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Chronic and acute infection of the gall bladder by *Salmonella* Typhi: understanding the carrier state

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Abstract

Despite major treatment and prevention efforts, millions of new typhoid infections occur worldwide each year. For a subset of infected individuals, *Salmonella enterica* subsp. *enterica* serovar Typhi colonizes the gall bladder and remains there long after symptoms subside, serving as a reservoir for the further spread of the disease. In this Progress article, we explore recent advances in our understanding of the mechanisms by which *Salmonella* spp. — predominantly *S.* Typhi — colonize and persist in the human gall bladder.

Typhoid or enteric fever is caused primarily by *Salmonella enterica* subsp. *enterica* serovar Typhi and is a human-specific disease for which there are an estimated 21 million new infections every year, resulting in approximately 200,000 deaths[1]. Typhoid is an acute illness often characterized by high fever, malaise and abdominal pain[2]. Globally, children are disproportionately affected, especially in south central Asia, Southeast Asia, Latin America and Southern Africa, where the incidence of antibiotic resistance exacerbates the morbidity and mortality associated with the disease[1,3]. Serious complications include intestinal perforation, septicaemia and meningitis, with the highest incidence of these being found in paediatric and immunocompromised patients. These complications are life threatening and require advanced medical care that is often not available in typhoid-endemic regions[4–6].

Typhoid is most commonly spread by ingestion of contaminated water or food. Following entry into the small intestine, the bacteria cross the intestinal epithelial barrier (probably by invasion of microfold (M) cells in the Peyer's patches and lymphoid-associated tissues), are phagocytosed by macrophages and spread systemically, producing acute disease[7–10]. The most common sites of infection are the ileum, liver, spleen, bone marrow and gall bladder.

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The bacteria reach the gall bladder through the vasculature or the ducts that emanate from the liver[2,11]. Over the past decade, the incidence of antibiotic resistance among *S.* Typhi isolates has risen dramatically in endemic regions. Strains that are refractory to almost every available first-line antibiotic have been recovered, and up to 60% of all strains isolated exhibit multidrug resistance[12,13]. Fortunately, with adequate treatment, most patients recover from the acute phase of typhoid; however, 3–5% of individuals who are infected with *S.* Typhi develop a chronic infection in the gall bladder[14,15]. Because *S.* Typhi is a human-restricted pathogen, these chronic carriers form a crucial reservoir for the further spread of the disease through bacterial shedding in faeces and urine[16–18]. Chronic *S.* Typhi infections can persist for decades, and although infected individuals are highly contagious, they are typically asymptomatic, making the identification of carriers difficult[19,20]. The situation is further complicated by the fact that approximately 25% of carriers experience no clinical manifestations during the acute phase of the disease[2].

Epidemiological studies conducted in endemic regions have indicated that there is a strong link between the development of the chronic carrier state and the presence of gallstones; in fact, approximately 90% of chronically infected carriers have gallstones[21,22]. In addition, the typhoid carrier state, both with and without the occurence of gallstones, has been implicated as the crucial predisposing factor for the development of gall bladder cancer[23–26]. It has been proposed that bacterial degradation of bile salts and chronic cholecystitis (gall bladder inflammation) related to gallstones could promote the development of gall bladder carcinomas[27]. This impact on human health, combined with the high incidence of typhoid fever in many parts of the world, highlights the importance of understanding the mechanisms involved in *S.* Typhi carriage. In this Progress article, we summarize recent findings on the mechanisms of gall bladder infection by *Salmonella* spp., with an emphasis on biofilm formation on gallstones as a hallmark of typhoid carriage. For a historical perspective of typhoid carriers, see BOX 1.

Acute gall bladder disease

Cholecystitis is primarily caused by obstruction of the biliary tract due to the presence of gallstones (BOX 2). Typically, this obstruction causes distention, bile stasis (lack of bile flow to and from the gall bladder), inflammation and infection of the gall bladder[28,29]. A range of bacteria have been identified by culture or by PCR in the gall bladders of patients with cholecystitis and cholelithiasis (gallstones in the gall bladder), including *Escherichia coli, Klebsiella pneumoniae, Citrobacter freundii, Salmonella* spp., *Helicobacter* spp., *Enterobacter* spp., *Enterococcus* spp., *Pseudomonas aeruginosa, Bacteroides fragilis, Staphylococcus aureus, Proteus* spp. and *Acinetobacter* spp.[29–35]. In the case of *Helicobacter* spp., a prospective study showed a higher frequency of gallstone formation in mice that were fed a lithogenic (gallstone-inducing) diet and inoculated with bacteria than in uninfected controls fed the same diet[36]. However, whether these microorganisms have a primary role in causing cholecystitis or cholelithiasis, or are merely colonizers of a previously damaged gall bladder, is still unknown. Acalculous cholecystitis (inflammation in the absence of gallstones) is less common (~10% of acute cholecystitis cases) and occurs predominantly in critically ill or injured patients or after complicated surgery[37].

Despite the fact that cholecystitis and sonographic gall bladder abnormalities have been reported in acute and chronic typhoid fever patients[4,17,31,38], little is known about the interaction of *S.* Typhi with the gall bladder. Because *S.* Typhi is a human-restricted pathogen, *in vivo* studies of *S.* Typhi pathogenesis typically involve a mouse model of infection using *Salmonella enterica* subsp. *enterica* serovar Typhimurium. The pathological features of the course of mouse infection with *S.* Typhimurium are similar to those of human infection with *S.* Typhi[39]. Typically, susceptible mice lacking a functional copy of the

natural resistance-associated macrophage protein 1 gene (*Nramp1*; also known as *Slc11a1*) are used for *S.* Typhimurium infections. NRAMP1 is a crucial factor in controlling the replication of intracellular bacteria[40].

To begin to examine host–pathogen interactions during acute infection, Menendez *et al.*[41] used *S.* Typhimurium infection of *Nramp1*$^{-/-}$ mouse strains. A total of 3×10^7 to 5×10^7 bacteria and 5×10^2 bacteria were inoculated orally or intravenously, respectively, and the number and localization of bacteria were assessed for up to 120 hours after infection. *S.* Typhimurium was found in the gall bladder 48 hours after infection, with the highest bacterial burden present at 120 hours post-infection in both the gall bladder lumen and tissue. Bacteria were present in the intestine and shed in the faeces throughout the entire course of infection. Active invasion by *S.* Typhimurium into the gall bladder epithelium followed by efficient replication and intracellular survival was also observed. *S.* Typhimurium localized to the cytoplasm of gall bladder epithelial cells within a *Salmonella*-containing vacuole (SCV). Interestingly, the bacteria did not translocate to the lamina propria or the mucosa.

S. Typhimurium colonization of the gall bladder induced a localized inflammatory response that was mediated by neutrophils and led to loss of epithelial integrity, thickening of the mucosa and tissue damage (Fig. 1). Invasion-deficient bacteria could not infect gall bladder tissue or elicit an inflammatory response and pathological damage, although they replicated efficiently in the gall bladder lumen, indicating that only invasive intracellular bacteria are responsible for the inflammatory process. This study corroborated the traditional implication of *S.* Typhi as a cause of acute cholecystitis and its potential role during acute typhoid disease. The events that must occur in chronic carriers to effect the transition from an acute, strong pro-inflammatory response to a relative lack of symptoms and pathology are of great interest to researchers.

Biofilms and chronic carriage

In addition to the serious health impact of acute typhoid disease, the chronic state is also a public health problem, as chronically infected individuals can intermittently shed the bacteria in their faeces and urine, and thus contribute to transmission of the pathogen. Antibiotic treatment has not proved effective in the resolution of chronic *S.* Typhi colonization of the gall bladder, in contrast to treatment of acute infection. Even prolonged, high-dose antibiotic therapy resolves less than two-thirds of chronic infections, and treatment with ampicillin has been shown to be effective only in patients without gallstones[42,43]. Complete resection of the gall bladder (cholecystectomy) increases the cure rate, but it does not guarantee elimination of the carrier state[44]. Additional foci of infection can persist in the biliary tree, mesenteric lymph nodes or liver[26,45,46]. Although not available to many patients, the most effective treatment is a combination of surgery and antibiotics[47]. However, as studies that demonstrate the rapid acquisition of multidrug resistance emerge, it is becoming increasingly difficult to apply previous findings to current strains.

The clinical observations relating to carriers — with regard to their resistance to antibiotic treatment, the confinement of the infection to the gall bladder, gall bladder removal being the most successful therapy and the evidence for long-term evasion of the immune response — are consistent with a biofilm-related disease[28]. Biofilms are communities of microorganisms that adhere to each other and to inert or live substrates and are encased in an extracellular matrix[48]. Typically considered as a response to stress, biofilms have been implicated in many chronic and acute infections[49]. *S. enterica* is known to form matrix-encased biofilms on abiotic and biotic surfaces[50,51]. The gall bladder is the site of bile

storage and, as such, is an environment that is habitable exclusively by organisms that are resistant to bile's detergent-like properties[52,53]. Salmonellae, along with other enteric pathogens, not only are highly resistant to bile, but also respond to the presence of bile by regulating the expression of many resistance-related genes[54]. Early studies to investigate the ability of *S. enterica* to form biofilms on human gallstones and cholesterol-coated surfaces (see below) indicated that formation of a robust biofilm on cholesterol is dependent on the presence of bile[50]. Interestingly, in *S.* Typhimurium bile also seems to have an effect on several known global gene regulation pathways in a manner that is independent of traditionally implicated stress or biofilm mediators such as RpoS (also known as RNA polymerase sigma factor σ^{38}) and AgfD (also known as CsgD)[55,56]. Bile has also been shown to downregulate the expression of *Salmonella* pathogenicity island 1-encoded genes, which are involved in host cell invasion. However, this is likely to be a spatiotemporal response that does not interfere with invasion after the bacteria have penetrated the mucous layer of the epithelia, where a decreased apparent bile concentration would be encountered[57]. Bile also slightly downregulates the expression of motility genes, but this transcriptional regulation does not have a dramatic effect on the number of flagella per bacterium or on motility[58].

In vitro biofilm models

Various static and dynamic systems have been used to examine *Salmonella* spp. biofilms. Early studies of biofilms in relation to chronic *S. Typhi* carriage used gallstones that had been removed from patients during cholecystectomy. Bacteria incubated with gallstones for 7–14 days formed dense matrix-encased biofilms; however, in controls using an alternative substrate of similar size and shape, no such biofilms were formed[50]. As an *in vitro* surrogate for gallstones, the tube biofilm assay (TBA) was developed for the study of biofilm formation on cholesterol[56]. This method involves coating siliconized microcentrifuge tubes with cholesterol. Bacteria are incubated in these tubes for a period of 24 hours, after which the culture is aspirated and non-adherent (non-biofilm) bacteria are removed by washing with PBS. using this method, the role of bile in the enhancement of *S. enterica* biofilm formation was confirmed and specific binding to cholesterol was observed[56]. Bilirubin, a major component of pigment stones (see BOX 2), has also been evaluated in the TBA. *S. enterica* formed biofilms poorly on calcium bilirubinate compared with on cholesterol, further indicating the specificity of *S. enterica* binding to, and subsequent biofilm formation on, cholesterol-coated surfaces. The use of the TBA eliminates the dependence on human gallstones and allows for assay standardization, as the cholesterol composition of gallstones is variable[56]. However, although the TBA is both economical and reproducible, the flow through system is probably more representative of the gall bladder environment. This system consists of bile-containing media flowing at a specific rate through chambers containing coverslips made of plain glass or cholesterol-coated glass. This method has recently been used to study biofilms on cholesterol-coated surfaces, corroborating and extending the results observed in the TBA (J.S.G., unpublished observations).

Biofilm initiation on gallstones

For successful biofilm formation on gallstones, *Salmonella* spp. must first access and colonize the gall bladder or biliary tract. The bacteria must then attach to the surface of gallstones as well as persist in the presence of natural host defences[59]. It is thought that bile stasis, which can occur in the presence or absence of gallstones, contributes to successful colonization[28]. Several known bacterial biofilm-associated factors have been investigated to determine which are crucial for the formation of mature *S. enterica* biofilms on the surface of gallstones and on cholesterol-coated surfaces[50,60]. Flagella and fimbriae are two bacterial appendages that have been implicated in various species as being important for the initial stages of biofilm formation on a range of surfaces[61,62]. In *S.* Typhimurium, the presence of

flagellar filaments but not motility (verified by a mutation in the motility protein A gene (*motA*) that results in a bacterium that cannot rotate the flagellum) was necessary for biofilm formation on gallstones. By contrast, motility was required for biofilm formation on glass under different assay conditions[60].

To build on this work and examine the factors that are required specifically for the formation of biofilms on cholesterol, a pool of transposon mutants was examined in the TBA with daily passage of planktonic (non-adherent) bacteria[58]. Using this method, 49 mutants deficient in cholesterol binding and subsequent formation of biofilms were obtained. Many of the non-adherent mutants represented transposon insertions in flagellum biosynthesis genes. Specifically, the flagellin subunit (FliC) was demonstrated to be necessary for initial binding to cholesterol-coated surfaces. Loss of outer-membrane protein C (OmpC) also negatively affected binding to cholesterol as well as subsequent biofilm formation. In addition, 18 of the transposon library mutants showed insertions in *fim W*. This gene encodes a negative regulator of the type 1 fimbriae operon, and its deletion confers a constitutively expressed type 1 fimbrial phenotype. Further analysis demonstrated that a hyper-fimbriate phenotype negatively affected the initial stages of biofilm formation on cholesterol. Thus, the initial attachment phase of biofilm formation in *S. enterica* might involve a combination of flagella and outer-membrane proteins that can be masked by overexpression of surface fimbriae[58].

Biofilm maturation on gallstones

After the initial attachment phase, biofilm development typically involves the formation of microcolonies followed by the development of the mature biofilm. Both stages are characterized by the presence of extracellular polymeric substances (EPS) that aid in biofilm structure and cell–cell interaction[63]. The components of EPS that have been identified in *Salmonella* spp. biofilms include cellulose, colanic acid, the Vi antigen, curli fimbriae, the O antigen capsule and biofilm-associated proteins[51,64,65]. Deletion of the genes encoding the *S.* Typhi Vi antigen does not affect biofilm formation[50]; furthermore, this antigen is not present in *Salmonella enterica* subsp. *enterica* serovar Enteritidis or *S.* Typhimurium, but these serovars can still form robust biofilms on cholesterol-coated surfaces. Cellulose and colanic acid are important for *S.* Typhimurium biofilm formation on biotic and abiotic surfaces, including Hep-2 cells, chicken intestinal tissue and plastic[51]. However, although an *S.* Typhimurium double mutant for cellulose and colanic acid was negatively affected in biofilm formation on plastic and glass, biofilm formation on gallstones was unaffected. These results demonstrate that various components of EPS are required for *S. enterica* surface-dependent biofilm development[60].

Gibson and colleagues[65] identified the polysaccharide O antigen capsule in *S.* Enteritidis This capsule has been shown to be important for environmental persistence and for attachment to, and colonization of, plants[65,66]. Mutation of *galE*, the gene responsible for the synthesis of galactose — which is used in construction of the lipopolysaccharide (LPS) outer core and LPS O antigen and is putatively involved in synthesis of the O antigen capsule — has been shown to yield mutants that are unable to form biofilms on gallstones[50]. Conversely, mutations in *rfaD* (also known as *hldD*), a gene that is involved in synthesis of the LPS outer core and O antigen alone, exhibit no such defect[60]. In addition, mutation of the genes putatively associated with O antigen capsule synthesis negatively affects *S. enterica* biofilm formation on cholesterol-coated surfaces and gallstones. Furthermore, bile has been shown to upregulate the expression of genes involved in the formation of the O antigen capsule and enhance capsule expression, further suggesting an important role for the O antigen capsule in *S. enterica* biofilms on gallstone surfaces[56].

Mouse model of typhoid carriage

Most *S.* Typhimurium pathogenesis studies have been conducted in susceptible BALB/c or C57BL/6 mice and so have provided valuable data on acute-phase infection but have yielded little data relating to chronic infection[39]. Supported by previous investigations[67,68], a 2004 study proposed the use of the 129X1/SvJ *Nramp1*$^{+/+}$ mouse for analysis of chronic infection, demonstrating that *S.* Typhimurium was detectable in the tissues of this mouse for 1 year following oral infection[69]. This model was recently adapted for *in vivo* studies of chronic gall bladder infection. After 6–8 weeks of a lithogenic diet (1% cholesterol and 0.5% cholic acid), mice developed cholesterol gallstones. *S.* Typhimurium infection in mice harbouring cholesterol gallstones resulted in enhanced colonization of gall bladder tissue and bile compared with colonization in infected mice lacking gallstones[70]. These infected mice with cholesterol gallstones exhibited a 3-log increase in faecal shedding of *S.* Typhimurium compared with similarly infected mice lacking gallstones, possibly as a result of an increased bacterial load in the gall bladder. Electron microscopy analysis of the gallstones removed from infected mice revealed a dense bacterial biofilm covering more than 50% of the surface. This work strongly supports the hypothesis that biofilms on gallstone surfaces mediate the typhoid carrier state. Furthermore, the greatly increased faecal shedding in these carriers supports the importance of carriers to the spread of typhoid fever. The development of this mouse model of a cholesterol gallstone-mediated chronic carrier state will facilitate further *in vivo* studies[70]. A model for biofilm formation on gallstones in the gall bladder is shown in Fig. 2.

Finally, humanized mouse models are currently being developed, such as mice that are deficient in v(D)J recombination-activating protein 2 (RAG2) and the interleukin receptor common γ-chain (γc) — so are immunodeficient — and are engrafted with human fetal liver haematopoietic stem and progenitor cells[71]. Such models may be useful in the future study of chronic infections, allowing *S.* Typhi to be studied directly.

Human studies of typhoid carriage

As previously stated, many studies have shown an association between the presence of gallstones and typhoid carriage[21,22,42]. To further investigate the role of gallstones in chronic *S.* Typhi colonization, otherwise asymptomatic patients from an endemic region who were presenting for cholecystectomy due to the presence of gallstones were screened for the presence of *S.* Typhi[70]. Multiplex PCR and traditional plating assays revealed the presence of *S.* Typhi in five of the 103 patients with cholelithiasis (~5% of patient samples). The gallstones from all five patients were positive for *S.* Typhi, two of the five harboured *S.* Typhi in the gall bladder epithelial tissues and only one had a positive culture of bile. Electron microscopy analysis revealed that gallstones from three out of four of these patients exhibited 80–90% surface coverage with a dense bacterial biofilm. The gallstone from the patient who lacked biofilm formation was thought to be a pigment stone, with calcium bilirubinate and not cholesterol as the main constituent. This supports previous *in vitro* findings that calcium bilirubinate is not the preferred surface for *S. enterica* biofilm formation on gallstones. However, gallstones from patients harbouring *E. coli* in the absence of *S.* Typhi (13% of patient samples collected) exhibited no such biofilms[70]. *E. coli* is a common inhabitant of a poorly functioning gall bladder but was not found to co-infect gall bladders with *S.* Typhi[70].

These clinical data correlate with previous *in vivo* observations and support the hypothesis that chronic carriage of *S.* Typhi is mediated by biofilm formation on gallstones. In addition, they demonstrate the presence of a subset of healthy carriers in an endemic population and

highlight the importance of the development of methods to identify and successfully treat such patients.

Summary and comments

There is now a considerable body of data to support the notion that *S.* Typhi can persist in the gall bladder of typhoid carrier patients, primarily associated with gallstones. However, as the picture of chronic *S.* Typhi infection becomes more complete, it is possible that other locations (such as the gall bladder epithelium (Fig. 2), other organs or organ systems, or specialized host cells) could be identified as alternative niches. In many cases, persistence can be fostered by the establishment of a biofilm on gallstones. Further characterization of the factors involved in biofilm formation on gallstones and the interaction of *S. enterica* with the gall bladder epithelium is ongoing, but many questions remain. At the epidemiological level, comprehensive studies in endemic areas are required to produce information on the incidence of chronic typhoid carriers, the incidence of gallstones in these patients and potential non-gall bladder sites of *S.* Typhi persistence. Such information would provide additional insight into the impact of the chronic carrier state with respect to both transmission and efficient prevention strategies. Similarly, prospective studies should be carried out using animal models to examine strategies of gallstone biofilm prevention. Furthermore, the immunological factors involved in the interaction between *S.* Typhi, the gall bladder epithelium and gallstones must be investigated to determine whether *S.* Typhi is a primary contributor to chronic infection as a promoter of gall bladder damage, or whether the bacterium has a secondary role, taking advantage of existing gall bladder inflammation and damage. Finally, investigation of cholesterol biofilm inhibitors such as those that target EPS production or the flagella–cholesterol interaction could lead to the development of promising therapies to eliminate typhoid carriage, especially in areas of high endemicity. These studies will further the ultimate goal of the reduction or elimination of the global burden of typhoid fever.

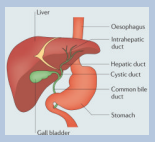
Box 1 | Typhoid Mary

Salmonella enterica subsp. *enterica* serovar Typhi is a human-restricted pathogen, making healthy carriers crucial in the infectious cycle. This role was famously illustrated by Mary Mallon, or Typhoid Mary, a cook in New York City (USA) in the early twentieth century. She is reported to have infected at least 54 people. Other carriers were also identified in New York City around this time, many of who spread the infection to more people than Mary Mallon did. Around the same time, in Folkstone (UK), another chronic carrier referred to as ‘Mr N. the milker’ infected more than 200 people over the course of 14 years. In these cases, public health officials ultimately stepped in and requested that the carriers remove themselves from food service, and although Mr N. and others agreed, Typhoid Mary refused, ultimately leading to her arrest and involuntary lifelong quarantine on North Brother Island in New York. Typhoid Mary was the first identified healthy carrier of an infectious disease in the United States, as she was symptom free and was not documented as experiencing a bout of typhoid fever[72–74].

This confinement practice continued even after Mary’s death. In 2008, it emerged that 43 female typhoid carriers were quarantined in the Long Grove Asylum in Epsom (Surrey, UK) between 1907 and 1992 and some were held for more than 40 years until the asylum closed in 1992 (REF. 75), well after the widespread use of antibiotics had begun and increased medical knowledge of the typhoid fever carrier state was gained.

Box 2 | The gall bladder and gallstones

The biliary tract consists of the gall bladder, cystic duct, common bile duct and intrahepatic ducts (see the figure). The role of the gall bladder is to store bile until it is needed for the emulsification of lipids after the ingestion of food[76]. The term cholelithiasis refers to the presence of gallstones (or choleliths) in the gall bladder or the bile ducts and is one of the most prevalent of those medical conditions that require surgery[77]. The composition of gallstones determines their classification as cholesterol gallstones (more than 70–80% cholesterol), pigment gallstones (40–60% calcium bilirubinate) or mixed gallstones (30–70% cholesterol)[78]. Cholesterol gallstone formation depends on a combination of factors, including the super saturation of bile with cholesterol, alteration of gall bladder contractility and hypersecretion of mucin. Patient factors that predispose for gallstone formation include age, obesity, female gender, unknown genetic determinants and chronic bacterial colonization[77,79,80]. For an extensive review on gallstone formation, see REFS 79,81.



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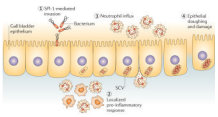

Figure 1. *Salmonella* spp. acute infection of the gall bladder

Following systemic infection, *Salmonella* spp. colonize the gall bladder from the liver. Bacteria can replicate extracellularly in the lumen or can actively invade the gall bladder epithelium in a *Salmonella* pathogenicity island 1 (sPI-1)-dependent manner (step 1). Although the bacteria can replicate inside the epithelial cells, in the *Salmonella*-containing vacuole (sCV), they do not translocate to the lamina propria and mucosa. This intracellular infection leads to a local inflammatory response (step 2) mediated by neutrophils (step 3), with subsequent tissue damage and epithelial sloughing (step 4). This could lead to the release of *Salmonella* spp. cells into the lumen for invasion of new epithelial cells. Based on data from REF. 41.

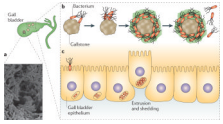

Figure 2. Model of *Salmonella enterica* subsp. *enterica* serovar Typhi biofilm formation on cholesterol gallstones

a | Electron micrograph of *Salmonella enterica* subsp. *enterica* serovar Typhi in a biofilm on the surface of a human gallstone. **b** | *S.* Typhi probably gains access to the gall bladder during the acute phase of infection and initially attaches to gallstone surfaces through a specific interaction between flagellin and cholesterol. This initial binding could be facilitated by outer-membrane protein C (ompC). Subsequent attachment of bacteria is aided by the presence of, but not motility mediated by, flagella[59,62]. On cholesterol, biofilm formation is dependent on the presence of exopolysaccharide (green), probably including the o antigen capsule[55]. Detachment of bacteria from the biofilm would allow entry into the intestine via bile, followed by shedding in the faeces and urine[70]. **c** | A possible alternative strategy by which *S.* Typhi persists in the gall bladder is through invasion of gall bladder epithelial cells. In this model, invasive bacteria replicate intracellularly, and shedding could occur as a part of epithelial regeneration, wherein gall bladder epithelial cells containing *S.* Typhi would be extruded to the lumen, and released bacteria could infect new cells or be shed into the intestine via bile[82].