prevention of severe NTS bacteraemia.

This thesis postulates a short–term, reversible or within–host mode of complement resistance rather than a permanent genetic change preceding transmission of complement resistant strains, consistent with reports in current literature from observations in other pathogenic bacteria (Pishko *et al.*, 2003).

This study is an initial description of the "serum resistome" and transcriptome of an S. Typhimurium ST313 isolate. These analyses have elucidated potential mechanisms for growth and survival of iNTS strains in serum and also identified a wider array of possible genes which modulate antibody/complement resistance phenotype. Other membrane proteins conventionally known to be associated with antibody and complement resistance in S. Typhimurium, however, did not seem to have a noticeable contribution to this phenotype. The different responses to complement lysis in the

S. Typhimurium strains in this study could be attributable to differences in the relative expression of surface polysaccharides.

5.5. Research Questions for Further Study and Future Directions

There is growing evidence for a human reservoir for NTS in Africa (Kariuki *et al.*, 2006b), and post–bacteraemic children have been hypothesized to be a reservoir for invasive strains (Msefula, 2009). Since complement resistance can be acquired in vivo (Pishko *et al.*, 2003) even following vaccination (Williams *et al.*, 2001), this raises the question as to what changes that occur in bacterial populations following carriage and subsequent immunity to S. Typhimurium. Additionally, the question as to whether complement resistance might become favorable for the bacteria following infection under immune selection, and whether these strains spread to and proliferate within dominantly susceptible host populations may be a question worth further investigation. Analyzing changes in complement sensitivity and gene expression of isolates serially passaged in immune sera or in vivo could be one way to examine this.

Since much of what is known about S. Typhimurium ST313 is from patient isolates, genetic, and phenotypic characterization of complement resistant community isolates from healthy or immune individuals also might provide a more comprehensive picture this phenotype in iNTS, and better predict changes in S. Typhimurium ST313 populations following the introduction of a vaccine, and the potential impact of host immunity.

While the goal of this study was to examine the relationship between genotype and phenotype, the absence of a distinct genetic feature and common transcriptome profiles suggest epigenetic control of complement resistance factors among the strains in this study. If this is the case, a contribution of epigenetic factors, particularly those associated with short–term expression of surface components during serum incubation with the potential to modulate complement susceptibility could also be investigated further. This could be achieved, for instance, by assaying promoter activity of genes regulated by two–component systems, e.g., *pmrAB* in complement resistant strains.

Genome–wide experiments have also identified several new putative factors and possibly compensation mutations that could possibly be involved in S. Typhimurium fitness during exposure to serum/complement, which could be assayed further. A benefit of testing these genes individually,

for example, by defined gene knockouts or low–throughput gene expression studies would offset a drawback of high–throughput experiments (Reynolds *et al.*, 2011). It would isolate random or pleiotropic effects associated with assaying thousands of genes from those specifically associated with complement bactericidal activity.

Specifically, this study has demonstrated that the CA polysaccharide expression increases significantly during exposure of *S.* Typhimurium to serum and might have a role in modifying responses to complement. Since the absence of *wca* enhances immunogenicity of S. Typhimurium antigens (Wang *et al.*, 2013), the possibility of CA polysaccharide interacting with O–antigen and whether this modifies the associated immune responses may be worth considering for future studies, or their effects on NTS vaccines and vaccine immunity, especially of those not based on purified polysaccharides.

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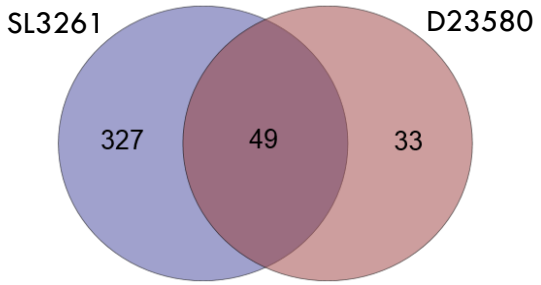
APPENDIX I

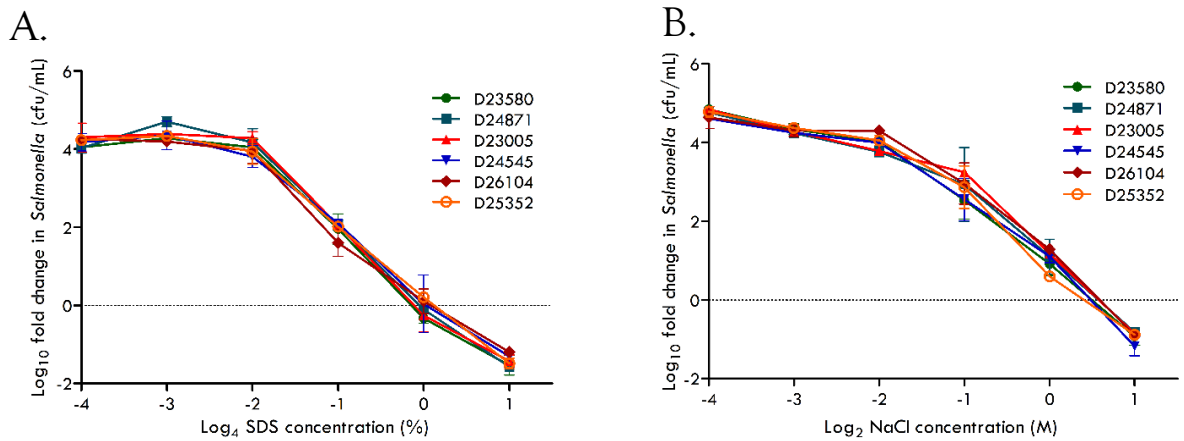
Comparison of serum–susceptibility determinants (genes associated with mutants that were significantly over–represented in output vs input pools) genes identified by TraDIS. This list includes genes that were common to experiments that used SL3261 and D23580 mutant libraries screened in immune human serum (all had p values <0.05).



GeneID	Description of product function
mraZ	cell division protein MraZ
pdhR	pyruvate dehydrogenase complex repressor; activates lctPRD operon
proB	glutamate 5 (gamma glutamate)–kinase
proA	gamma–glutamyl phosphate reductase
mrdA	penicillin–binding protein 2; similar to *E. coli* cell elongation e phase protein
ybeA	rRNA large subunit methyltransferase
ybeB	conserved hypothetical protein (similar to ACR plant lojap protein)
nagA	N–acetylglucosamine–6–phosphate deacetylase
himD	integration host factor beta–subunit (IHF–beta)
SL1344_0979	predicted bacteriophage protein
mdoG	glucan biosynthesis protein G
mdoH	glucans biosynthesis glucosyltransferase H
plsX	glycerol–3–phosphate acyltransferase PlsX
fabF	3–oxoacyl–(acyl carrier protein) synthase II
pykF	pyruvate kinase
sapA	peptide transport periplasmic protein SapA precursor
sapB	peptide transport system permease protein SapB
sapC	peptide transport system permease protein SapC
sapD	peptide transport system ATP–binding protein SapD
sapF	peptide transport system ATP–binding protein SapF
zwf	glucose–6–phosphate 1–dehydrogenase
wcaJ	hypothetical extracellular polysaccharide biosynthesis protein
lysP	lysine–specific permease
rcsC	hybrid sensory kinase in two–component regulatory system with RcsB and YojN
crr	pts system protein
yfgA	cytoskeletal protein RodZ
yfhA	hypothetical transcriptional regulator
amiC	N–acetylmuramoyl–L–alanine amidase
dctP	periplasmic dicarboxylate–binding protein
yraO	DnaA initiator–associating protein DiaA
yrbA	conserved hypothetical protein (BolA family)
arcB	aerobic respiration control sensor protein ArcB
mreD	rod shape–determining protein; part of cell wall structural complex MreBCD
mreC	rod shape–determining protein MreC

rsmB	16S rRNA (cytosine967–C5)–methyltransferase
sapG	potassium transporter peripheral membrane protein
crp	cyclic AMP receptor protein
pitA	hypothetical low–affinity inorganic phosphate transporter
rfaY	heptose (II) phosphotransferase
rfaQ	heptosyltransferase III
cyaA	adenylate cyclase
tatC	sec–independent twin–arginine protein translocation system subunit
trkH	trk system potassium uptake protein
glnG	two–component system protein
glnA	glutamine synthetase
pfkA	6–phosphofructokinase
metB	cystathionine gamma–synthase
metL	bifunctional aspartate kinase II/homoserine dehydrogenase II
alr–b	alanine racemase

APPENDIX II



Susceptibility of six invasive *S.* Typhimurium strains to detergent and osmotic stress. Log_{10} change in viable bacterial counts of *S.* Typhimurium iNTS strains following incubation in LB broth containing varying concentrations of either **A.** SDS, or **B.** NaCl, at 37°C for 8 hours. No statistically significant difference in minimum inhibitory concentration was observed for any strain. Starting bacterial concentrations were 2.5×10^3 cfu/ml. Data represent means of two independent experiments. Error bars: SEM.

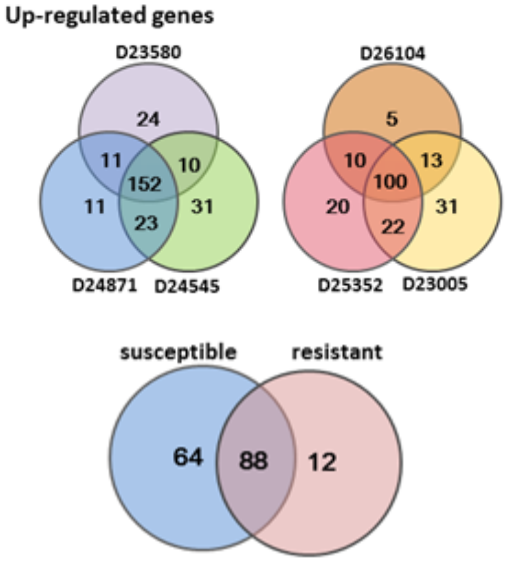
APPENDIX III

SNP's distinguishing 5 highly serum sensitive isolates in clade II.

Position in alignment	SL1344	SNP base	gene name	description
1224532	G	T	*rne*	ribonuclease E
3241318	C	A	*pepP*	proline aminopeptidase
4276246	T	C	*SL1344_4416*	alcohol dehydrogenase

APPENDIX IV

Differentially expressed genes in response to serum exposure in six iNTS strains. Venn diagrams showing numbers of differentially expressed genes determined by RNA–seq from growth in serum compared to LB (log_2 fold difference 1.5 or more and adjusted p–value <0.05) in the six strains. (Sensitive strains: D23580, D24871, D24545, Resistant strains: D26104, D25352, D23005).



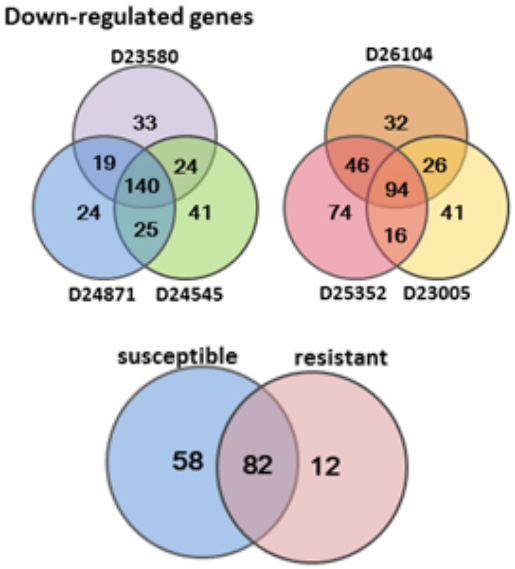
Genes significantly upregulated in sensitive strains only

Locus tag	Gene name	Description
STMMW_00401	*nhaA*	pH-dependent sodium/proton antiporter
STMMW_00411	*nhaR*	transcriptional activator protein NhaR
STMMW_00821		hypothetical lipoprotein
STMMW_04481	*yaiY*	hypothetical membrane protein
STMMW_04841	*yajI*	hypothetical lipoprotein
STMMW_05281	*cof*	conserved hypothetical protein
STMMW_06021	*purE*	phosphoribosylaminoimidazole carboxylase catalytic subunit
STMMW_08481	*bioC*	biotin synthesis protein BioC
STMMW_08491	*bioD*	dethiobiotin synthetase
STMMW_10981	*yccA*	hypothetical membrane protein
STMMW_12201	*ndh*	NADH dehydrogenase
STMMW_12211	*ycfJ*	hypothetical secreted protein
STMMW_12521	*envE*	hypothetical lipoprotein
STMMW_12561	*pliC*	periplasmic lysozyme inhibitor of c-type lysozyme
STMMW_13151		eriplasmic protein induced by stress response via Cpx and BaeSR system
STMMW_13511	*ydiV*	diguanylate cyclase/phosphodiesterase domain 1
STMMW_14501	*mliC*	membrane-bound lysozyme inhibitor of c-type lysozyme
STMMW_14911		hypothetical ABC transporter ATP/GTP-binding protein
STMMW_14921		hypothetical ABC transporter membrane protein
STMMW_14931		hypothetical ABC transporter periplasmic binding protein
STMMW_14941		hypothetical ABC transporter membrane protein
STMMW_15141	*ydeJ*	competence damage-inducible protein A
STMMW_15591	*yddX*	biofilm-dependent modulation protein
STMMW_16431	*hslJ*	heat shock protein (hslJ)
STMMW_17211	*trpB*	tryptophan synthase beta chain
STMMW_17251	*yciE*	conserved hypothetical protein
STMMW_18301		hypothetical exported protein
STMMW_18641	*purT*	phosphoribosylglycinamide formyltransferase 2
STMMW_19611	*fliR*	flagellar biosynthetic protein FliR
STMMW_19621	*rcsA*	colanic acid capsullar biosynthesis activation protein A
STMMW_21101	*wzzB*	lipopolysaccharide O-antigen chain length regulator
STMMW_21351	*cpsG*	phosphomannomutase in colanic acid gene cluster
STMMW_21361	*cpsB*	mannose-1-phosphate guanylyltransferase
STMMW_21381	*gmm*	hypothetical O-antigen biosynthesis protein
STMMW_21441	*wcaD*	colanic acid polymerase
STMMW_21481	*wzc*	tyrosine-protein kinase
STMMW_21711	*yegS*	conserved hypothetical protein
STMMW_24241	*lpxP*	encodes the cold shock-induced palmitoleoyl transferase
STMMW_24311	*ypeC*	conserved hypothetical protein
STMMW_24951	*ypfG*	hypothetical exported protein

Genes significantly upregulated in resistant strains only

Locus tag	Gene name	Description
STMMW_04341	*foxA*	ferrioxamine B receptor precursor
STMMW_08241	*gpmA*	phosphoglycerate mutase 1
STMMW_22301		hypothetical regulatory protein
STMMW_25051	*purC*	phosphoribosylaminoimidazole-succinocarboxamide synthase
STMMW_32381	*yhaN*	conserved hypothetical protein
STMMW_32391	*yhaO*	hypothetical membrane transport protein
STMMW_35511	*livM*	high-affinity branched-chain amino acid transport system permease protein
STMMW_38841	*ilvC*	ketol-acid reductoisomerase
STMMW_42621		predicted bacteriophage protein
STMMW_42631		conserved hypothetical protein
STMMW_42641	*rtsB*	Regulator of flhDC

STMMW_25161	*purM*	Phosphoribosylformylglycinamidine cyclo-ligase
STMMW_26581	*rseB*	sigma-E factor regulatory protein RseB precursor
STMMW_26591	*rseA*	sigma-E factor negative regulatory protein
STMMW_26601	*rpoE*	RNA polymerase sigma-E factor (sigma-24)
STMMW_27681	*ygaC*	conserved hypothetical protein
STMMW_29681	*ygdR*	possible lipoprotein
STMMW_30261	*yggE*	periplasmic immunogenic protein
STMMW_30381	*yggG*	conserved hypothetical protein (contains chaperone function)
STMMW_30681	*yggN*	hypothetical protein
STMMW_31641	*ygiM*	hypothetical signal transduction protein
STMMW_31651	*yobH*	tRNA nucleotidyltransferase
STMMW_33091	*yrbC*	possible exported protein
STMMW_33911	*yhdV*	possible lipoprotein
STMMW_34671	*nirC*	hypothetical nitrite transporter
STMMW_35491	*livF*	high-affinity branched-chain amino acid transport ATP-binding protein
STMMW_35501	*livG*	high-affinity branched-chain amino acid transport ATP-binding protein
STMMW_36351	*yiaD*	hypothetical outer membrane protein
STMMW_36511	*bax*	hypothetical exported amidase
STMMW_37511	*cigR*	hypothetical inner membrane protein
STMMW_41721	*yjbF*	hypothetical lipoprotein
STMMW_41731	*yjbG*	hypothetical exported protein
STMMW_42191	*yjcD*	hypothetical xanthine/uracil permeases family protein
STMMW_44001	*treR*	trehalose operon repressor



Genes significantly downregulated in sensitive strains only

Locus tag	Gene name	Description
STMMW_06231		hypothetical membrane protein
STMMW_07271	*gltL*	glutamate/aspartate transport ATP-binding protein
STMMW_07301	*gltI*	ABC transporter periplasmic binding protein
STMMW_07911	*sdhA*	succinate dehydrogenase flavoprotein subunit
STMMW_08821	*dps*	DNA protection during starvation protein
STMMW_10831	*ompA*	outer membrane protein A
STMMW_11011		conserved hypothetical inner membrane protein
STMMW_11021	*pipC*	cell invasion protein
STMMW_11301	*ymdF*	conserved hypothetical protein
STMMW_11401		hypothetical secreted protein
STMMW_12541	*pagD*	hypothetical outer membrane virulence protein
STMMW_12891	*yeaJ*	conserved hypothetical membrane protein
STMMW_13971	*spiR*	two-component sensor kinase
STMMW_14341	*sodB*	superoxide dismutase
STMMW_14691	*fumA*	Fumarate hydratase class I, aerobic
STMMW_15291		outer membrane protein
STMMW_15391		hypothetical secreted hydrolase
STMMW_15781	*steA*	Type III secretion system effector protein
STMMW_17741	*ychH*	hypothetical exported protein
STMMW_18431		hypothetical protein
STMMW_19061	*motA*	motility protein A
STMMW_19401	*fliD*	flagellar hook associated protein (FliD)

Genes significantly downregulated in resistant strains only

Locus tag	Gene name	Description
STMMW_15371	*hyaB2*	uptake hydrogenase-1 large subunit
STMMW_15961	*ugtL*	hypothetical membrane protein
STMMW_22361	*fruA*	PTS system, fructose-specific IIBC component
STMMW_24331	*nupC*	nucleoside permease NupC
STMMW_28811	*ygbJ*	conserved hypothetical protein
STMMW_34281	*rpsS*	30S ribosomal subunit protein S19
STMMW_34291	*rplB*	50S ribosomal subunit protein L2
STMMW_34301	*rplW*	50S ribosomal subunit protein L23
STMMW_35001	*comF*	competence gene-DNA binding and transport
STMMW_35011	*yhgI*	conserved hypothetical protein
STMMW_38441		hypothetical shikimate 5-dehydrogenase
STMMW_43851		hypothetical protein

STMMW_20691	*pduA*	hypothetical propanediol utilization protein
STMMW_20701	*pduB*	hypothetical propanediol utilization protein PduB
STMMW_22921	*ompC*	outer membrane protein C
STMMW_23081	*glpA*	anaerobic glycerol-3-phosphate dehydrogenase subunit A
STMMW_23091	*glpB*	Anaerobic glycerol-3-phosphate dehydrogenase subunit B
STMMW_23101	*glpC*	anaerobic glycerol-3-phosphate dehydrogenase subunit C
STMMW_23111	*sseL*	Type III secretion system effector protein, deubiquitinase
STMMW_23631		hypothetical transketolase N-terminal section
STMMW_25661	*asrB*	anaerobic sulfite reductase subunit B
STMMW_27561	*csiD*	Protein csiD.
STMMW_29231	*gudD*	probable glucarate dehydratase 1
STMMW_29241	*ygcY*	probable glucarate dehydratase
STMMW_29251	*gudT*	probable glucarate transporter
STMMW_30121		hypothetical protein
STMMW_31121		methyl-accepting chemotaxis protein
STMMW_31561	*ribB*	3,4-dihydroxy-2-butanone 4-phosphate synthase
STMMW_31581	*glgS*	glycogen synthesis protein GlgS
STMMW_32171		methyl-accepting chemotaxis protein II
STMMW_32181	*aer*	aerotaxis receptor protein
STMMW_32471	*garK*	conserved hypothetical protein
STMMW_32481	*garRb*	2-hydroxy-3-oxopropionate reductase
STMMW_32491	*garL*	5-keto-4-deoxy-D-glucarate aldolase
STMMW_32501	*garD*	D-galactarate dehydratase
STMMW_33371	*nanT*	hypothetical sialic acid transporter
STMMW_33381	*nanA*	N-acetylneuraminate lyase
STMMW_35371		conserved hypothetical protein
STMMW_35661	*tcp*	methyl-accepting chemotaxis citrate transducer
STMMW_35811	*yhiP*	hypothetical PTR2 family transport protein
STMMW_36001	*yhjH*	conserved hypothetical protein
STMMW_36031	*dctA*	C4-dicarboxylate transport protein
STMMW_37091	*yibR*	conserved hypothetical protein
STMMW_38701	*rbsR*	ribose operon repressor
STMMW_42461	*adiY*	hypothetical AraC family regulatory protein
STMMW_43871		hypothetical membrane protein