Virulence factors of
Salmonella enterica serovar Enteritidis

Virulentiefactoren van
Salmonella enterica serovar Enteritidis

(met een samenvatting in het Nederlands)

Proefschrift

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"Is it not pleasant to learn

with a constant perseverance and application?"

Confucius (551-479 BC)

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General Introduction

1. INTRODUCTION

Bacteria belonging to the species *Salmonella* are a major cause of intestinal disease in humans and animals worldwide. In the Netherlands and most other western countries, two serovars of *Salmonella enterica*, serovar Enteritidis and serovar Typhimurium, have been identified as the prime etiologic agents of human salmonellosis. In Figure 1, it is shown how the incidence of human salmonellosis caused by these serovars evolved in the Netherlands during the past two decades. Efforts to control *Salmonella* contamination have resulted in a drop in the total number of recorded *Salmonella* infections in humans. Remarkably however, this drop could entirely be attributed to a decrease in serovar Typhimurium infections. Infections caused by serovar Enteritidis dramatically increased during this period and now are similar or exceed the number of serovar Typhimurium cases. A similar trend was observed in other European countries and in the United States (55, also see: http://www.rivm.nl:80/ bibliotheek/rapporten/ 216852003.pdf).

During the serovar Enteritidis pandemic, the majority of outbreaks was traced back to foods containing raw or undercooked poultry products (55,17). Since the mid 1980s, serovar Enteritidis has emerged as a major *Salmonella* serotype in poultry in many countries (69). An immunological survey conducted in 1993 in the Netherlands indicated that approximately 10-15% of the Dutch layer flocks tested were infected with serovar Enteritidis (68). It has been suggested that the steady increase of serovar Enteritidis infections in chicken flocks in recent decades is associated with the eradication of *Salmonella enterica* serovar Gallinarum from poultry between 1950 and 1970 (7). Both serovars share a distinct surface antigen (the O9-antigen) and mathematical models predict that cross-immunity against this antigen will lead to between-serovar competition which previously was at the advantage of the avian-adapted serovar Gallinarum. The eradication of serovar Gallinarum may thus have created a niche for serovar Enteritidis (43, 55). Serovar Typhimurium does not possess the O9-antigen. This may explain why the occurrence of serovar Typhimurium remained unaffected by the eradication of serovar Gallinarum (33).

An additional reason for the increase in outbreaks of salmonellosis caused by serovar Enteritidis may be that infection of chicken is usually symptomless (68) and that carriership remains unnoticed (47). The important role of contaminated
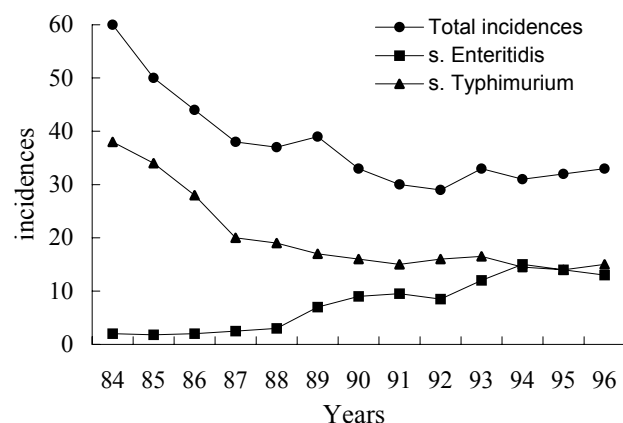

Figure 1: Estimated incidences of typed human *Salmonella* isolates in the Netherlands between 1984 and 1996. Adopted from A.W. van der Giessen (68).

poultry in the infection chain implies that most effective infection intervention can be expected from the control of the spread of the bacteria and the prevention of carriership in poultry. In order to develop novel infection intervention methods, detailed knowledge of the interaction between serovar Enteritidis and its host, both at the level of transmission of *Salmonella* between birds in poultry flocks and at the level of the individual animal, is imperative. Considering the importance and economic impact of the serovar Enteritidis problem, it is particularly remarkable that despite numerous studies in the related serovar Typhimurium, little is known about the molecular interactions between serovar Enteritidis and the infected animal. The research described in this thesis was designed to address this topic and specifically focussed on systematic investigation of virulence determinants that are involved in serovar Enteritidis colonization of poultry. The identification of genes encoding for virulence factors may provide valuable insights that can be utilized in the control of serovar Enteritidis infections, for example by vaccination.

2. CLASSIFICATION OF *SALMONELLA*

The genus *Salmonella* is a member of the family of *Enterobacteriaceae* which also includes the genera *Escherichia* and *Yersinia* (70, 48).

Analysis of the genetic relatedness of *Salmonella* strains using multi-locus enzyme electrophoresis has revealed that this genus can be divided into two species *Salmonella enterica* (*S. enterica*) and *Salmonella bongori (S. bongori)*, with *S. enterica* subdivided into six subspecies (*enterica, salamae, arizonae, diarizonae, indica,* and *houtenae* (57). According to this classification system, the correct names for the formerly called *Salmonella enteritidis* and *Salmonella typhimurium* are *S. enterica* subsp. *enterica* serovar Enteritidis and serovar Typhimurium, respectively. In this thesis, these strains will be referred to serovar Enteritidis and serovar Typhimurium.

Identification of the various serovars of *Salmonella* is historically based on the presence of lipopolysaccharide (somatic or O antigen), flagella (H antigen, phase I and II) and capsular (Vi) antigen on the bacterial cell surface as determined by serum agglutination. Each *Salmonella* serogroup has a group-specific O-antigen. Within each O-group, different serovars are distinguished by the combination of O and H antigens that are present. Other methods such as phage typing, biotyping, determination of antibiotic resistance patterns or plasmid profile analysis are used to identify isolates beyond the level of serovars and are valuable additional tools in epidemiological studies (4). Genetic analysis of *Salmonella* serovars indicates that their divergence in orthologous genes ranges from 3.8-4.6%. At the deduced amino acid level, serovars differ from beween 0.7 and 1.3% (60). On the basis of these findings, it is assumed that the different serovars may have a common ancestor that diverged mainly by the acquisition via horizontal gene transfer of distinct genetic regions from other microorganisms (8).

3. PATHOLOGY OF *SALMONELLA* INFECTION

Serovar Enteritidis and serovar Typhimurium can colonize both humans and chicken. In humans, the infection can manifest as a non-bloody diarrhea with abdominal pain, nausea, vomiting and fever. The disease (non-typhoidal fever) is usually self-limiting and recovery follows within a few days to a week but, occasionally, systemic infection may occur in vulnerable human patients such as infants and elderly people, leading to serious syndromes (40). In the course of an infection, *Salmonella* are thought to first colonize the intestine and penetrate the intestinal barrier (25). Invasion of the intestinal mucosa results in an extrusion of the infected epithelial cells into the intestinal lumen and a destruction of microvilli, which leads to a loss of absorptive surface. The invasion of epithelial cells elicits the production of the pro-inflammatory cytokines (19), which stimulate the influx of polymorphonuclear leukocytes into the infected mucosa. Experiments in mice suggest that bacterial entry and destruction of M cells play a major role in the invasion process (36, 38). Via this route, the bacteria can reach the Peyer's patches (53). Here, the bacteria can be taken up by macrophages that are believed to carry these engulfed bacteria to systemic sites through the lymphatic system. In the mouse liver, *Salmonella* can be found inside macrophages (58).

As mentioned above, infections of adult chicken with serovar Enteritidis are generally symptomless. These symptomless carriers are difficult to trace which leads to problems in the control of the infection in a chicken flock (47). *Salmonella* infections in chicken are mostly caused by the intake of contaminated food, although vertical transmission in which the bacteria are directly transferred from the mother to the offspring via the egg has been described (28, 35, 61). Once ingested, the bacteria first need to pass the acidic environment of the stomach and thus bacterial resistance against these acidic conditions plays an important role in infection (23). Upon reaching the alimentary tract of the chicken, the bacteria are able to colonize the gut without causing disease. At this time, it remains enigmatic as to why serovar Enteritidis appears to exhibit commensal behaviour in adult chicken while it behaves as a pathogen in humans.

In newly hatched chicken, serovar Enteritidis can cause diarrhea and septicemia with invasion and infection of a variety of internal organs of the hen including liver, spleen, peritoneum, ovaries and oviducts (28). When the animals become infected with serovar Enteritidis, extensive interstitial oedema of the lamina propria and the submucosa can be observed within one day of infection, followed by a rapid influx of granulocytes and macrophages (18). In general, the recruitment of inflammatory cells is much faster and stronger in day-old chicken than in month-old chicken. Once the bacteria have reached the macrophages, they may use these cells as vehicles to disseminate to distinct organs. In this regard, the course of serovar Enteritidis infection in young chicken resembles that in susceptible humans.

4. SALMONELLA VIRULENCE DETERMINANTS

Differences in virulence among *Salmonella* serovars and in the course of *Salmonella* infections in various host species, have been attributed to the variable acquisition and evolvement of virulence genes (21). In serovar Typhimurium, at least 80 different virulence

genes have been identified. A large part of these genes are clustered on the chromosome in distinct regions, called *Salmonella* pathogenicity islands (SPIs). At this time, five SPIs have been identified (Figure 2). In addition, several smaller clusters of virulence genes have been identified that are located in so-called pathogenicity islets (49). Finally, a number of virulence genes appear to exist randomly dispersed in the genome (49). From the total repertoire of virulence genes, the acquisition of distinct SPIs is particularly considered to have extended the adaptive abilities of the different *Salmonella* serovars enabling the crossing of host barriers and the occupation of niches in novel hosts (43). Acquisition of SPI-1, for instance, has been associated with the ability of *Salmonella* to penetrate the epithelium, while SPI 2 to 4 appear to

Figure 2: Genetic organization of the currently known *Salmonella* Pathogenicity Islands (SPIs). Adapted from Marcus (49).

facilitate the invasion of and survival within macrophages (49). At this point, it should be noted that most knowledge about SPIs and other *Salmonella* virulence genes is based on observations with serovar Typhimurium and that thus far only a fraction of these genes have been identified in other serovars including serovar Enteritidis.

4.1 BACTERIAL ADHESION AND MOTILITY

Bacterial adhesion to host mucosal surfaces is often a first step in the establishment of an infection. Bacteria attach to target cells via firm interaction of bacterial surface components with distinct host receptors. Many bacterial pathogens use subcellular surface appendages that radiate from the bacterial surface for initial adherence. Typical examples are the bacterial pili (fimbriae) and flagella. In a study on bacterial adherence to chicken gut explants, non-flagellated mutants of serovar Enteritidis were found to be less adherent than the wild type strain (1). However, in a

study using cultured human Caco-2 cells, a non-flagellated mutant of serovar Enteritidis adhered as well as the wild type strain (67). Contradictory results have also been reported on the role of fimbriae in *Salmonella* adhesion. For example, the PE fimbriae of serovar Typhimurium have been demonstrated to be involved in bacterial adhesion to the murine small intestine (10), while SEF14 fimbriae in serovar Enteritidis appeared to primarily play a role in the bacterial uptake by macrophages (20). Awareness is growing that bacterial adhesion to eukaryotic cells is a highly specific process and that the various *Salmonella* serovars may have evolved a number of sophisticated adhesins, each optimally designed to facilitate bacterial interaction with the multitude of cell types encountered in the various hosts (20, 56). Detailed molecular analysis of the various adhesion events is necessary to identify the critical bacterial and host components that confer the interaction. This knowledge may provide novel attractive targets for infection intervention.

Flagella also play a major role in *Salmonella* motility. In many studies, bacterial motility was found to be essential for adherence or invasion (2, 39, and 41). It can be imagined that in many systems, flagella provide the driving force that enable the bacteria to penetrate the host mucus layer more rapidly and reach the host cell surface. The importance of flagella for *Salmonella* virulence is perhaps best illustrated by the highly sophisticated machineries that bacteria have developed to propel themselves. The flagella apparatus is encoded by a large array of genes, many of which are tightly regulated by environmental signals (15).

4.2 BACTERIAL INVASION

Bacterial penetration of the intestinal mucosa is often an important step in the establishment of an infection as it enables the microorganisms to pass the epithelial barrier. In mice, serovar Typhimurium has been demonstrated to invade the mucosal tissue rapidly. Both enterocytes and M cells can be invaded, but the subsequent destruction of M cells is thought to contribute to spreading of the infection and the occurrence of systemic disease (38).

Salmonella invasion of murine intestinal mucosal cells including M cells, has been demonstrated to be mainly conferred by the type-III-secretion system (TTSS) located on SPI-1 (10, 49, 53). This pathogenicity island is approximately 40 kb in size and contains at least 29 ORFs (16). The TTSS structural genes (including *invG*, *prgH* and *prgK*) encode proteins that can form a needle-like structure (Figure 3) which appears to partly resemble the structure of bacterial flagella (44). When *Salmonella* adheres to a target cell, this needle-like structure is assumed to form a channel with its base anchored in the cell wall and its tip puncturing the membrane of the host cell. Through this channel, several bacterial proteins (SipB, SipC, SptP SopE and AvrA) can be secreted into the cellular cytoplasm. These injected proteins trigger host cell signaling events that cause local rearrangement of the actin cytoskeleton leading to cell membrane ruffling, and the active uptake of bacteria by the host cell (23, 24, 73). This array of events is tightly regulated by environmental conditions including oxygen concentrations, osmolarity and growth phase. The importance of TTSS penetration of the intestinal epithelium was further underlined by the observation that strains with a defect in SPI-1 were greatly reduced in their ability to cause murine typhoid fever by an oral route of infection, while they

were fully virulent when introduced directly into the peritoneal cavity (53), although recently these findings were disputed (51). At this time, TTSS of SPI-1 is considered to be the primary machinery responsible for *Salmonella* invasion of eukaryotic cells. However, it should be emphasized that observations made for serovar Typhimurium in a distinct host do not necessarily hold for other serovars or other hosts. The recent finding that *Salmonella* pathogenicity island 2 (SPI-2) but not *Salmonella* pathogenicity island 1 (SPI-1) is sufficient for virulence of serovar Gallinarum in chicken (37), illustrates that serovar and/or host specificity may be present.

4.3 INFECTION OF MACROPHAGES

Once *Salmonella* has breached the epithelial barrier they come into contact with cells of the reticuloendothelial system, in particular resident macrophages that are intimately associated with M cells. *Salmonella* can invade and survive within macrophages. In recent years, research has gained important insight into the mechanisms that serovar Typhimurium employs to encounter the hostile environment created by the macrophages. Serovar Typhimurium bacteria reside within macrophages in membraneous vacuoles. It has been demonstrated that the bacteria can block the maturation of phagosomes into phagolysosomes (26, 27) and that the vacuoles have a reduced acidity (pH >5.0)(26). The adjustment of the intravacuolar pH by the microorganisms has been demonstrated to be essential for the activation of distinct *Salmonella* virulence genes (3).

The molecular mechanisms by which *Salmonella* modulates the intracellular trafficking remain largely to be defined, but it has been demonstrated that the *Salmonella* pathogenicity islands are required. The *Salmonella* pathogenicity island 2 (SPI-2) (Figure 2) was identified as a gene cluster required for survival of serovar Typhimurium inside host cells (52). Wild type serovar Typhimurium starts to grow four hours after invasion of macrophages, but mutants in SPI-2 failed to grow to the same extent as the wild type strain (32). SPI-2 is 40 kb in size and contains more than 40 genes. SPI-2 has a mosaic structure and can be divided into at least two segments, which may have been obtained by two separate events of horizontal gene transfer (31). SPI-2 carries genes that encode for a second type III secretion system (TTSS-2) that is structurally and functionally distinct from the TTSS that is encoded by SPI-1. Genetic homology of the *Salmonella* TTSS-2 with the *Yersinia* TTSS suggests that serovar Typhimurium delivers proteins into *Salmonella*-containing-vacuoles or through the vacuolar membrane into the host cytosol. This process influences intracellular trafficking and contributes to the intracellular survival of *Salmonella* in macrophages (66).

SPI-3 and SPI-4 also play a role in the survival of *Salmonella* in macrophages (49). SPI-3 is a 17 kb segment on the chromosome and contains ten ORFs that have no mutual relationship in their functions, indicating that SPI-3 is a mosaic similar to SPI-2 (12). The *mgtC* and *mgtB* genes of SPI-3 encode for proteins that allow serovar Typhiumurium to acquire magnesium under low magnesium conditions. These genes were shown to be required for survival of the bacterium in macrophages and for virulence in mice (11).

SPI-4 contains 18 putative ORFs and some of these ORFs are homologous to the Type-I secretion system. Therefore it is expected that SPI-4 is involved in secretion of a cytotoxin (71). For at least one locus of SPI-4, it was demonstrated that it is required for intramacrophage survival (9, 49).

The survival of *Salmonella* within macrophages is generally considered to be essential for the translocation of bacteria from the gut-associated lymphoid tissue to the

Figure 3: Needle complex structure of type III secretion system of *Salmonella*, electronmicroscope image (left) and structure illustration (right). Adapted from Kimbrough (42).

mesenteric lymph nodes and the liver and spleen. The observation that *Salmonella* with a defect in intramacrophage survival is attenuated for virulence in mice when inoculated intraperitoneally (52) supports the role of macrophages for the dissemination of *Salmonella*.

4.4 METABOLIC ADAPTATION

During the various stages of an infection, *Salmonella* encounters a variety of environmental challenges, such as nutrient starvation, oxidative stress and digestive enzymes. *Salmonella* is equipped with a series of adaptive mechanisms that enable it to survive these challenges. Apart from the various tightly controlled *Salmonella* pathogenicity islands that function at various stages of the infection, a sophisticated regulation of bacterial metabolism appears to exist. One group of genes that play a role in metabolic adaptation are the starvation-stress response genes (62). These genes encode for certain metabolic functions that are required for *Salmonella* to survive at a certain stage of infection and thus can be considered as determinants of virulence. One example is the serovar Typhimurium *aroA* gene, which is involved in the synthesis of 5-enolpyruvylshikimate 3-phosphate that is required for synthesis of aromatic amino acids (14). Mutation of the *aroA* gene results in attenuated virulence of *Salmonella*, and this property forms the basis for the widespread use of *aroA* mutants as vaccine strains. The equivalent *aroA* mutant has also been tested in serovar Enteritidis for effective protection (33). Other examples of metabolic genes that influence the adaptive abilities of *Salmonella* are the *narZ* gene that encodes a cryptic nitrate reductase important for the virulence of serovar Typhimurium in the mouse model (64), the *fadF* gene which is specifically induced in cultured epithelial cells and encodes an acyl-CoA dehydrogenase (63), and the *eutE* gene that probably allows the bacteria to utilize ethanolamine as a nitrogen and carbon source and that is important for virulence in mice (65). The identification of genes that are required for bacterial survival at a certain stage of infection deserves more attention as they may provide opportunities to develop attenuated strains with vaccine potential.
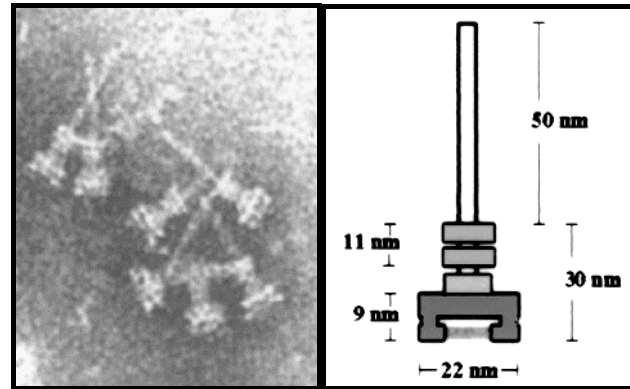
5. HOST RESISTANCE AGAINST *SALMONELLA*

The interaction of *Salmonella* with the host varies considerably with the age and type of host. In general, resistance to salmonellosis increases as the animal ages. For example, 1-day-old chicks are readily infected with 10^2 *Salmonella*, whereas 10^6 *Salmonella* are needed to induce infection in 50% of 8-week old chicken. Studies in mice have demonstrated profound effects of the genetic background of the host on the resistance against *Salmonella* infection. Genes that affect both the sensitivity to endotoxin and that influence the type of immune response that is elicited by *Salmonella* have been identified (34).

The specific immunity elicited by *Salmonella* plays a major role in the containment of the infection, in particular at the later stages of infection. Both humoral and cell-mediated immunity are considered to contribute to clearance of the bacteria. Specific antibodies are common in the sera of animals that are exposed to *Salmonella* in the environment and adults passively transfer antibodies to their offspring (59). Secretory IgA may also be found in the intestine of animals that have recovered from disease. These antibodies may be protective by binding to bacterial surface components involved in the adhesion to or penetration of the mucosal barrier. It should be noted that antibody levels in sera not necessarily correlate with protection against infection, perhaps in part because of the antigenic diversity among serotypes. The exact nature of the protective antigens is not known, although serum IgG antibodies to the O-chain of the lipopolysaccharide are generally protective against infection with the same serotype.

As *Salmonella* can reside in macrophages, cell-mediated immunity is of considerable importance against salmonellosis. Transfer of sensitized T cells confers protection. In poultry, clearance of *Salmonella* has been correlated primarily with cell-mediated immunity (45), although it remains unclear how cell mediated immunity contributes to intestinal clearance. Studies on the immunogenicity of *Salmonella* are complicated by the virulence- and infection dose-dependence of the response. For example, it has been demonstrated that virulent strains were more quickly eliminated from the chicken gut than less virulent strains (5). This effect has been related to the more severe transitory lymphocyte depletion and atrophy of the bursa induced by highly virulent strains. In addition, it is important to realize that in young chicks, *Salmonella* infections can manifest as an infection of the reticuloendothelial system with little intestinal involvement (e.g. serovar Enteritidis) and as an extensive intestinal infection with various degrees of invasion (e.g. serovar Gallinarum). The different involvement of macrophages in the infection process may have direct implications for the nature and effectiveness of the elicited immune response.

6. CONTROL AND VACCINATION

The major source of serovar Enteritidis infections in humans is contaminated poultry products such as eggs and meat. The increase in the number of contaminated farms coincides with the increased incidence of serovar Enteritidis infections in humans and this has led to a

number of efforts to control *Salmonella* infection in chicken flocks. One strategy to achieve this goal is to raise chicken flocks under *Salmonella*-free conditions (17). However, since serovar Enteritidis is wide spread in the environment and can easily be transmitted to chicken, the prevention of the spread of *Salmonella* by high cost housing, tight control of feed quality, hygiene and management only, is a difficult task.

Vaccination is another approach used to control *Salmonella* in poultry (72). In recent years, a number of inactivated *Salmonella* vaccines have been used with variable efficacy (29). At this time, live attenuated, orally administered *Salmonella* vaccines are considered to have the highest vaccine potential. The latter have been associated with the ability of live vaccines to induce a more effective cell-mediated immune response when compared with killed bacteria. Several attenuated strains have been tested as vaccines, including a serovar Gallinarium 9R strain, serovar Enteritidis rough and/or *aro*A mutants (6), and an *aro*A mutant of serovar Typhimurium (46). The vaccines stimulated both humoral and *Salmonella* specific cell-mediated immunity. For example, vaccination experiments with AroA⁻ mutants of serovar Typhimurium in mice and sheep showed a strong induction of *Salmonella* specific serum IgM and IgG and high levels of IgA (13). As secretory IgA can protect mice against an oral challenge with serovar Typhimurium by blocking bacterial adherence and invasion (50, 54) and maternal IgA and IgG antibodies are transferred to the progeny via the egg (30), this response may be protective. Indeed, some of the vaccine strains were capable to protect chicken against exposure to virulent strains and some of them significantly reduced shedding of *Salmonella*. However, these methods all failed to eliminate *Salmonella* from chicken probably in part because of the antigenic diversity among serotypes. Development of more effective vaccines clearly awaits a better understanding of the *Salmonella* virulence determinants that direct the course of an infection.

7. AIM AND SCOPE OF THIS THESIS

The emergence of serovar Enteritidis as one of the most prominent food-borne pathogens in humans demands the development of novel infection intervention strategies. As contaminated poultry products are the main source of serovar Enteritidis infections, strategies to eradicate *Salmonella* from poultry have a high priority. This requires detailed knowledge of the interaction of *Salmonella* with animal cells. Despite the importance of the *Salmonella* problem for human health and economy, the understanding of the molecular interaction of serovar Enteritidis and chicken cells, is still rudimentary. The experiments described in this thesis were designed to better understand the interaction between serovar Enteritidis and chicken macrophages.

The approach followed in this thesis consisted of a systematic search for *Salmonella* virulence genes, followed by a detailed *in vitro* and *in vivo* analysis of the most interesting virulence determinants. In Chapter 2, it is described how a mutant library of the serovar Enteritidis genome was constructed by random insertional mutagenesis using Transposon mini-Tn*10* (Kan^R). The genome of serovar Enteritidis is 4.5 mb in size, and contains about 4000 genes. To cover most genes, a library containing nearly 8000 mutants was constructed. The library was used to search for mutants that were impaired in their ability to infect macrophages,

which are target cells of *Salmonella* in chicken. In Chapters 2, 3 and 4, novel virulence genes important for *Salmonella* infection of macrophages were analyzed in detail and their possible role *in vivo* was evaluated by assessing their behaviour in experimental infections in chicken. In Chapter 5, the discovery of a new *Salmonella* Pathogenicity Island (SPI-6) important for *Salmonella* infection of macrophages, is described. The significance of our findings for infection intervention is discussed in Chapter 6.

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Chapter 2

Identification of virulence genes affecting *Salmonella enterica* serovar Enteritidis infection of macrophages using a Tn*10* mutant library approach

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ABSTRACT

A mini-Tn*10* mutant library of *Salmonella enterica* serovar Enteritidis strain CVI-1 was generated to search for novel virulence genes. Screening of 7680 individual mutants for their ability to invade and survive inside the chicken macrophage cell line HD-11 yielded, after 2 rounds of selection, 37 mutants that were chosen for further analysis. Motility assays indicated that 14 of the mutants were non-motile, explaining their invasion deficient phenotype. Inverse PCR and sequencing of the regions flanking the transposon confirmed that the transposon had inserted into genes involved in bacterial motility. Genetic analysis of the remaining 23 motile mutants revealed 3 mutants with defects in genes (*invG*, *invI* and *spaS*) that in *S. enterica* serovar Typhimurium encode components of the type III secretion apparatus located in the *Salmonella* pathogenicity island 1. 20 mutants had defects in genes likely to be involved in gene regulation, LPS biosynthesis, metabolism, or with unknown function. Further genetic characterization of one of the mutants (SEM35) indicated that the affected gene shared homology with *pbpA* (penicillin binding protein) genes that may be involved in peptidoglycan synthesis. Complementation of the knockout strain with an intact copy of the affected gene restored the wild type infective phenotype. The gene was designated as *pbpA2* as the *S. enteritidis* genome appeared to contain two *pbpA*-like genes of which only the one that was disrupted in the mutant (*pbpA2*) was able to successfully complement SEM35.

INTRODUCTION

Salmonella poses a major problem to public health causing diseases ranging from gastroenteritis to typhoid fever. Infections are usually acquired by ingestion of contaminated food or water. The majority of cases is caused by two serovars: *Salmonella enterica* serovar Typhimurium (serovar Typhimurium) and *Salmonella enterica* serovar Enteritidis (serovar Enteritidis). Since the mid-1980s, the incidence of serovar Enteritidis infections is steadily increasing and this serovar has now replaced serovar Typhimurium as the primary etiologic agent of *Salmonella* infections in many countries (35). The reason for the emergence of serovar Enteritidis as a pathogen is not known but is likely related to the increased presence of serovar Enteritidis in poultry used for consumption (11, 35). In adult chicken, serovar Enteriti*dis* usually manifests itself in symptomless carriership. Strategies aimed at eradication have thus far not been very successful and await more knowledge of the molecular mechanisms underlying the pathogen-host interaction.

Extensive research on virulence determinants of serovar Typhimurium has led to the identification of a large array of virulence genes and the molecular mechanisms explaining the behaviour of the bacteria are rapidly being delineated. Many (more than 80) of the virulence genes of serovar Typhimurium are apparently randomly positioned in the genome (17). Other virulence genes are located in small gene clusters (islets) or in larger clusters containing a series of genes and operons (*Salmonella* Pathogenicity Islands, SPIs) (29). SPI-1 encodes a type III secretion apparatus that secretes peptides that are directly involved in induction of bacterial invasion into epithelial cells (9, 23). SPI-2 (7, 22, 32), SPI-3 (6), and SPI-4 (51) are required for bacterial survival in macrophage cells. The genes located in the newly discovered SPI-5 may contribute to enteric rather than to systemic disease (52) but their exact function remains to be defined. It should be noted that most of the data on serovar Typhimurium virulence are based on experiments in a mouse infection model, or with murine or human cell lines.

Despite the large number of virulence genes that have been found in serovar Typhimurium, knowledge of the virulence repertoire of serovar Enteritidis is scarce. The serovars Enteritidis and Typhimurium are up to 95% identical at the genetic level and share many phenotypic characteristics. Thus it is assumed that both pathogens have many virulence mechanisms in common. Genes that have been confirmed to be present and functional in serovar Enteritidis include several *inv* genes (34), *pagC* (31), LPS biosynthesis genes (41), *tolC* (42), *hns* (21), and a novel ABC transporter system, encoded by the *sfbABC* locus appears to be specific for serovar Enteritidis (33). Inactivation of the *sfbA* gene results in a strain that is avirulent and induces protective immunity in BALB/c mice (33). Subtle but potentially important differences between the serovars Typhimurium and Enteritidis that have been noted involve the role of the O-antigen in virulence (18), the presence of a unique pathogenicity islet encoding fimbriae (8, 13) and the function of the regulatory gene *rpoS* (1).

In the present study we followed a systematic approach to identify serovar Enteritidis determinants important for the various steps of a cellular infection notably adhesion, invasion and intracellular survival. The work involved the generation of a mini-Tn*10* mutant library

which was employed to screen for cellular infection deficient mutants in a chicken macrophage infection model. This approach resulted in the identification of a series of mutants with defects in homologues of serovar Typhimurium virulence genes as well as in previously unrecognized virulence factors.

Materials and methods

Bacteria strains, cell line and culture conditions. *Salmonella enteritidis* strain CVI-1 and the chicken macrophage cell line HD-11 were obtained from ID-DLO (Lelystad, The Netherlands). The strain CVI-1 was typed as phage type 1, which corresponds to phage type 4 of the United Kingdom phage typing system (47). The characteristics of the non-flagellate, non-motile mutant serovar Enteritidis Se857*fliC*- have been previously described (46). Bacteria were routinely grown on LB agar or in LB broth. When appropriate, ampicillin (100 µg/ml), chloramphenicol (20 µg/ml) or kanamycin (50 µg/ml) were added. HD-11 cells were grown in RPMI 1640 medium (Gibco-BRL) supplemented with 10% FCS, 0.02% 2-mercaptoethanol, penicillin (100 U/ml) and streptomycin (100 µg /ml).

Construction of the mini-Tn*10* delivery system. An approximately 5 kilobase (kb) DNA fragment containing mini-Tn*10* and the transposase gene IS*10*-R was isolated from plasmid pLOF/KM (12) after digestion with *Eco*RI. The *Eco*RI sticky ends were filled in with Klenow and the DNA fragment was ligated into the *Sma*I-digested plasmid pKO3. This suicide vector replicates in a temperature-dependent fashion and confers resistance to chloramphenicol in *Salmonella* strain CVI-1. The ligation mixture was introduced into *E. coli* DH5α by electroporation and transformants carrying plasmid mini-Tn*10*-pKO3 (Fig. 1A) were selected on LB plates containing kanamycin and chloramphenicol.

Construction of the mutant library. Serovar Enteritidis strain CVI-1 was transformed with mini-Tn*10*-pKO3 by electroporation. The bacteria were then transferred to LB broth containing chloramphenicol and grown at 30°C for 2.5 hours. This time frame has previously been demonstrated to be sufficient for Tn*10* transposition (28). After 2.5 h, the transformants were plated onto LB plates containing kanamycin and grown at 42°C to select for transposon mutants and counterselect for transformants carrying pKO3 (pKO3 replicates at 30°C but not at 42°C). After 16 h of growth, colonies were individually collected in 100 µl of LB broth containing kanamycin in 96-well plates. After growth, a duplicate was made from each plate that was stored at -80°C. The remaining plates were used in the infection assays. The absence of the plasmid was confirmed in 50 randomly picked transformants grown in media containing chloramphenicol.

Identification of transposition loci. The mini-Tn*10* insertion sites were determined by sequencing of the flanking chromosomal DNA obtained by inverse PCR. Chromosomal DNA was digested with *Hin*dIII or *Nsi*I, re-ligated and used as a template in a PCR with the primer combinations KML (5'-ATGAGTGACGACTGAATCCGGTGA G-3'), and PIS903 (5'-CACTGATGAATGTTCCGTTGCG-3'), and KMR (5'-AGATGAGATGGTCAGACTAA ACTGG-3') and PIS903 (Fig. 1B). The PCR consisted of 2 min denaturation at 94°C followed by 35 cycles of 30 s denaturation at 94 ^{o}C, 30 s annealing at 60 ^{o}C, and 210 s extension at 72 ^{o}C. PCR products were purified using the QIAquick PCR purification Kit (QIAGEN Inc.) and directly sequenced on an ABI310 automated sequencer (Perkin Elmer Co.) with primer PIS10 (5'-TATGACAAGATGTGTATCCACC-3'). Sequencing data were processed with the LASERGENE software package (DNA STAR Inc.). BLAST algorithms were used to search for similar sequences in the EMBL/Genbank databases and in the database of the unfinished serovar Typhimurium genome (http://www.ncbi.nlm.nih.gov).

Complementation of SEM35. Intact copies of the *pbpA* and *pbpA2* genes were obtained by PCR on chromosomal DNA of CVI-1 with the primers 5'-GCAGTAACGCCAGCAGAATGAGTAG-3' and 5'-TCAAACGGGATTTAC GGAATATCTG-3' and 5'-CTGAAACTGGGTCTGGATACGTTGG-3' and 5'-TGCCTACGGTATTCTTAAAGC GGTG-3', respectively. The approximately 2.5 kb products were cloned into the pGEM-Teasy vector (Promega Inc.), sequenced and recloned into plasmid pWSK29 (48). After electrotransformation of the plasmid, complemented SEM35 was selected on LB agar containing ampicillin. Complementation was verified with PCR and restriction analysis.

Motility assay. Bacterial motility was assayed in 0.4% LB soft agar U-shape tubes as described (46).

Infection experiments. The screening procedure for cellular infection impaired mutants was based on the widely used protocol for measurement of *in vitro* invasion/survival of *Salmonella* species in cultured eukaryotic cells (46). Screening of the mutant library was performed with HD-11 cells grown in 96-well microtiter plates. After growth of the individual mutants on plates, the bacteria were transferred with the help of a 96 cylinder copier (Sigma) to the cells and incubated for 30 min (37°C, 5% CO_2). After removal of the nonadherent bacteria, the infected cells were incubated (2 h, 5% CO_2, 37°C) in medium containing colistin (100 µg/ml) to kill all remaining extracellular bacteria. After removal of the colistin and two rinses with PBS, the macrophages were lysed (1 min, 22°C) with 0.1% Triton X-100 in medium and the suspension was copied onto LB plates to select potential cellular infection-defective mutants. Potentially interesting mutants (i.e. mutants that were recovered in low quantities) were retested using HD-11 cells grown in 35 mm tissue culture wells. In these experiments, bacteria were first grown in 10 ml of LB medium with the appropriate antibiotics in 50 ml Falcon tubes at 37°C. After reaching mid-logarithmic phase, the bacteria were collected by centrifugation, washed once with PBS, and added to HD-11 chicken macrophage cells maintained in 2 ml of RPMI 1640 in 6-well plates. After 30 min of incubation (5% CO_2, 37°C), non-adherent bacteria were removed by two washes with medium, exposed to colistin as described above, and plated at various dilutions onto LB agar plates. The number of viable intracellular bacteria was estimated by CFU counting after 24 h of growth. In the infection experiments, the non-motile, non-invasive mutant Se857*fliC-* (46) served as a negative control, while the parent strain CVI-1 served as positive control.

FACS analysis. FACS analysis was applied to determine the number of intracellular *Salmonella* independent of the CFU recovery assay described above. For this purpose, selected mutants were transformed with the pEGFP vector (Clontech Inc.) which carries the gene encoding the enhanced green-light fluorescent protein under the control of a *lacZα* promoter. Fluorescent bacteria grown to mid-logarithmic phase were used in infection experiments as described above, except that after removal of the non-adherent bacteria at 30 min of incubation, cell bound bacteria were released by 0.25% trypsin / 0.05% EDTA in PBS. The released macrophages were diluted in 1 ml of PBS and analyzed in a FACS Caliber (Beckton-Dickinson) with combined measurement of light scatter and fluorescence. Data were processed using CellQuest software. FACS results were from three independent experiments.

RESULTS

Construction of a *serovar Enteritidis* transposon mutant library

A mutant library of serovar Enteritidis was constructed by transforming strain CVI-1 with plasmid pKO3 carrying the mini-Tn*10* and the kanamycin resistance gene (kan^R) (Fig. 1A). After induction of the transposition event by growing the bacteria at reduced temperature (30°C)

for 2.5 hours in the presence of chloramphenicol, transposon mutants were selected at 42°C on LB plates containing kanamycin. Eventually, 7680 kanamycin resistant colonies were individually collected in eighty 96-well plates. Southern blotting using *EcoR*I and *Hin*dIII digested chromosomal DNA isolated from thirty randomly selected mutants and the kan[R] gene as a probe showed unique hybridization patterns for each of the mutants (data not shown), suggesting random insertion of the transposon in the genome.
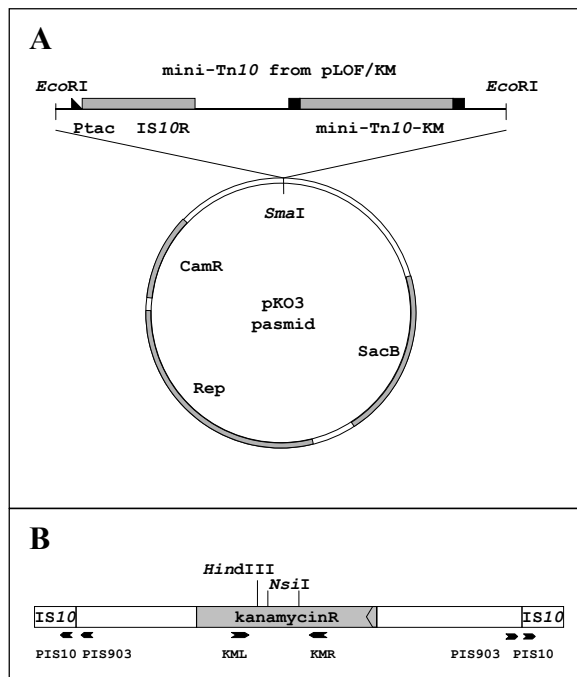

Fig. 1: (A) Map of the mini-Tn*10*-pKO3 vector. An *Eco*RI fragment carrying the mini-Tn*10* from pLOF/KM was inserted into the *Sma*I site of plasmid pKO3; (B) Map of the mini-Tn*10* transposon and the positions of various primers used in this study.

Isolation of mutants deficient in invasion and intracellular survival

In search for virulence determinants of serovar Enteritidis, all 7680 mutants were tested individually for their ability to invade or survive in the chicken macrophage HD-11 cell line. Preliminary experiments indicated that maximal invasion of the wild type serovar Enteritidis strain CVI-1 occurred within the first 30 min of infection (data not shown). Potentially interesting mutants were selected by plate counting of the number of intracellular bacteria recovered after 30 min of infection followed by 2 h of treatment with colistin (100 μg/ml) which selectively kills extracellular microorganisms. After initial screening in a 96-well format and additional rescreening of a selected number of mutants using 6-well plates, we identified 37 clones that consistently gave reduced (5-50% of the wild type) levels of cellular infection (Table 1). These mutants were chosen for further analysis.

Characterization of the mutants

It has been described that motility of *Salmonella* is essential for *in vitro* invasion of epithelial cells (46). Experiments with the aflagellate mutant Se857*fliC-* demonstrated that also cellular infection of chicken macrophages required motile bacteria (data not shown). Analysis of the 37 selected mutants for their behavior in U-shaped tubes containing 0.4% LB soft-agar demonstrated that 14 mutants (38%) were non-motile, while 23 mutants (62%) exhibit normal motility. The non-motile mutants are listed as group I in Table 1.

The poor recovery of the motile mutants (group II in Table 1) from the macrophages may result from a defect in the ability to enter the cells and/or from a reduced ability to survive intracellularly. In order to discriminate between these possibilities, we performed FACS analysis on the infected macrophages. This technique allows direct measurement of the number of ingested microorganisms provided that they carry a fluorescent marker (44). Fluorescent bacteria were obtained by transforming each of the mutants and the positive (CVI-1) and negative (Se857*fliC*-) control strains with plasmid pEGFP which carries the EGFP gene under the control of a *lacZα* promoter. FACS analysis on infected macrophages confirmed that

Group	s.E. Mutant	Blast Identities	Insertion locus	Putative function
I	07,14,15,16	86%(X)	*fliD*	Flagella hook associate protein 2
	05,21,28	98%	*flhD*	Transcriptional activator for motility genes
	33	98%	*flhC*	Transcriptional activator for motility genes
	36	97%	*fliA*	Flagella-specific promoter
	25	98%	*fliL*	Flagella structure protein
	41	97%	*fliN*	Flagella switch protein
	38	98%	*fliP*	unknown
	43	94%	*motA*	Flagella motor protein
	37	94%	*flgI*	Flagella P-ring
II	06	94%	*invG*	SPI-1, type III secretion system
	13	98%	*invI*	SPI-1, type III secretion system
	27	90%	*spaS*	SPI-1, type III secretion system
	08	95%	*rfbI*	LPS side chain synthesis
	42	91%	*rfaI*	LPS outer core synthesis
	02	97%	*rfaJ*	LPS outer core synthesis
	01	98%	*pmrB*	Regulator of *pmr* gene expression
	09,30	92%	*pmrF*	Lipid A modification
	19,24	99%	*phoP*	Regulator of virulence genes
	44	84%	*oxyR*	Sensor and regulator of oxidative stress
	12	100%	*barA*	Sensor and regulator
	35	97%	*pbpA2*	Peptidoglycan synthesis
	10,11,17,23	97%	*ugd*	UDP-glucose dehydrogenase
	20	92%	*tdh*	Theonine dehydrogenase
	31	36%	*cat2*	4-hydroxybutyrate coenzyme A transferase
	03	90%	*icd*	Isocitrate dehydrogenase
	26	81%	*yegQ*	Putative protease precursor
	29	-	-	-

Table 1. Identification and characterization of *S. enteritidis* mutants impaired in their ability to invade and/or survive in chicken macrophages. Group I are non-motile mutants, while group II are motile mutants. Percentages of sequence identity were obtained by BLASTN analysis of obtained sequence flanking the mini-Tn*10* using several databases (see Materials and Methods). (X) indicates that the BLASTX algorithm instead of BLASTN was used. For mutant 31, similarity with *cat2* was found by BLASTX analysis of the *S. typhimurium* homologue carrying the identical sequence as that flanking the transposon in SEM31. For mutant 29, only plasmid sequence was obtained suggesting that in this mutant the vector carrying mini-Tn*10* may have been integrated into the genome.

successful discrimination of the wild type and non-invasive bacteria could be achieved by this method. As shown in Fig. 2, infection of the macrophages with the invasive fluorescent bacteria (strain CVI-1) resulted in 73% fluorescence positive macrophages (peak M2, Fig. 2A), while infection with the non-invasive control (Se857*fliC*-) gave less than 5% of positive cells (Fig. 2B). Quantitation of the absolute number of intracellular bacteria however, was difficult as fluorescence microscopy indicated variation in the intensity of the fluorescence signal within the population of *Salmonella* (data not shown). FACS analysis on the 23 individual motile mutants indicated that only two of them (SEM6 and 13, Fig. 2C) did not invade the macrophages. The other 21 mutants exhibited low to the wild type levels of invasion, suggesting that they were impaired in invasion and /or intracellular survival.

Genetic characterization of the mutants

In order to identify the insertion site of the transposon within the chromosome of the various mutants, we followed an inverse PCR strategy using outward oriented transposon primers. This procedure involved dig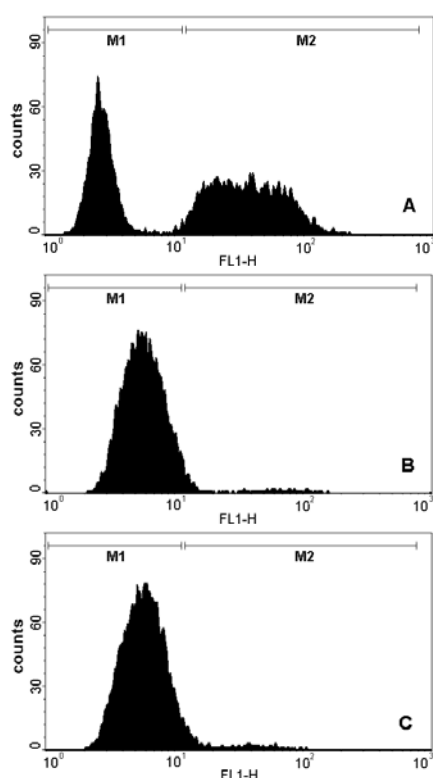estion of the chromosomal DNA, followed by ligation and DNA amplification using the primer PIS903 in combination with primer KMR or KML (Fig. 1B). Sequencing of the PCR products yielded in general 150-500 bp of DNA sequence on each site of the transposon. Comparative analysis with sequences in Genbank and with the unfinished whole genome sequence of serovar Typhimurium (available at htttp://www.ncbi.nlm.nih.gov), revealed transposon insertion sites for 36 of the 37 mutants (Table 1). The data showed that in the 14 non-motile mutants (group I), nine different genes had been disrupted which all have been implicated to function in the construction, assembly, regulation or function of flagella or motility. In the group of motile mutants (group II), a more diverse set of genes appeared to have been affected. Three of the mutants from this group (SEM6, 13 and 27) carried the transposon in serovar Enteritidis homologues of *invG*, *invI* and *spaS*. These three genes are located in the pathogenicity island 1 (SPI-1) of serovar Typhimurium and encode part of a type III secretion system that has been demonstrated to be important for invasion of host cells. From the remaining twenty mutants, six appeared to have defects in genes involved in LPS synthesis (*rfaI*, *rfaJ* and *rfbI*) or lipid A modification (*pmrB* and *pmrF*), one in

Fig. 2: FACS fluorogram of macrophages after 30 min of infection with GFP-labeled *Salmonella enteritidis* wild type strain CVI-1 (A), the invasion-deficient mutant SE857*fliC*- (B), and SEM13 (*InvI*-)(C). The M2 fraction represents the cell fraction containing fluorescent bacteria. Data are from a representative experiment.

peptidoglycan synthesis (*pbpA*), four in putative homologues of global regulatory genes (*phoP*, *oxyR*, *barA*), seven in putative metabolic genes (*ugd*, *tdh*, *icd*, *cat2*) and one in a homologue of the *E. coli yegQ* gene. In one mutant, the plasmid appeared to have integrated into the genome.

Closer analysis and complementation of SEM35

In order to further verify the validity of the followed strategy, one of the survival deficient mutants (SEM35)(Fig. 3) was investigated in more detail. Homology searches based on the obtained nucleotide sequence flanking the transposon indicated that the gene affected in this mutant likely was the serovar Enteritidis homologue of the putative *pbpA* gene of serovar
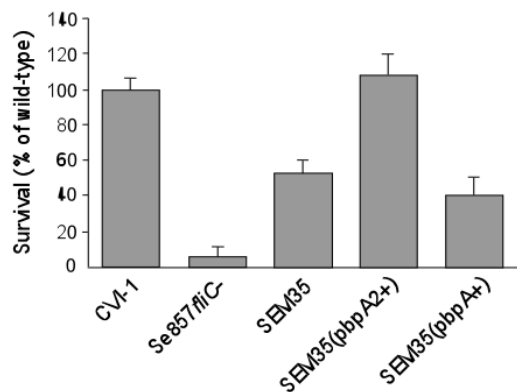

Fig 3: Infection assay demonstrating the intracellular survival of the SEM35 in HD-11 macrophages before and after complementation with the *pbpA* or *pbpA2* gene. Intracellular survival was estimated by counting of the number of viable bacteria recovered from the macrophages after 30 min of infection followed by 2 h of colistin treatment. Tested strains included the wild type strain CVI-1(positive control), Se857*fliC*- (negative control), SEM35 (*pbpA2* mutant), SEM35 carrying pWSK29::*pbpA2*, and SEM35 carrying pWSK29:: *pbpA*. Note that *pbpA2* but not *pbpA* was able to restore the defect in SEM35. Data are the mean ± SD of three experiments.

Typhimurium (accession number: U62714) (45) which, at the predicted amino acid level, is highly homologous to the *E. coli* penicillin binding protein-2 (PBP-2) (5). In order to ascertain this finding, we PCR amplified the entire putative *pbpA* of serovar Enteritidis strain CVI-1 using primers based on the serovar Typhimurium genome sequence. Comparative analysis of the obtained sequence indicated that the serovar Enteritidis gene was identical to the serovar Typhimurium *pbpA* gene located adjacent to the *argS* locus. At the predicted protein level, the serovar Enteritidis *pbpA* showed additional homology to the *E. coli pbpA* which is located next to the *rodA* operon. Further analysis of the unfinished serovar Typhimurium genome sequence indicated that this serovar carried two related *pbpA* sequences, one adjacent to the *argS* locus (which we designated as *pbpA2*) and one next to the putative *rodA* locus. In serovar Typhimurium, the *pbpA* gene adjacent to the *argS* locus (i.e., *pbpA2*) has been identified as an acid-inducible gene (45) but thus far this gene has never been implicated in intracellular survival.

To ascertain that the mutant phenotype of SEM35 was caused by disruption of the serovar Enteritidis *pbpA2* gene, the intact copies of both the *pbpA* (next to *rodA*) and *pbpA2* (next to *argS*) were PCR amplified, cloned onto plasmid pWSK29, and introduced into SEM35. Bacterial survival assays demonstrated that complementation in trans with the intact *pbpA2* gene completely restored the wild type phenotype, while the *pbpA* gene was unable to complement the defect (Fig. 3). This finding indicates that the mutant phenotype was caused by a disruption of *pbpA2* and that the two *pbpA* homologues in serovar Enteritidis are functionally different.

DISCUSSION

The emergence of serovar Enteritidis as the primary etiologic agent of human *Salmonella* infections has led to increasing efforts to control the disease via a variety of intervention strategies. Despite these efforts, success has been limited in part because of the limited knowledge of the molecular mechanisms that contribute to the spread and development of an infection. In this work, we addressed this topic by generating a serovar Enteritidis mutant library with the aim to systemically investigate virulence determinants. The method used to generate the library was based on the random transposition of the mini-Tn*10* transposon into the genome. This procedure has been proved to be successful for a number of pathogens but to our knowledge has never been adopted to construct an array of random serovar Enteritidis mutants. The success of the transposition event was evident from the apparently random insertion of the transposon into the genome as indicated by the unique hybridization pattern for a randomly selected set of mutants and the multitude of cellular infection defective mutants that were obtained. From the 37 mutants that were chosen for further analysis, the transposon had inserted into 32 different positions in 26 different genes. These data indicate the library as a powerful tool to unravel the machineries that contribute to serovar Enteritidis virulence.

In this work, we searched for mutants that were impaired in the ability to infect chicken macrophages. The HD-11 cell line was chosen as an infection model instead of the commonly used murine or human cell lines as chicken form a primary host for serovar Enteritidis and human consumption of contaminated chicken products is a major source of *Salmonella* infection in many countries. Preliminary studies showed that non-opsonized serovar Enteritidis can efficiently invade the macrophages provided that bacterial motility was maintained.

The mutant library was initially screened for cellular invasion impaired mutants using the classical bacterial survival assay in which at a distinct time point extracellular bacteria are killed by an antibiotic and intracellular survival is estimated by CFU counting of microorganisms recovered from the cells. This procedure resulted, after 2 selection cycles, in a selected group of cellular infection impaired mutants. Further analysis showed that one set of mutants was non-motile. This is consistent with observations in other intestinal pathogens such as *C. jejuni* (50) and the more closely related serovar Typhimurium (27) for which motility appears to be a requirement for rapid cellular invasion. Genetic analysis of the non-motile mutants showed that the transposon had inserted in a number of genes that shared considerable homology with genes documented to be involved in the assembly of flagella and bacterial motility in serovar Typhimurium. Although detailed analysis of each of the mutants is required

to ascertain the role of the individual genes, the consistent disruption of motility genes strongly confirms that this trait is important for serovar Enteritidis infection of macrophages.

In the second group of motile mutants, three of them carried the transposon in genes that were homologous to the genes that are part of the *Salmonella* pathogenicity island I (SPI-1). This region of genes and operons encodes the type III secretion apparatus that secretes peptides that translocate into the host cell membrane and promote bacterial uptake by host cells (16). The finding of homologous genes in our assay, suggests that a similar system operates in serovar Enteritidis entry into chicken macrophages. Serovar Enteritidis SPI-1 has previously been suggested to play a role in the cellular infection of mouse cells (16). Because of the apparent similarity with the well-documented function of SPI-1 in serovar Typhimurium, the mutants in this system were not further studied.

Other mutants in the second group carried the transposon in putative regulator genes, LPS biosynthesis genes, metabolic genes, or genes with unknown function. In other pathogens, regulator genes have been demonstrated to play a crucial role in virulence as they enable the microorganisms to adapt to different host environments (40). We obtained mutants with transposon insertions in homologues of serovar Typhimurium *phoP*, *oxyR* and *barA*. In serovar Typhimurium, PhoP is part of a two-component system that regulates virulence genes that promote intracellular survival in macrophages (14). OxyR regulates genes that react to oxidative stress and thus indirectly protects against oxidative killing (43). The BarA protein contains both an autophosphorylated histidine site and a phospho-accepting site and is involved in the adaptive response in *E. coli* (24). Recently, this gene was demonstrated to act as a regulator of virulence genes in serovar Typhimurium, although the set of genes that is subject to regulation remains to be defined (3). In our experiments, we did not find many structural virulence genes known to be regulated by either PhoP or OxyR in serovar Typhimurium. One explanation for this may be that the regulated genes show redundancy in which case only mutations in the global regulator and not in individual regulated genes will be selected in our screening assay.

Our data suggest that both the polysaccharide moiety and the lipid A constituent of LPS are important for serovar Enteritidis infection of the cultured macrophages. LPS has been well established as a virulence factor in serovar Typhimurium. The O-antigen confers protective immunity (38), while the polysaccharide and the lipid A contribute to intracellular survival (14, 19, 20). The isolation of a mutant in *pmrF*, a gene which in serovar Typhimurium is involved in the 4-aminoarabinose modification of lipid A, indicates that also in serovar Enteritidis structural modification of the lipid A is required for optimal cellular infection. The number of serovar Enteritidis LPS biosynthesis mutants that were isolated suggests that LPS may be an attractive target for infection intervention.

In addition to the regulator and LPS biosynthesis genes, a number of genes likely to be involved in bacterial metabolism were found to be disrupted in the mutants (Table 1). Among those genes is the homologue of the PhoP/PhoQ regulated *ugd (pagA)* gene, which in serovar Typhimurium has been demonstrated to be transcriptionally active inside macrophages and to be necessary for growth in a low magnesium environment (2, 44, 45). Additional mutated genes

were homologues of the *tdh* gene in *E. coli* and the *cat2* gene in *Clostridium aminobutyricum* which have been suggested to function as a threonine dehydrogenase (4) and a 4-hydroxybutyrate coenzyme A transferase (36), respectively. In *E. coli*, the *tdh* homologue is regulated by the *lrp* gene (15), which is one of the regulators of virulence genes in serovar Typhimurium (30). The function of the *tdh* gene in *Salmonella* has never been established. The identified serovar Enteritidis homologue may encode the enzyme isocitrate dehydrogenase. This gene is considered to have a housekeeping function and appears to be conserved among most *Salmonella* serovars (49). Although the exact role of various metabolic genes in cellular infection awaits further study, our results strongly indicate that metabolic adaptation is essential in the infection of chicken macrophages.

One of the mutants that was studied in more detail was SEM35 which carried the transposon in the *pbpA2* gene, which belongs to the penicillin binding protein gene family. This mutant showed reduced intracellular survival. The successful complementation of SEM35 with an intact copy of the gene confirmed that disruption of *pbpA* caused the mutant phenotype. Penicillin binding proteins play a role in cell wall synthesis and the resistance against antimicrobial peptides (37). Whether *pbpA2* has a similar function is unknown. In serovar Typhimurium, the promoter of the *pbpA2* gene is acid-inducible which would be consistent with a role in intracellular survival, but this has not been further investigated (45). At this point, it may be noteworthy that our analysis of the unfinished serovar Typhimurium whole genome sequence suggests that this species carries two *pbpA*-like genes at different positions in the genome. Only the *pbpA2* located adjacent to the *argS* locus, was able to complement the defect in serovar Enteritidis SEM35. The inability of the second gene (*pbpA*) which is positioned next to the *rodA* locus, to successfully complement the *pbpA2* mutant, indicates that the two *pbpA*-like genes have different functions.

The successful identification of a series of genes involved in cellular infection of macrophages indicates that this event is multifactorial. Our results suggest that application of the mutant library nicely complements the ongoing efforts to sequence the whole genome of this pathogen and is a powerful tool in the search for virulence genes. It can be expected that further detailed analysis of the isolated mutants will shed more light on the bacterial traits important for infection of macrophages and thus may provide the basis for the development of novel infection intervention strategies.

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Chapter 3

A *Salmonella enterica* serovar Enteritidis *cat2* mutant exhibits altered carbon utilization and attenuated behaviour *in vivo*

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ABSTRACT

Screening of a *Salmonella enterica* serovar Enteritidis mini-Tn*10* library for mutants with an impaired ability to infect chicken macrophages using a colistin survival assay, resulted in the identification of mutant SEM31. FACS analysis indicated that SEM31 showed reduced intracellular survival rather than a defect in cellular invasion. BlastX analysis indicated that the mutant harboured the transposon in a gene that exhibited 84% similarity (73% identity) with the *cat2* gene of *Yersinia pestis* and 56% similarity with the *cat2* gene of *Clostridium kluyveri*. Successful complementation of the defect was achieved by introduction of plasmid pWSK29 carrying an intact copy of the gene. In *Y. pestis*, *cat2* is located on the 102 kb unstable *pgm* locus and is assumed to encode an acetyl-CoA:4-hydroxybutyrate coenzyme A transferase involved in the fermentation of succinate. Experiments with SEM31 in M9 minimal medium unexpectedly showed that the mutant had acquired the ability to utilize succinate as a sole carbon source for growth. The parental strain maintained its stationary growth phase under these conditions. Infection experiments in day-old *Salmonella*-free chicken indicated that SEM31 was much more rapidly cleared from the liver and spleen than the wild type strain, indicating that the serovar Enteritidis *cat2* gene is a critical infection determinant *in vivo*.

INTRODUCTION

Salmonellae are considered as facultative intracellular pathogens. After contact with the host, the bacteria are assumed to traverse the intestinal mucosal layer, to disseminate to the liver and spleen, and to reside inside cells of the reticulo-endothelial system (24). *Salmonella enterica* serovar Typhimurium has been demonstrated to possess a number of sophisticated machineries that orchestrate the invasive behaviour of this bacterium. These include a type III secretion apparatus that facilitates translocation of effector proteins into the plasma membrane or the cytosol of host cells (7, 9, 24) and a variety of adaptive mechanisms that enable survival and multiplication in different environments including the intracellular compartment (19).

In recent years, *Salmonella enterica* serovar Enteritidis has emerged as a pathogen replacing serovar Typhimurium as principal cause of salmonellosis in many countries (13). This has been associated with the successful eradication of the avian adapted *Salmonella enterica* serovar Gallinarum from poultry. The resulting loss of immunity against *Salmonella* of serogroup D1 is assumed to have enabled the zoonotic serovar Enteritidis, which belongs to the same serogroup, to find its niche in poultry and to spread to human via the consumption of contaminated food (13). In comparison to serovar Typhimurium, research on the molecular mechanisms that contribute to the pathogenesis of serovar Enteritidis infections has been limited. Screening of a series of random Tn*pho*A insertion mutants indicated the existence of cell line specific invasion mechanisms (20). Furthermore, analysis of defined sets of mutants supported a role of bacterial motility (21), a type III secretion system (12), LPS (6), and fimbriae (14) in serovar Enteritidis infection. A separate group of determinants that likely contribute to serovar Enteritidis infection are factors that facilitate bacterial adaptation to the changing environments encountered in the various hosts. In serovar Typhimurium, a number of metabolic enzymes have been demonstrated to be critical for virulence and to be excellent targets for the construction of strains that exhibit attenuated behaviour but that still have vaccine potential (17).

In a systematic search for serovar Enteritidis virulence determinants, we screened a transposon library of strain CVI-1 for mutants with an impaired ability to invade and/or survive within chicken macrophages. Survival assays and FACS analysis using EGFP carrying mutant and the wild type bacteria demonstrated that mutant SEM31 was impaired in its ability to survive intracellularly. Identification of the gene disrupted by the transposon, complementation experiments and functional analysis, indicated that the mutant phenotype was caused by a disruption of the *Salmonella cat2* gene involved in succinate metabolism. Experimental infections in chicken demonstrated that SEM31 was attenuated in its ability to persist in the liver and spleen, suggesting that the identified gene contributes to *Salmonella* intracellular survival *in vivo*.

MATERIAL AND METHODS

Bacterial strains, cell lines and culture conditions. *Salmonella enterica* serovar Enteritidi*s* strain CVI-1 and the chicken macrophage cell line HD-11 were obtained from ID-DLO, Lelystad, The Netherlands. The strain CVI-1 was typed as phage type 1, which corresponds to phage type 4 of the United Kingdom phage typing system (23). The construction and characteristics of the non-motile, non-flagellate serovar Enteritidis strain Se857*fliC-* have been decribed previously (21). *Salmonella enterica* serovar Typhimurium strain 420/101 was kindly provided by Dr. P. Nuijten (Intervet, Boxmeer, The Netherlands). Bacteria were grow at 37°C in 10 ml of Luria-Bertani broth in 50 ml Falcon tubes at 180 rpm or onto LB agar, unless indicated otherwise. When appropriate, ampicillin (100 μg /ml), chloramphenicol (20 μg /ml), kanamycin (50 μg/ml) and/or nalidixic acid (100 μg/ml) were added. Nalidixic acid resistant CVI-1 was used in all experiments to facilitate recovery from infected animals (see below). M9 minimal medium was composed of 0.6% (w/v) Na_2HPO_4, 0.3% (w/v) KH_2PO_4, 0.05% (w/v) NaCl, 0.1% (w/v) NH_4Cl, 2 mM $MgSO_4$ and 0.1 mM $CaCl_2$ in distilled water. Carbon sources were added as indicated. The HD-11 chicken macrophage cell line was grown (37°C, 5% CO_2) in RPMI 1640 tissue culture medium (GIBCO Inc.) supplemented with 10% FCS, 0.02% 2-mercaptoethanol, 100 U/ml of penicillin and 100 μg/ml of streptomycin.

Construction of the mini-Tn*10* mutant library. An approximately 5 kb *Eco*RI fragment containing mini-Tn*10* and the transposase gene IS*10*-R were isolated from plasmid pLOF/KM (3). After filling in the sticky ends with Klenow, the DNA fragment was ligated with the *Sma*I-digested plasmid pKO3. This suicide vector replicates in a temperature-dependent fashion and confers resistance to chloramphenicol in *Salmonella* strain CVI-1. The ligation mixture was then used to transform *E. coli* DH5α. Transformants carrying plasmid mini-Tn*10*-pKO3 were selected on LB plates containing kanamycin and chloramphenicol.

Salmonella strain CVI-1 was transformed with mini-Tn*10*-pKO3 by electroporation. The bacteria were then transferred to LB broth containing chloramphenicol and grown at 30°C for 2.5 hours. This procedure has previously been demonstrated to be sufficient for mini-Tn*10* transposition (8). After 2.5 h, the transformants were plated onto LB plates containing kanamycin and grown at 42°C to select for transposon mutants and counterselect for transformants carrying pKO3 (pKO3 replicates at 30 °C but not at 42 °C). After 16 h of growth, single colonies were collected in 100 μl of LB broth containing kanamycin in 96-well plates. After growth, duplicate microtiter plates were stored at -80°C and the original plates were used further in the screening of mutants. Growth of 50 randomly picked transformants in media containing chloramphenicol confirmed that the mutants had lost the plasmid. Southern blotting of chromosomal DNA isolated from 30 individual mutants using a kanamycin probe indicated that mini-Tn*10* had randomly inserted into the chromosome.

Fig. 1: Map of the mini-Tn*10* transposon carrying the kanamycin resistance gene cloned between the inverse repeats of Tn*903*. Artificial IS*10* are 70 bp in size. Relative locations of *Hin*dIII and *Nsi*I are marked. Annealing position of the primers are indicated with arrows.

Genetic analysis of SEM31. The mini-Tn*10* insertion site in SEM31 was determined by sequencing of the flanking chromosomal DNA obtained by inverse PCR. Chromosomal DNA was digested with *Hin*dIII or *Nsi*I, re-ligated, and used as a template in a PCR with the primer combinations

KML (5'-ATGAGTGACGACTGAATCCGGTGAG-3') and PIS903 (5'-CACTGATGAATGTTCCGTTGCG-3'), and KMR (5'-AGATGAGATGGTCAGACTAAACTGG-3') and PIS903 (Fig. 1). The PCR consisted of 35 cycles of a 30 s denaturation at 94 ^{o}C, 30 s annealing at 60 ^{o}C, and 210 s extension at 72 ^{o}C. PCR products were purified using the QIAquick PCR purification Kit (QIAGEN Inc.) and directly sequenced on an ABI-310 sequencer (Perkin Elmer Co.) with primer PIS10 (5'-TATGACAAGATGTGTATCCACC-3'). Sequencing data were processed using the Lasergene software package (DNASTAR Inc.). Similarity searches were done using the BLAST algorithms against the GENBANK database and the database of the unfinished serovar Typhimurium genome (http://www.ncbi.nlm.nih.gov).

Complementation of SEM31. The intact copy of the *cat2* gene was obtained by PCR amplification using the primers PcatL (5'-GCACTGTACTCAGCAAACGAATGTC-3') and PcatR (5'-ACCTCTTCGTAGAATCTTATT GTGG-3') and chromosomal DNA of *Salmonella* strain CVI-1 as template. The PCR consisted of 120 s of denaturation at 94°C, followed by 35 cycles of 20 s of annealing at 65°C, 120 s of extension at 72°C, and 20 s of denaturation at 94°C. The PCR product was gel purified, cloned into the pGEM-T easy vector (Promega) in *E. coli* DH5α, verified by sequencing, and then cloned into plasmid pWSK29 (25) using the *not*I restriction sites. The plasmid was introduced into *Salmonella* mutant SEM31 by electroporation, kanamycin and ampicillin resistant transformants were selected.

Construction of EGFP-producing strains. EGFP producing *Salmonella* were obtained by electroporating the plasmid pEGFP (Clontech) into strains CVI-1, Se857*fliC*- and SEM31, followed by selection on kanamycin and/or ampicillin.

Infection assay. Cellular infection of HD-11 cells was carried out as described (21) with some modifications. Bacteria grown for 16 h in LB medium were diluted (1/100) in 10 ml of fresh LB medium in 50 ml plastic Falcon tubes, grown for 2 h (37°C, 180 rpm), collected by centrifugation (15,000 x g, 1 min, 22°C), washed once with PBS, and resuspended in 1 ml of cell culture medium. Bacteria were added to HD-11 cells maintained in 2 ml of pre-warmed RPMI 1640 in 35 mm 6-well tissue culture plates at a m.o.i. of 10. After 30 min of incubation (5% CO_2, 37°C), the cells were rinsed twice with 5 ml of tissue culture medium to remove non-adherent bacteria and further incubated (2 h, 5% CO_2, 37°C) in tissue culture medium containing 100 µg/ml of colistin to kill all remaining extracellular bacteria. After removal of the colistin and rinsing of the cells twice with PBS, the macrophages were lysed (1 min, 22°C) in PBS containing 0.1% Triton X-100, and plated at various dilutions onto LB agar. The number of viable intracellular bacteria was estimated by CFU counting after 24 h of growth. In the infection assays, the non-motile, non-invasive mutant Se857*fliC*- (21) was used as negative control, while the parent strain CVI-1 served as a positive control. Infection experiments were repeated at least three times.

FACS analysis of EGFP labeled *Salmonella*. FACS analysis was applied to determine the location of *Salmonella* after infection of the macrophages. For this purpose, selected mutants were transformed with plasmid pEGFP (Clontech Inc.) which carries the gene encoding the green-light fluorescent protein under the control of a *lacZα* promoter. Infection experiments were carried out as described above except that at 30 min of incubation, the non-adherent bacterial cells were released by treatment with trypsin/EDTA in PBS. The trypsin treatment also released the macrophages. These were diluted in 1 ml of PBS and analyzed by FACS Caliber (Beckton-Dickinson) with combined measurement of light scatter and fluorescence. Data were processed using CellQuest software. FACS results were from three independent experiments.

Bacterial growth in M9 minimal media. To assess bacterial utilization of distinct carbon sources, *Salmonella* were grown in 10 ml of LB medium for 16 h, collected by centrifugation (1,600 x *g*, 6 min, 20°C), rinsed twice with PBS, resuspended into 10 ml of PBS, and diluted (1/100) in 10 ml of M9 minimal medium with the appropriate carbon sources. At 2, 4, 6, 8 and 24 h of incubation (37°C, 180 rpm), bacterial growth was estimated by measurement of the optical density at 590 nm. When appropriate, aliquots were plated onto LB agar to monitor changes in bacterial viability.

Chicken infection experiments. Day-old broilers (Ross) were obtained from a *Salmonella* culture-negative parent flock. The animals were divided into two groups and housed in isolators. The day after hatching, $1x10^5$ *Salmonella* in 0.25 ml phosphate buffered saline were orally administered to 45 animals per strain. *Salmonella* strains used for the inoculation of the animals were grown overnight in Brain Heart Infusion Broth without antibiotics. After inoculation, five chicken per group were euthanized by cervical dislocation at days 2, 5, 8, 12, 15, 19, 23, 26 and 29 after inoculation. Serial dilutions of caecal contents and of liver and spleen homogenized in peptonwater, were made in saline. One hundred microliters of each dilution was plated on Brilliant Green Agar supplemented with 100 μg/ml nalidixic acid to facilitate isolation of *Salmonellae*. Red colonies were counted after 18 hours of incubation at 37°C. A random set of colonies of the mutant strain was checked by PCR for the presence of the insert. All chicken were cared for in accordance with accepted procedures of the Dutch law of animal welfare. The ethical committee of the institute approved all experimental procedures applied to the animals.

RESULTS

Isolation of mutant SEM31 impaired in intracellular survival

In search for *Salmonella* serovar Enteritidis virulence determinants, a mini-Tn*10* mutant library of strain CVI-1 was tested for mutants that were impaired in their ability to infect chicken macrophage cells HD-11. Selection was achieved by comparing the number of bacteria that could be recovered from the intracellular compartment after 30 min of infection followed by 2 h of colistin treatment. This procedure has previously been shown to effectively kill all extracellular microorganisms (21). One of the mutants that was selected after screening of 7680 individual mutants

Fig. 2: FACS fluorogram of HD-11 macrophages infected with GFP-labeled *Salmonella*. Cells were infected with the non-flagellate mutant SE857*fliC*- as negative control (A), the parental strain CVI-1 as positive control (B), and the mutant SEM31 (C). After 30 min of infection, the infeceted cells were analyzed by FACS. The M2 peak represents macrophages that contained fluorescent bacteria. The M1 peak represents non-fluorecent macrophages. Data are from one of three experiments.

was SEM31. In a series of experiments, this mutant consistently showed a recovery of 40-50% compared to the parental strain (Fig. 2).

To further assess the nature of the defect in SEM31, we applied FACS analysis on macrophages infected with EGFP producing SEM31. Fluorescence of the bacteria was obtained by introduction of the pEGFP plasmid which replicated in *Salmonella*. Control experiments showed that the synthesis of EGFP did not influence the behaviour of the strains towards macrophages (data not shown). This was further proved by the results from experiments with the well-characterized invasion-deficient mutant Se857*fliC*- producing EGFP. For this mutant, no M2 peak (representing macrophages carrying intracellular microorganisms) was found in the FACS analysis (Fig. 2A). FACS analysis on 30 min infected macrophages revealed comparable signals for the wild type strain (Fig. 2B, peak M2) and mutant SEM31 (Fig. 2C), indicating that both strains were present in macrophages and suggesting that the mutant was most likely impaired in intracellular survival.
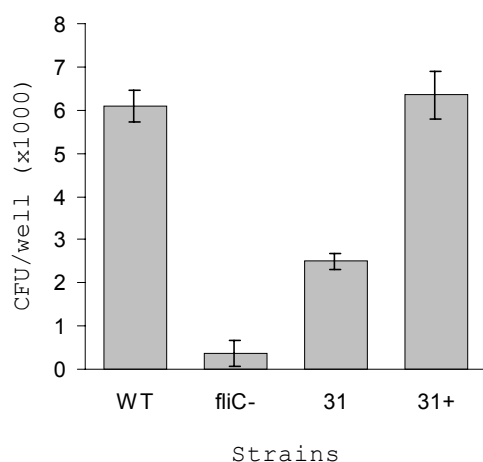
Sequence analysis of SEM31

In order to gain more information about the nature of the defect in SEM31, we determined the location of the mini-Tn*10* within the genome. This was achieved by inverse PCR on digested and re-ligated chromosomal DNA using outward oriented primers annealing in the mini-Tn*10* (Fig. 1). This procedure resulted in one PCR product. Cloning and sequencing of the product yielded 164 bp of *Salmonella* sequence flanking the transposon. BlastX analysis indicated that the obtained sequence as well as the homologous gene in serovar Typhimurium exhibited 84% similarity (73% identity) to ORF52 (putative *cat2*) in *Y. pestis* (Genbank accession number: AL031866) and 56% similarity (37% identity) to the *cat2* gene in *Clostridium kluyveri* (Genbank accession number: L21902) as well as with *cat2* homologues in other *Clostridia* species. Sequence comparison with the *cat2* gene of serovar Typhimurium indicated that the transposon had inserted at position 514-516 of the putative serovar Enteritidis *cat2* gene. The relationship with the *cat2* family was further strengthened by the presence of the VTExG motif at amino acid position 399-403 (Table 1), which appears to be a signature of *cat2* homologues (5).

Species	Sequence	Genbank accession
Salmonella	RTDTHWI**VTEFG**SVNLKGLSSTE	AF376036
Y. pestis	RIDTHYI**VTEFG**AVNLKGLSSTE	AL031866
C. kluyveri	RNEVDYV**VTEYG**VAHLKGKTLRD	L21902
C. aminobutyricum	RNDADYV**VTEYG**IAEMKGKSLQD	AJ250267
C. difficile	RNDVDIV**VTEYG**IAELKGKSLRE	www.sanger.ac.uk
Synechocystis sp.	RGDIHYV**VTEYG**IAYLHGKNIRE	D90908
C. elegans	RAHVHYV**VTEYG**IAQLWGKNMRQ	U28928

Table 1: Presence in the *Salmonella* VTExG motif present in all (putative) acetyl-CoA:4-hydroxybutyrate CoA transferases.

Complementation of SEM31

To ascertain that the mutant phenotype of SEM31 was caused by disruption of the putative *Salmonella cat2* gene, the mutant was complemented with an intact copy of the gene. The intact *cat2* gene was amplified from serovar Enteritidis strain CVI-1 by PCR using primers designed on the basis of the DNA sequence of the homologous gene in serovar Typhimurium strain LT2 as derived from the (unfinished) LT2 whole genome database. This procedure yielded a product containing the intact *cat2* gene plus 189 bp of upstream and 95 bp of downstream sequence, respectively. The product was cloned into pWSK29 behind the *lacZα* promoter (Wang, 1991) and introduced into SEM31 by electroporation.

Infection of HD-11 cells with the complemented mutant indicated that the introduction of the intact copy of *cat2* restored the survival defect observed for SEM31. In the colistin survival assay, comparable numbers of complemented and the wild type bacteria were recovered, while SEM31 showed the mutant phenotype (Fig. 3). The successful complementation confirmed that the reduced survival of SEM31 was exclusively caused by disruption of the *cat2* gene.

Fig. 3: Cellular infection of HD-11 cells with serovar Enteritidis. Cells were infected (30 min followed by 2 h of colistin treatment) with the parental strain CVI-1 (WT), the aflagellate mutant SE857*fliC-* (fliC-), mutant SEM31 (31), and the complemented mutant (31+). The number of intracellular bacteria recovered after 30 min of infection followed by 2 h of colistin treatment was determined by CFU counting. Data are the mean ± SD of three experiments.

Putative function of the *cat2* gene

In *Clostridium kluyveri*, the *cat2* (*orfZ*) gene encodes the enzyme acetyl-CoA:4-hydroxybutyrate CoA transferase that has an important metabolic function in the fermentation of succinate and succinyl-CoA (15, 18, 21). This function led us to test the ability of SEM31 and CVI-1 to utilize distinct carbon sources for bacterial growth. In this assay, bacteria were grown in minimal M9 medium enriched with 0.4% of sodium pyruvate, sodium succinate, sodium citrate, potassium acetate, sodium malate, γ-aminobutyrate or 4-hydroxybutyrate, for up to 24 h. The validity of this assay was established by growing the strain in M9 minimal media in the absence and presence of glucose. Both strains grew well when glucose was added to the medium (Fig. 4). Systematic analysis of the effect of the other carbon sources demonstrated that both strains could utilize pyruvate and, to some extent acetate, but were unable to utilize malate, γ-aminobutyrate, or 4-hydroxybutyrate for growth (data not shown). Unexpectedly however, addition of succinate or citrate to the M9 medium resulted in efficient bacterial growth of SEM31, while the wild type was unable to utilize these carbon sources for growth (Fig. 4). The

growth of the wild type could be restored by the addition of as little as 0.02% of glucose to the M9 medium containing succinate (data not shown), indicating that the succinate did not have a general inhibitory effect on the wild type strain. Analysis of the viability of the bacteria showed that the wild type strain remained viable in M9 medium for up to 16 h of incubation, irrespective of the presence of succinate (data not shown). Together, the data can be interpreted as if the *cat2* locus enabled the bacteria to remain in stationary growth phase under starving conditions with succinate as sole carbon source. In the mutant, this regulatory effect appeared to be lost, resulting in succinate mediated bacterial growth.
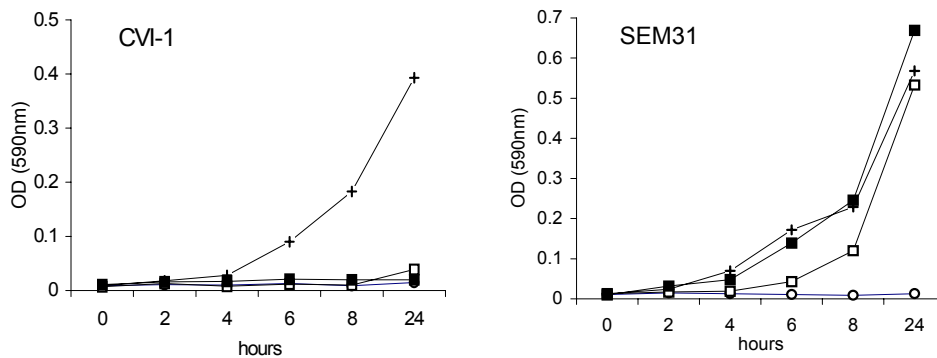

Fig. 4: Growth curves of the parental *Salmonella* strain CVI-1 and the mutant SEM31 in M9 minimal media in the absence (O) and presence of 0.4% of glucose (**+**), succinate (■), or citrate (□). Note that the mutant but not the wild-type can utilize succinate and citrate for growth. Data are from one of six experiments.
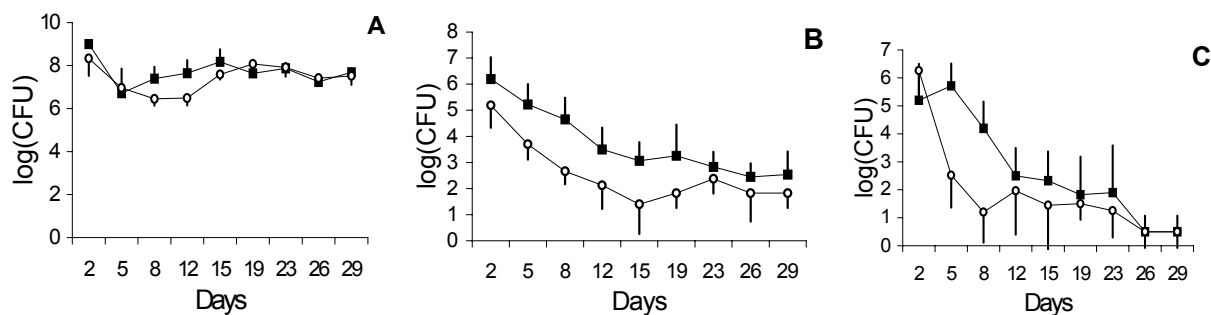
Survival of the *cat2* mutant in chicken

The significance of a functional *cat2* gene for the survival of *Salmonella in vivo* was evaluated in a chicken infection model. In these experiments, 10^5 parental strain CVI-1 or mutant SEM31 bacteria were orally administered to two groups of day-old chicks. At regular intervals, five birds from each group were sacrificed to determine the level of colonization and bacterial invasiveness. Culturing of the caecum content revealed similar numbers of the wild type and mutant bacteria during the entire infection period of 29 days, indicating that intestinal colonization was not influenced by disruption of the *cat2* gene (Fig. 5A). The level of invasiveness was determined by measuring the recovery of *Salmonella* from the liver and spleen. As shown in Fig. 5B and C, SEM31 efficiently infected the liver and spleen but was much more rapidly cleared from both the liver and spleen than the parent strain. At days 5 and 8 post-inoculation, the wild type levels isolated from the liver exceeded the number of mutant bacteria by a factor of 100-1000. Together, these data suggest that the *cat2* gene contributes to the spread and persistence of *Salmonella* by positively influencing bacterial survival in the liver and spleen.

DISCUSSION

In this work, we identified a previously unrecognized *Salmonella* gene that contributes to the ability of the pathogen to survive inside chicken macrophages *in vitro* and that enhances

bacterial virulence in chicken. This gene, designated *cat2*, was identified by screening of a mini-Tn*10* mutant library of serovar Enteritidis strain CVI-1 followed by identification of the transposon insertion site. Complementation experiments indicated that the disrupted gene was

responsible for the intracellular survival deficient mutant phenotype.

Genetic analysis indicated that the deduced amino acid sequence of the *Salmonella cat2* gene was 84% similar to the ORF52 (Cat2) of *Y. pestis*, and 56% similar to the Cat2 (OrfZ) of *Clostridium kluyveri*. In *Y. pestis*, the *cat2* gene is located in the 102 kb unstable *pgm* locus (4). This region is composed of two distinct parts: the high-pathogenicity island HPI which carries virulence genes involved in iron acquisition, and the pigmentation segment that carries the *hms* (hemin storage) virulence locus as well as many genes of unknown function (2). The function of the *Y. pestis cat2* gene, which is located in the pigmentation segment of the region adjacent to a putative two-component regulatory system (2), has not been investigated yet. In *Clostridia*

Fig. 5: Survival of the wild type *Salmonella* strain CVI-1 (■) and the mutant SEM31 (O) in chicken. Data represesent the number of bacteria (CFU) that were recovered from the ceacum content (A), the spleen (B) and the liver (C) at various times after infection of day-old chicks. Data are the mean ± SD of five chicken.

species, the *cat2* gene product has been reported to encode the enzyme acetyl-CoA:4-hydroxybutyrate CoA transferase that catalyzes the formation of 4-hydroxybutyryl-CoA and acetic acid from 4-hydroxybutyrate and acetyl-CoA (5, 21). This enzyme reaction is an important step in the utilization of several metabolites including succinate and succinyl-CoA which is a central intermediate in the tricarboxylic acid cycle. The observed strong sequence homology including the presence of the conserved VTExG motif (5), with the *cat2* gene in *Clostridia* species, suggests that in *Salmonella*, Cat2 may have a similar metabolic function and thus may exert its effect by influencing the adaptive abilities of the pathogen under various growth conditions.

An intriguing observation during the characterization of SEM31 was that the mutant appeared to have acquired the ability to utilize succinate and, to a lesser extent, citrate as single carbon sources. This finding was unexpected considering that Cat2 has been demonstrated to function in the fermentation of succinate (5, 18). The growth of the *Salmonella cat2* mutant on succinate may indicate that in the parent strain, Cat2 plays a regulatory role in energy

metabolism. It can be imagined that, dependent on Cat2 enzyme activity, metabolic intermediates are redirected into alternate metabolic pathways, thus preventing (in the presence of Cat2) or enabling (in the absence of Cat2) the utilization of distinct carbon sources for growth. In other words, under conditions of nutrient stress, *Salmonella* may prefer to maintain its stationary growth state and utilize distinct carbon sources that are available for other purposes than bacterial growth. The inhibition of growth is not detrimental to the bacteria as the carbon-starved bacteria maintained viable for up to 24 h and resumed growth directly after the addition of glucose to the minimal medium (data not shown). There is one report that serovar Typhimurium is able to utilize succinate as sole-carbon-source when grown in morpholine propanesulfonic acid (MOPS) medium (11). Analysis of the unfinished serovar Typhimurium whole genome sequence indicated that the serovar Typhimurium LT2 strain carries a copy of the *cat2* gene in its genome. In our hands, serovar Typhimurium (strains LT2 A and 421/101) were unable to grow in M9 minimal medium supplemented with succinate as sole-carbon-source (data not shown). Clearly, detailed investigation of the activity of the various metabolic pathways in different media with variable carbon sources is necessary to clarify the regulatory mechanism(s) by which *Salmonella* Cat2 exerts its effect on growth.

FACS analysis indicated that the reduced recovery of SEM31 from the infected macrophages was caused by a defect in survival rather than inefficient uptake of the bacteria by the eukaryotic cells. Intracellular survival of serovar Typhimurium requires a series of complex, tightly regulated adaptive changes that enable the bacterium to survive in the intracellular compartment and resist oxidative challenge and killing by cationic antimicrobial peptides (19). At this point, it is of interest to note that recently succinate has been demonstrated to protect stationary phase grown *E. coli* and *Salmonella* from killing by the cationic antimicrobial peptide P2 (1). Succinate was demonstrated to exert this effect by reducing the P2 mediated inhibition of O_2 consumption, although the exact mechanism of action remains to be defined. Based on these findings, one might expect that a strain that is able to utilize succinate for growth under conditions of nutrient stress (i.e. the *Salmonella cat2* mutant) is less able to survive an encounter with hostile antimicrobial peptides than a strain that cannot grow on succinate and sustains the stationary growth phase (i.e. wild type *Salmonella*). Thus, it can be speculated that the observed reduced intracellular survival of the *Salmonella cat2* mutant is related to the acquired ability of the mutant to utilize distinct carbon sources for growth. This property would reduce the local concentration and thus the protective effect of the compound, and enable the bacteria to enter the exponential growth phase in which they become highly susceptible to antimicrobial peptides and oxidative killing (1, 10, 16). However, it should be emphasized that this attractive scenario remains hypothetical as long as the exact role of Cat2 in *Salmonella* metabolism has not been elucidated.

The relevance of the *cat2* gene for *Salmonella* virulence was evaluated in a more natural setting by monitoring the behaviour of the wild type and mutant during experimental infection of chicken. This *in vivo* approach demonstrated that SEM31 was more rapidly cleared from the liver and spleen from the infected animals than the parent strain. The fact that the intestinal colonization and persistence in the gut, and the migration to the internal organs were not affected in the mutant, indicates that the *cat2* gene primarily contributes to virulence at a later

stage of the infection. In the liver and spleen, *Salmonella* is assumed to utilize macrophages as a niche of infection (26). The more rapid clearance of the *cat2* mutant from the liver and spleen thus may point to a reduced ability of the mutant to escape the killing by macrophages which is fully in line with the results of the *in vitro* experiments. The finding of a *Salmonella* mutant with an apparent defect in its metabolic adaptive abilities and attenuated behaviour in chicken, further illustrates the importance of the adaptive machinery for the virulence of this pathogen. The *in vivo* data also indicate that the bacterial adaptive requirements vary between different infection niches in a single host. Although the mutation in the *cat2* gene appears to be more critical at the invasive than colonization stage of the disease, our findings may boost the efforts to engineer serovar Enteritidis strains with limited adaptive abilities that may have vaccine potential.

ACKNOWLEDGEMENTS

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Chapter 4

The *yegQ* gene of *Salmonella enterica* serovar Enteritidis is required for chicken macrophage infection

(Manuscript in preparation)

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ABSTRACT

A *Salmonella enterica* serovar Enteritidis mutant impaired in the ability to infect chicken macrophages was selected from a mini-Tn*10* mutant library. Characterization of the mutant (SEM26) using inverse PCR and sequence analysis indicated that the transposon had inserted into a gene that showed 93% similarity with the *yegQ* gene of *Escherichia coli*. Complementation experiments confirmed that the mutant phenotype was caused by a defect in the *Salmonella yegQ* gene. Microscopy on infected macrophages indicated that the mutant was impaired in bacterial invasion rather than intracellular survival. Challenge of day-old chicks with the SEM26 resulted in unaltered colonization of the caecum but much more rapid (100-1000-fold) clearance of the mutant from the liver and spleen than the parental strain CVI-1. Western blot analysis of culture supernatants of a recombinant CVI-1 that produces YegQ with a FLAG-peptide reporter indicated that the protein was secreted into the environment. Together, our results indicate that *Salmonella* YegQ is a novel virulence determinant that is particularly important at the systemic stage of the infection.

INTRODUCTION

In recent years, *Salmonella enterica* serovar Enteritidis has emerged as the primary cause of Salmonellosis in humans in the western hemisphere (14, 19). One plausible explanation for the dramatic increase in serovar Enteritidis disease is the increase in asymptomatic infection in chicken that appeared to have followed the successful eradication of the host-adapted *S. enterica* serovar Gallinarum from the birds (2, 19). The occupation of this open niche by the more broad-host range serovar Enteritidis has led to a large reservoir from which the pathogen easily spreads to human through the consumption of contaminated chicken products. The emergence of serovar Enteritidis as a major bacterial food-borne pathogen has boosted efforts to control carriership and disease both by sanitation measures and vaccination, thus far with limited success.

Infection of eukaryotic cells is considered a major virulence trait of *Salmonella*. The pathogen has been demonstrated to colonize the intestine, to penetrate and traverse the mucosal barrier, to infect the mesenteric lymph nodes and to disseminate to more distant locations, in particular the liver and spleen (10, 23). At the cellular level, the serovar Enteritidis-host interaction is generally assumed to resemble that of the closely related serovar Typhimurium, although differences have been noted. Bacterial virulence determinants that have been implicated in *Salmonella* pathogenesis include hair-like organelles with adhesive function (fimbriae) (4, 15), flagella (15, 17), lipopolysaccharide (LPS) (11), several type III secretion systems that allow translocation of effector molecules into host cell compartments (9), and a series of adaptive systems that enable the pathogen to survive the changing environments encountered in the host (6, 18). One genetic element that may be unique to serovar Enteritidis and other closely related *Salmonella* comprises the SEF14 locus which encodes fimbriae that appear to confer interaction of the bacteria with macrophage cells (5). A major future challenge is to position the various virulence determinants in the setting of the natural infection in the various hosts. This knowledge appears imperative to develop more effective infection intervention strategies that are based on targeted inhibition of critical infection events.

In the present study, we searched for new virulence determinants that are important for the serovar Enteritidis infection of chicken macrophages. Repeated screening of a *Salmonella* transposon mutant library for mutants impaired in their ability to infect HD-11 chicken macrophage cells resulted in the isolation of SEM26. The gene disrupted in this mutant is similar to the *yegQ* of *Escherichia coli* and was found to be secreted into the environment. The factor was demonstrated to be required for efficient bacterial invasion of the macrophages and to contribute to persistence of *Salmonella* in chicken.

MATERIALS AND METHODS

Bacterial strains and cell culture. *Salmonella* serovar Enteritidis strain CVI-1 and the chicken macrophage cell line HD-11 (3) were obtained from ID, Lelystad. The non-flagellate, non-motile mutant Se857*fliC-* was kindly provided by van Asten (20). Bacteria were grown either in Luria-Bertani (LB) broth or on LB agar plates with the appropriate antibiotics (100 µg/ ml of ampicillin, 20 µg/ml of chloramphenicol, 50 µg/ml of kanamycin, and/or 100

µg/ml of nalidixic acid). HD-11 cells were grown in RPMI 1640 medium (GIBCO Inc.) supplemented with 10% FCS and 0.02% 2-mercaptoethanol.

Construction and screening of the mini-Tn*10* mutant library. The construction of the mini-Tn*10* mutant library of strain CVI-1 has been previously described (26). The library comprised 7680 mutants that were each individually tested for their ability to infect HD-11 cells. SEM26 was isolated after repeated screening of the library for mutants that showed poor invasion and/or survival as measured by the recovery of bacteria from the intracellular compartment after 30 min of infection and 2 h of colistin (100 µg/ml) treatment as described (26).

DNA manipulation. The site of insertion of mini-Tn*10* in SEM26 was determined by inverse PCR (2 min denaturation at 94$^{\text{o}}$C, followed by 35 cycles of 30 s of denaturation at 94$^{\text{o}}$C, 30 s of annealing at 60$^{\text{o}}$C, and 210 s of extension at 72$^{\text{o}}$C) with the primer combinations KML (5'-ATGAGTGACGACTGAATCCGGTGAG-3'), and PIS903 (5'-CACTGATGAATGTTCCGTTGCG-3'), and KMR (5'-AGATGAGATGGTCAGACTAAACTGG-3') and PIS903, and *Hin*dIII (or *Nsi*I)-digested and re-ligated chromosomal DNA as template. DNA sequencing was performed on an ABI Prism 310 genetic analyzer (Applied Biosystems Inc.) using the ABI dye terminator cycle sequencing kit. DNA and protein data were analyzed with the Lasergene software (DNAstar, Inc.). The BLAST algorithm (1) was used to compare sequences to databases available at the National Center for Biotechnology Information.

The y*egQ* gene of strain CVI-1 was PCR amplified with the primers PQL (5'-ATTGAGCGAAGCGGTA TGTG-3') and PQR (5'-TGAAATCGTTATGGACTGCC-3'). The PCR consisted of 35 cycles of 15 s at 94$^{\text{o}}$C,15 s at 60$^{\text{o}}$C and 120 s at 72$^{\text{o}}$C using Taq DNA polymerase (Promega). The PCR product was cloned into the pGEM-T Easy vector (Promega Inc.) in *E. coli* DH5α and re-cloned into the *not*I site of plasmid pWSK29 under the control of the *lac*Zα promoter (24). This plasmid, which replicates in *Salmonella*, was introduced into SEM26 by electroporation. Transformants were selected on kanamycin and ampicillin plates.

FLAG-tagged YegQ was constructed by PCR amplification of the *yegQ* gene as described above but with the primers pyegL (5'-CGAAGCTTATGTTTAAACCAGAACTCCT-3') and pyegR (5'-GGAGATCTATTCTTA GCATGGGGGGTTAC3') that contained extended *Hin*dIII and *Bgl*II sites, respectively, and that amplified the gene without the stopcodon. The amplified DNA was digested with *Hin*dIII and *Bgl*II and cloned into plasmid pFLAG-CTC (Sigma Inc.) that was predigested with the same enzymes. This resulted in a recombinant *yegQ* gene that carried the tag-encoding sequence at its C-terminal end. The plasmid pYegQ-FLAG was introduced into strain CVI-1 by electroporation, and ampicillin / kanamycin resistant transformants were selected.

Infection assays. For use in infection experiments, *Salmonella* was grown to exponential growth phase (2 h, 37$^{\text{o}}$C, 210 rpm) in 5 ml of LB broth containing the appropriate antibiotics. Bacteria were collected by centrifugation (1,600 x *g*, 1.5 min, 22$^{\text{o}}$C), resuspended in 5 ml of RPMI 1640 tissue culture medium, and added to HD-11 cells maintained in 1 ml of RPMI 1640 in 35 mm tissue culture dishes at an m.o.i of 10. At 30 min of incubation (37C, 5% CO_2 in air), the cells were rinsed twice with 1 ml of RPMI 1640 to remove non-adherent bacteria and further incubated in medium containing 100 µg/ml of colistin which selectively killed the extracellular microorganisms. After 2 h, the antibiotic was removed and the cells were lysed with 1 ml of 1% Triton X-100 in DPBS (1 min, 22$^{\text{o}}$C). The whole cell lysates were diluted in DPBS and plated onto LB agar to estimate the number of viable intracellular bacteria. In all infection assays, the invasion-defective strain Se587*fliC*- (20) served as negative control.

For microscopy, infection assays were performed in 1 ml of HEPES buffer (20 mM of HEPES, 5. 145 mM of NaCl, 5 mM of KCl, 5 mM of $Na_2HPO_4.2H_2O$, 5 mM of KH_2PO_4, 1 mM of $CaCl_2$, 1 mM of $MgCl_2$, 5 mM of glucose, pH 7.4) with cells grown on 13 mm circular glass coverslips as previously described (21). Bacterial adherence and invasion were determined by light microscopy, as described (22). Infection assays were repeated at least three times.

Localization of YegQ. In order to evaluate the cellular distribution of YegQ-FLAG, CVI-1 bacteria carrying pYegQ-FLAG were grown to mid-logarithmic phase in 50 ml Falcon tubes containing 9 ml of LB medium plus ampicillin. At 2 h of growth (37°C, 210 rpm), 1 ml of bacteria was pelleted by centrifugation (16,000 x g,1 min, 22°C) and lysed in 75 μl of loading buffer. The supernatant of the remaining culture was collected by centrifugation (5,500 x g, 10 min, 4°C) and proteins were precipitated with 5 vol of chilled (-20°C) aceton. After 20 min at -20°C, the precipitate was collected by centrifugation (5,500 x g, 20 min 4°C), air-dried, dissolved in 150 μl of loading buffer, and boiled for 10 min. Proteins (10 μl sample per lane) were separated by SDS-PAGE (12.5% gel) and transferred onto Hybond C nitrocellulose (Amersham). Blots were blocked (1 h, 22°C) in 10 ml of PBS containing 0.05% Tween 20, 2% milk powder and 1% BSA (fraction V, Sigma). After blocking, the blots were washed in 20 ml of PBS, incubated (30 min, 22°C) in 1 ml anti-FLAG peptide monoclonal antibody M2 (1/1000 dilution, Sigma,) in PBS containing 0.5% BSA. After washing of the blots twice with the same buffer, the blots were incubated with HRP-conjugated anti-mouse IgG (1/10,000 dilution in PBS + 0.5% BSA; Amersham) and developed using SuperSignal (Pierce).

Chicken infection experiments. Day-old broilers (Ross) were obtained from a *Salmonella* negative parent flock. The animals were divided into two groups and housed in isolators. The day after hatching, 45 animals per treatment group were orally inoculated with 10^5 *Salmonella* bacteria in 0.25 ml phosphate buffered saline. *Salmonella* strains for the inoculation of the animals were grown overnight in Brain Heart Infusion Broth containing 100 ug/ml of nalidixic acid. Five birds per group were euthanized by cervical dislocation at days 2, 5, 8, 12, 15, 19, 23, 26 and 29 after inoculation. Serial dilutions of caecal contents were made in saline. Liver and spleen were homogenized in 3 ml of peptonwater. Serial dilutions of these homogenates were made in saline. Hundred microliters of each dilution was plated onto Brilliant Green Agar supplemented with 100 ug/ml of nalidixic acid. Plates were incubated for 18 h at 37°C. Red colonies representing *Salmonella*, were counted. A random sample of colonies of the mutant strain was subcultured and checked by PCR for the specific insert. Data are presented as the mean +/-SD of five chicken, unless indicated otherwise. All chicken were cared for in accordance with accepted procedures of the Dutch law of animal welfare. The ethical committee of the institute approved all experimental procedures applied to the animals.

RESULTS

Isolation of a *Salmonella* mutant defective in intracellular survival in chicken macrophages

Screening of a mini-Tn*10* mutant library of *Salmonella* strain CVI-1 for mutants impaired in their ability to invade and/or survive inside the cultured chicken macrophage HD-11 infection model resulted in the isolation of mutant SEM26. The number of SEM26 bacteria recovered from the intracellular environment after 30 min of infection followed by 2 h of colistin treatment to kill the extracellular microorganisms, was reduced by about 70% compared to the parent strain (Fig. 1). The nature of the defect in SEM26 was further investigated by
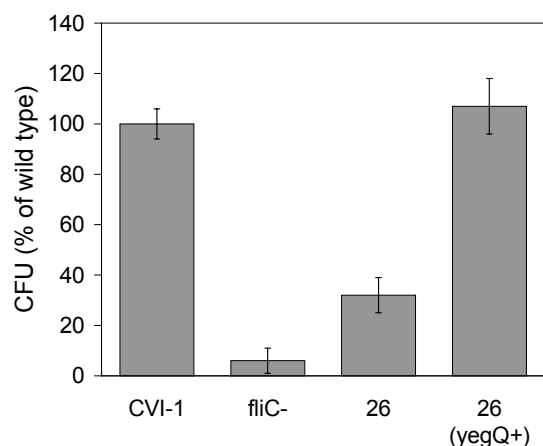
Fig. 1: Invasion of serovar Enteritidis strains CVI-1, SEM26 (26) and complemented SEM26 (26(yegQ+)) into HD-11 chicken macrophages as determined by colistin survival assay. Se857*flic-* (fliC-) is a non-flagellate, non-motile mutant which was used as a negative control. Data are the mean ± SD of five experiments.

microscopic analysis on the infected macrophages. Comparison of the number of the wild type *Salmonella* and SEM26 inside the macrophages demonstrated that the mutant was impaired in bacterial invasion rather than intracellular survival (Fig. 2).

Nature of the genetic defect in SEM26

In order to locate the insertion site of the transposon in SEM26, chromosomal DNA was digested with *Hin*dIII, re-ligated and used in a PCR with outward oriented kanamycin primers. This inverse PCR strategy yielded a fragment of approximately 250 bp. Sequencing of the product gave 166 bp of *Salmonella* sequence upstream of the transposon. Searching of the EMBL/Genbank database with the Blast algorithm indicated that the *Salmonella* DNA sequence that flanked the transposon showed 90% similarity with the *yegQ* genes of *Escherichia coli* and *Haemophilus influenzae*. Searching the database of the (unfinished) serovar Typhimurium LT2 genome (http://www.ncbi.nlm.nih.gov) yielded 97% similarity with a thus far unidentified gene.

To verify that the mutant phenotype of SEM26 was caused by disruption of the *Salmonella yegQ* homologue, we complemented the mutant *in trans* with a low copy plasmid (pWSK29) that carried an intact copy of the *yegQ* gene under the control of the *lacZα* promoter. The intact *yegQ* gene was obtained from strain CVI-1 by PCR using primers based on the homologous serovar Typhimurium sequence. This procedure resulted in the desired gene with an additional 339 bp and 66 bp up- and downstream sequence.
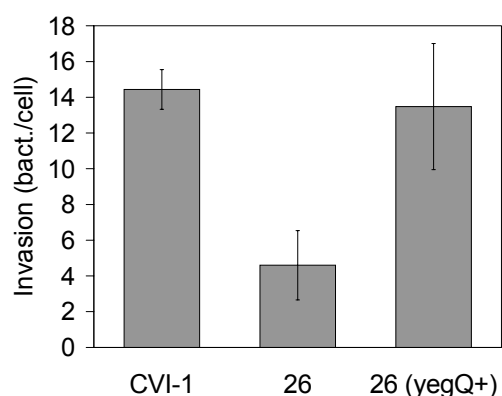

Fig. 2: Invasion of serovar Enteritidis strains CVI-1, SEM26 (26), and complemented SEM26 (26(yegQ+)) into HD-11 chicken macrophages as determined by microscopy. Data are the mean ± SD of five experiments.

Infection assays with complemented SEM26 performed as described above, showed successful restoration of the bacterial invasion to the wild type levels (Fig. 1). These data strongly suggest that the defective *yegQ* was exclusively responsible for the attenuated behaviour of SEM26 towards the chicken macrophages.

Attenuated behaviour of SEM26 in chicken

The potential relevance of the *yegQ* gene for *Salmonella* infection was evaluated in challenge experiments in chicken. Groups of five *Salmonella*-free 1-day old chicken were fed
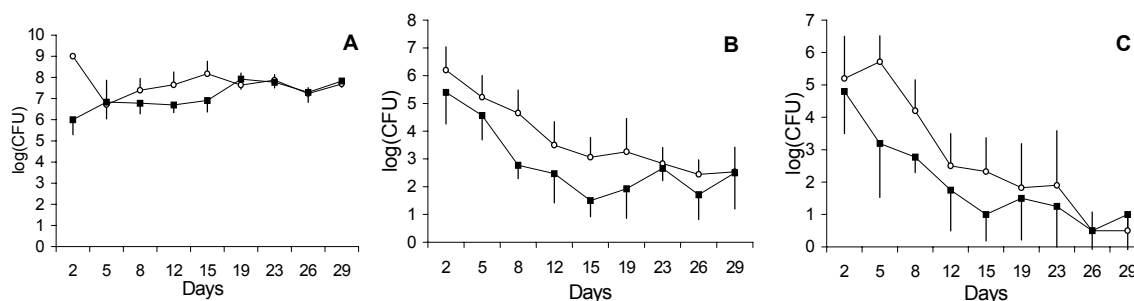

Fig. 3: Infection of one-day-old chicken with serovar Enteritidis strains CVI-1 (O) and SEM26 (■). At the times indicated, the number of bacteria recovered from the (A) cecum, (B) spleen and (C) liver were determined by plate counting (see Materials and Methods). Data are the mean ± SD of five chicken.

with approx. 10^5 of SEM26 or the wild type *Salmonella*. At 2, 5, 8, 12, 15, 19, 23, 26 and 29 days post-inoculation, 5 chicken of each group were sacrificed and the caecum content, liver and spleen were analyzed for the presence of *Salmonella*. As shown in Fig. 3, both the wild type and mutant strain efficiently colonized the chicken intestine with a mean number of bacteria of 10^8 per g caecum content throughout the entire study period. Monitoring of the number of bacteria present in the spleen and liver showed a much more rapid clearance of the mutant from these organs. This effect was particularly evident at 5 and 8 days of the infection when the mutant was recovered from these organs in numbers 100-1000-fold less than the parental CVI-1 (Fig. 3). These data suggest that the *yegQ* gene is important for *Salmonella* virulence in chicken and that

Species	Sequence	Genbank accession
Salmonella	QVPDMEI**EI****F**VH**GALC**MAYS**GR****CLLS**GYINKRD	–
E. coli	QVPDMEI**EI****F**VH**GALC**MAYS**GR****CLLS**GYINKRD	P76403
P. multocida	QVPEMEI**EV****F**VH**GALC**MAYS**GR****CLLS**GYINKRD	AE0067057
H. influenzae	QVPDMIL**EI****F**VH**GALC**MAYS**GR****CLLS**GYINKRD	U32725
V. cholerae	KCPDNTI**EV****F**VH**GALC**MAYS**GR****CLLS**GYINKRD	AE004158
P. aeruginosa	QVPDMEI**EV****F**VH**GALC**MAYS**GR****CLLS**GYINKRD	AE004956
N. meningitidis	QCPDIEL**EV****F**IH**GALC**IAYS**GR****CLLS**GYINKRD	AL162757
H. pylori	ALPNLEL**EI****F**VH**GSMC**FAFS**GR****CLIS**ALQKGRV	AE000537
P. gingivalis	KGHPVRI**EM****F**AH**GALC**MAVS**GK****CYLS**LHEHNTS	M60404
P. mirabilis	QSSPVPL**EV****F**AF**GSLC**IMAE**GR****CYLS**SYLTGES	AF088981

Table 1: Presence in the *Salmonella* YegQ of the signature motif conserved among members of the U32 peptidase family. In *Salmonella* YegQ, the motif (`ExFxxG[S/A][LIVM]CxxxxGxCx[LIVM]S`, indicated in bold) is located between positions 162 –180.

the molecule is required for bacterial survival in the liver and spleen but not for colonization of the intestine or migration towards the internal organs.

Salmonella yegQ is secreted into the environment

The YegQ protein of Salmonella shares 93% of its deduced amino acid sequence with the YegQ putative protease of *E. coli*. This protein has been suggested to belong to the U32 peptidase family. The Salmonella YegQ carried the signature motif characteristic of this family (Table 1). Other functionally characterized members of this family include the *prtC* gene in *Porphyromonas gingivalis* encoding a type I collagenase precursor (8) and the STM-protease A gene encoding the B2 protein in *Proteus mirabilis* that encodes a secreted protease (25). These genes showed 75% and 28% similar with the *Salmonella yegQ*. On the basis of these findings, we investigated the synthesis and processing of YegQ in *Salmonella*. For this purpose, we constructed a recombinant *yegQ* gene in which 24 nucleotides encoding the FLAG epitope, were inserted just in front of the stopcodon. This construct was cloned onto plasmid pWSK29 under control of the *lacZα* promoter, and introduced into Salmonella strain CVI-1. Western-blot analysis of whole cell lysates of recombinants carrying the FLAG-tagged YegQ protein showed expression of a FLAG-positive protein band of the expected size of 54 kDa as well as a number of smaller products (Fig. 4). The 54 kDa FLAG-positive band (but not the smaller proteins) was also clearly visible in the culture supernatant (Fig. 4) These data strongly suggest that *Salmonella* YegQ is secreted into the environment similar to the *P. mirabilis* protease. We analyzed YegQ with the program SignalP V2.0 (12) for the presence of a signal peptide sequence and cleavage site. The program predicted a low likelihood for a signal peptide, indicating that YegQ was secreted by another route than the Sec-dependent pathway.
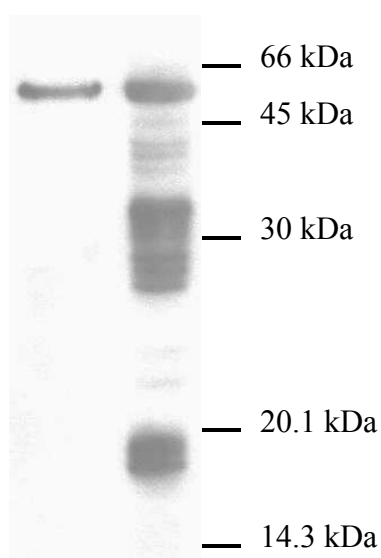

Fig. 4: Western blot demonstrating the presence of the flagged YegQ protein in whole cell lysates of serovar Enteritidis strain CVI-I carrying pWSK29::yegQ-FLAG (left lane), and concentrated culture supernant (right lane).

— 66 kDa
— 45 kDa
— 30 kDa
— 20.1 kDa
— 14.3 kDa

DISCUSSION

In this work, we identified the *Salmonella yegQ* gene as an important virulence determinant. This conclusion was based on the impaired ability of the mutant SEM26, which carried a defective *yegQ* gene, to infect macrophages *in vitro* and the attenuated behaviour of the mutant in chicken. The successful complementation of the defect with an intact copy of the gene confirmed *yegQ* as the key element responsible for the mutant phenotype.

Inverse PCR followed by sequencing indicated that in SEM26, the transposon had inserted in a gene that appeared to encode a 52 kDa protein. Computer-assisted sequence analysis indicated that this gene showed considerable similarity at the predicted protein level with genes belonging to the U32 family of peptidases. This homology and the presence of the signature sequence characteristic of the U32 peptidase family (http://www.expasy.ch), led us to designate the affected gene as the *yegQ* homologue of *Salmonella* in analogy with the *E. coli* nomenclature. In serovar Typhimurium, a gene with identical sequence to the serovar Enteritidis *yegQ* was found in the database of the unfinished LT2 genome, but thus far this gene has never been implicated in *Salmonell*a virulence.

Sequence analysis suggested that members of the U32 family of proteases with similarity to the *Salmonella* YegQ exist in many mucosal pathogens including *E. coli* (93% similarity), *Haemophilus influenzae* (85%), *Vibrio cholera* (81%), *Pseudomonas aeruginosa* (82%), *Neisseria meningitidis* (68%) and *Helicobacter pylori* (50%). The best characterized members of the family are the PrtC protein in *Porphyromonas gingivalis* and the B2 protein in *Proteus mirabilis*. The PrtC protein is located in the outer membrane fraction and has been demonstrated to exhibit collagenase-like activity which has been suggested to contribute to the periodontal lesions that may occur during *P. gingivalis* infection (8). The B2 protein of *P. mirabilis* was identified as a secreted protease during characterization of a series of signature-tagged mutants that were attenuated for virulence in a rodent model system (25). At this time, we have been unable to demonstrate proteolytic activity associated with the *Salmonella* YegQ using azocoll and gelatin as substrates.

Microscopy on infected macrophages showed that SEM26 was impaired in its ability to invade HD-11 cells. It is currently unknown how the YegQ protein may facilitate bacterial invasion into macrophages. The experiments with the FLAG-tagged *Salmonella* YegQ demonstrate that the protein may become secreted into environment. For *Salmonella* serovar Typhimurium, several proteins are secreted or directly translocated into the host cells, facilitating bacterial uptake and/or intracellular survival. Earlier studies suggest SPI-2 may export a protease or a protein that modifies the activity of a secreated protease, as consequence the mutant of *spiA* and *spiR* of serovar Typhimurium forms pre-mature flagellin (13). SPI-2 encodes another type III secretion systems, and it is essential for intramacrophage survival of *Salmonella* (7, 13). Analysis of the unfinished serovar Enteritidis genome (http://www.ncbi.nlm.nih.gov) indicates that the *Salmonella yegQ* gene is not located in one of the pathogenicity islands encoding type III secretion systems. However, we cannot exclude that YegQ is exported by one of these secretion machineries.

An important aspect of our work is the evidence that YegQ contributes to *Salmonella* infection in chicken. Infection experiments with the YegQ mutant indicated that YegQ deficiency leads to much more rapid clearance of the bacterium from the spleen and the liver but that the protein is not required for colonization of the caecum. This *in vivo* behaviour is in line with the unaltered *in vitro* growth characteristics and impaired ability to invade HD-11 chicken macrophages. In the natural infection, macrophages are assumed to play a critical role in *Salmonella* infection, in particular at the later stages of the disease when the liver and spleen

become colonized (16). The more rapid clearance but unaltered intestinal colonization of the mutant suggests that YegQ primarily acts at the later stages of infection and thus may be a potential intervention target to prevent persistent systemic disease.

Salmonella possesses a large repertoire of virulence genes that allow this pathogen to adapt to the various hosts and the changing environments encountered during the various stages of infection. The *yegQ* gene appears to be a critical element for infection of chicken macrophages. Further study directed towards resolving the machinery by which YegQ exerts its effect may help us to better understand the molecular mechanisms by which *Salmonella* manipulates the chicken macrophages, as well as the function of the U32 family of peptidases.

ACKNOWLEDGEMENTS

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Chapter 5

Identification of *Salmonella* Pathogenicity Island 6 in *Salmonella enterica* serovar Enteritidis required for cellular infection of chicken macrophages

(submitted to Molecular Microbiology)

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ABSTRACT

The *cat2* gene of *Salmonella enterica* serovar Enteritidis plays a role in regulation of bacterial growth on succinate and has been demonstrated to contribute to bacterial survival inside macrophages and *Salmonella* virulence in chicken. To further investigate this virulence gene, we assessed the ability of 12 different *Salmonella* serovars to grow with succinate as a sole carbon source. Two serovars (*S. enterica* serovars Heidelberg and Paratyphi A) were able to grow on succinate. Hybridization experiments showed that these species lacked the *cat2* gene, confirming the involvement of *cat2* on growth with succinate. The region flanking the *cat2* gene in serovar Enteritidis was cloned and comparative sequence analysis indicated that *cat2* was part of a 7.4 kb region that was unique to most *Salmonella* serovars but absent from the *Salmonella* serovars Heidelberg, Paratyphi A and Typhi. The *cat2* region comprised 7 ORFs (ORF1-7), from which 4 ORFs share homology with a putative pathogenicity island of *Y. pestis*. It had a lower than average GC content and was flanked by a *tRNA*-pheV gene at 68.2 cs on the serovar Typhumirum LT2 genome map. Infection experiments with defined ORF2, ORF4 and ORF6 (*cat2*) mutants showed impaired intracellular survival in chicken macrophages for all three mutants. In contrast to the ORF6 (*cat2*) mutant, the ORF2 and ORF4 mutant strains were not able to grow with succinate as single carbon source. On the basis of its appearance, and genetic and functional characteristics, we designated the *cat2* region as *Salmonella* pathogenicity island 6 (SPI-6).

INTRODUCTION

Salmonellae comprise a group of facultative intracellular pathogens that can cause diseases ranging from enteritis to typhoid fever. Infections are frequently associated with the ingestion of contaminated water or food. The manifestation of the infection varies largely dependent on the bacterial serovar and the susceptibility of the host. For *Salmonella*, more than 2,200 serovars have been recognized. The most well-known pathogens include *S. enterica* serovar Typhi which exclusively infects humans and higher primates, and the more broad-host-range non-typhoidal *S. enterica* serovars Typhimurium and Enteritidis. The non-typhoidal types form a major economic problem causing an estimated 1.4 million cases and hundreds of deaths in the United States every year (26).

It has been suggested that *Salmonella* virulence has evolved by the acquisition of genetic elements from other species by horizontal gene transfer (3, 4). The fact that a large proportion of the *Salmonella* virulence genes is clustered within the chromosome supports this concept. At present up to five so-called *Salmonella* Pathogenicity Islands (SPIs) have been recognized in addition to a considerable number of smaller sets of virulence genes located in distinct regions, termed pathogenicity islets (20). Typically, the SPIs are stably inserted into the chromosome, have an unusual GC content, are associated with mobile elements, and contain complex virulence determinants that appeared to have been acquired in a single event (12). Elucidation of the function of the genes encoded by the various SPIs has greatly contributed to the understanding of the molecular mechanisms that enable *Salmonella* to survive in a changing host environment.

In previous work aimed at identifying virulence determinants of *Salmonella* serovar Enteritidis, we identified a mini-Tn*10* mutant of strain CVI-1 (designated SEM31) that was impaired in its ability to survive within macrophages and exhibited attenuated behaviour in chicken (34). Characterization of the affected gene (designated *cat2*) indicated that it may have a regulatory function as the parental strain remained in stationary growth phase in media that contained succinate or citrate as sole carbon source, while the *cat2* mutant was able to grow under these conditions. In the present study, we further analyzed the *cat2* gene by investigating its conservation among *Salmonella* species. The results indicate that *cat2* is part of a cassette of virulence genes that is conserved among most but not all *Salmonella* species, and contributes to bacterial survival within chicken macrophages. The gene cassette may have been required by horizontal gene transfer and was designated as *Salmonella* pathogenicity island 6 (SPI-6).

MATERIALS AND METHODS

Bacterial strains, cell line and culture conditions. All bacterial strains and plasmids used in this study are listed in table 1. Bacteria were routinely grown on LB agar plates or in LB broth at 37°C. When appropriate, ampicilin (100 µg/ml) and/or kanamycin (50 µg/ml) were added. The chicken macrophage cell line HD-11 was obtained from ID, Lelystad, The Netherlands. The cells were grown in RPMI 1640 medium (GIBCO-BRL) supplemented with 10% FCS, 0.02% 2-mercaptoethanol, 100 U/ml penicillin 100 µg/ ml streptomycin.

Bacterial Strains	Genotype	Source or reference
E. coli DH5α	-	Lab collection
s. Enteritidis Se857	*fliC*::kan	Van Asten, 2000
s. Enteritidis SEM31	*cat2*::mini-Tn*10*	Zhao, 2001
s. Enteritidis Sm1	ORF2::kan	This paper
s. Enteritidis Sm2	ORF4::kan	This paper
s. Enteritidis CVI-1	Wild type	Van Zijderveld, 1992
s. Typhimurium LT2	Wild type	Lab collection
s. Typhimurium 421/101	Wild type	Intervet bv
s. Pullorum	Wild type	Intervet bv
s. Gallinarum	Wild type	Intervet bv
s. Dublin	Wild type	Lab collection
s. Paratyphi A	Wild type	Lab collection
s. Paratyphi B	Wild type	Lab collection
s. Heidelberg	Wild type	Lab collection
s. Infantis	Wild type	Lab collection
s. Brandenburg	Wild type	Lab collection
s. Panama	Wild type	Lab collection
s. London	Wild type	Lab collection

Table1. Characteristics of strains used in this study.

DNA cloning and sequencing. The DNA region containing the *cat2* gene was PCR amplified from serovar Enteritidis strain CVI-1 in three different overlapping fragments. Primers were based on serovar Typhimurium sequences available from the (unfinished) LT2 genome project (http://www.ncbi.nlm.nih.gov) and were used in the combinations P1a (5'-GCTCTATACGTCACAGATAATCAAG-3') and P1b (5'-GCACTGTACTCAGCAAACGA ATGTC-3'), P2a (5'-GGGACACGGTGTGAATAAAGGTATC-3') and P2b (5'-ATCTGAAAGACCGACACTGC CAATG-3'), and P3a (5'-TTTCGTCAGTCATTAACCTTGTGTC-3') and P3b (5'-GCTCAGACAGGATTTGCT AAATAAC-3'). PCRs consisted of 35 cycles of a 30 s of denaturation at 94°C, 30 s of annealing at 60°C, and 210 s of extension at 72°C. PCR products (designated 1 to 3 respectively) were cloned into *E.coli* DH5α using the pGEM-T easy vector (Promega), and sequenced in an ABI 310 sequencing device (Perkin Elmer). Sequence were assembled and analyzed using Lasergene software (DNA STAR Inc). All primers were obtained from Pharmacia Biotech.

Construction of mutants. For the construction of ORF2 and ORF4 knockout mutants, plasmids carrying PCR products containing the appropriate genes (see above) were used. Kanamycin resistance cassettes were inserted into the unique *Pst*I sites present in the respective genes, and the DNA fragments were recloned into pKO3 plasmid, which is a conditional suicide vector that replicates at 37°C but not at 42°C (19). Plasmids purified from *E. coli* DH5α were introduced into serovar Enteritidis strain CVI-1 by electroporation and knockout mutants were obtained by selection on kanamycin at 42°C. The disruption of the target genes was confirmed by PCR.

Hybridization experiments. In the dot-blot experiments, *Salmonella* chromosomal DNA was extracted using the CTAB method (29), spotted (0.2 µg of DNA) onto Hybond N membrane (Amersham Pharmacia Biotech), and hybridized with ORF2, ORF4 and ORF6 specific probes. Probe 1 consisted of a gel-purified 950 bp fragment obtained from PCR clone 1 (see above) after digestion with *Sma*I and *Pst*I. Probe 2 (1.6 kb) was obtained from

clone 2 after digestion with *Acc*I and *Pst*I, and probe 3 (1.1 kb) was obtained from clone 3 after digestion with *Hind*III and *Eco*RI. All probes were labeled using the DIG-DNA labeling kit (Boehringer Mannheim). Blots were hybridized at 68^oC for overnight in DIG-DNA hybridisation buffer (5xSSPE, 1% bocking reagent, 0.1% N-lauroylsarcosine, 0.02% SDS), and washed for 30 min by 2 x SSPE, 0.1% SDS at 60^oC, followed by 5 min 0.1 x SSPE, 0.1% SDS at room temperature. Blots were developed with the DIG Nucleic Acid Detection Kit (Boehringer Mannheim). For Southern blotting, chromosomal DNA was cut with *Eco*RI, separated onto 1% agarose gels and transferred onto Hybond N membrane (Amersham Pharmacia Biotech). Hybridization was carried out as described above.

Cellular infection of cultured chicken macrophages. *Salmonella* infection of HD-11 chicken macrophages was essentially carried out as described (34). In brief, log-phase grown bacteria (2 h, 37^oC) added (m.o.i of 10) to HD-11 cells kept in 35 mm tissue cultures dishes in RPMI without serum or antibiotics. After 30 min the infected cells were rinsed twice with PBS to remove free bacteria and re-incubated in fresh RPMI containing colistin (100 µg/ml) to kill all extracellular microorganisms. After removal of the colisitin, cells were lysed in 0.1% Triton X-100 in PBS and plated at various dilutions onto LB agar plates. After overnight growth (37^oC), the number of colony forming units (CFU), representing the number of intracellular bacteria, was counted. In all experiments, the invasion-deficient mutant Se857*fliC*- (31) and the invasive wild type strain CVI-1, were used as controls. Infection experiments were repeated at least three times.

Bacterial growth in minimal media. For evaluation of growth in minimal medium, *Salmonella* grown in LB medium (16 h, 37^oC, 180 rpm), were collected by centrifugation (1,600 x g, 10 min, 20^oC), washed once with PBS to remove residual nutrients and diluted (1/100) into 10 ml of M9 minimal medium (composition: 0.6% Na_2HPO_4, 0.3% KH_2PO_4, 0.05% NaCl, 0.1% NH_4Cl, 2 mM $MgSO_4$ and 0.1 mM $CaCl_2$) with varying carbon sources. Bacterial growth (37^oC, 180 rpm) was measured at various time intervals by measuring the optical density of the culture at 590 nm.

Genbank accession number: The DNA sequence of SPI-6 is available at Genbank under accession number AF376036.

RESULTS

Salmonella species vary in their ability to grow with succinate as a sole carbon source

In previous work, we demonstrated that the *cat2* gene of serovar Enteritidis strain CVI-1 restricts growth in M9 minimal medium with succinate as a sole carbon source (34). In order to further establish the relationship between the *cat2* gene and succinate utilization, we monitored the growth of 12 different *Salmonella* serovars in M9 minimal media containing either no carbon source, 0.4 % glucose, or 0.4% succinate. The serovar Enteritidis *cat2* mutant was included as a control. All tested serovars grew in M9 medium containing glucose but not in M9 medium without carbon source. With succinate as a sole carbon source, only two serovars, Heidelberg and Paratyphi A, were able to grow in the presence of succinate and thus showed the same phenotype as the serovar Enteritidis *cat2* mutant (Table 2). Plating of overnight cultures of the serovars that did not grown on succinate for growth showed that the bacteria were still viable and thus remained in stationary phase during the culture period (data not shown).

S. enterica serovars	no-C	glucose	succinate	*cat2*
Enteritidis CVI-1	–	+	–	+
Typhimurium LT2	–	+	–	+
Typhimurium 421/101	–	+	–	**NT**
Pullorum	–	+	–	+
Gallinarium	–	+	–	+
Dublin	–	+	–	+
Paratyphi A	–	+	+	–
Paratyphi B	–	+	–	+
Heidelberg	–	+	+	–
Infantis	–	+	–	+
Brandenburg	–	+	–	+
Panama	–	+	–	+
Enteritidis Sm1	–	+	–	Δ ORF2
Enteritidis Sm2	–	+	–	Δ ORF4
Enteritidis SEM31	–	+	+	Δ ORF6

Table 2: Relationship between utilization of succinate as sole carbon source for growth (24 h, 37°C) and the presence of SPI-6. Bacteria were grown in M9 minimal medium in the absence (no-C) or presence of glucose, or with succinate as sole carbon source. The absence or presence of the *cat2* gene was determined by dot blot hybridization using a *cat2*-specific probe (see Fig. 1). NT: not tested.

Presence of the *cat2* gene among *Salmonella* serovars

The aberrant growth behaviour of the serovars Heidelberg and Paratyphi A described above, suggested that these strains either carried a non-functional *cat2* gene, lacked the gene, or were otherwise unable to maintain their stationary phase in the presence of succinate.
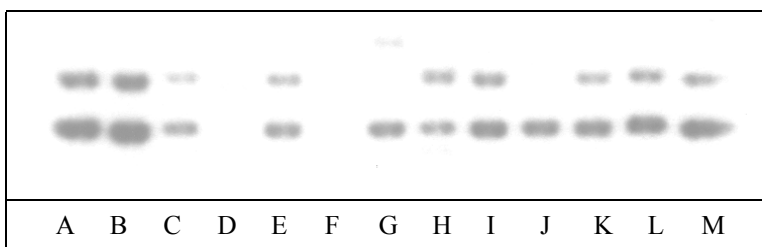

Fig. 1: Southern-blot of SPI-6 distribution among *Salmonella* serovars. Southern hybridisation was done with probe 3 that is flanking ORF6 (*cat2*) and part of ORF7. Chromosomal DNA was digested with *Pst*I and *Hind*III. Abbreviations: (A) s. Enteritidis CVI-1; (B) s. Typhimurium LT2; (C) s. Dublin; (D) s. Paratyphi A; (E) s. Paratyphi B; (F) s. Heidelberg; (G) s. Enteritidis SEM31; (H) s. Pullorum; (I) s. Gallinarium; (J) s. Infantis; (K) s. Brandenburg; (L) s. Panama; (M) s. London.

Hybridization experiments using DNA isolated from the 12 different serovars using the *cat2* gene of serovar Enteritidis as a probe, gave positive signals for 10 out of the 12 strains (Fig. 1). No hybridization was obtained for serovars Heidelberg and Paratyphi A, indicating that *cat2* is absent from these serovars (Table 2). These data together with the ability of these serovars to grow on succinate, strengthen the concept that the *cat2* gene is involved in sustaining the

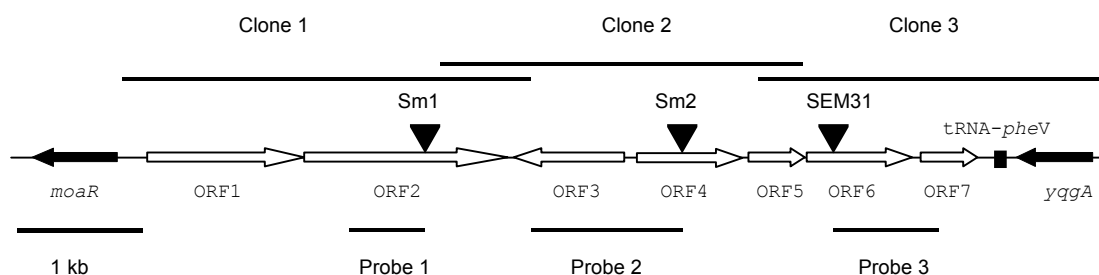stationary growth phase under the conditions employed.

Fig. 2: Genomic organization of SPI-6 (drawn on scale). Open arrows show the region unique to *Salmonella* serovar Enteritidis strain CVI-1 and Typhimurium strain LT2. Solid arrows and squares represent homologous region with *Salmonella* serovars Typhi and Paratyphi A. The solid box is the *tRNA*-pheV gene. Sequence data of the incomplete ORF 2 to ORF 7 can be accessed through Genbank accession number: AF376036. Solid triangles indicate the positions of the three mutations. The length of the bar represents 1 kilobase.

Identification of a serovar Enteritidis DNA region that is absent from the serovar Paratyphi A genome

To further investigate the potentially important difference between the *Salmonella* serovars, we characterized the DNA region flanking the serovar Enteritidis *cat2* gene. The region was cloned as three overlapping fragments that were obtained by PCR using primers
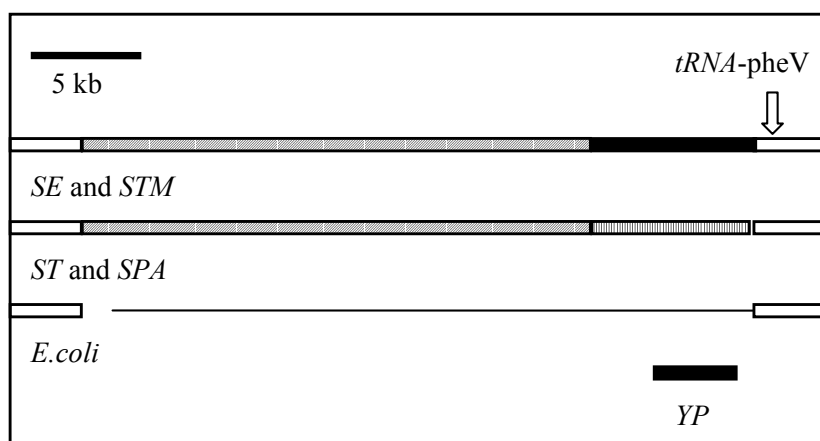

Fig. 3: Alignment of genome DNA sequences flanking the SPI-6 locus in several bacterial species. The position of the *tRNA*-pheV gene is marked with an open arrow. Filled boxes represent SPI-6 homologues in s. Enteritidis CVI-1 (*SE*) and s. Typhimurium LT2 (*STM*) and part of HPI in *Yersinia pestis* (*YP*). The box filled with vertical lines represents the region in s. Typhi (*ST*) and s. Paratyphi A (*SPA*) of the region of SPI-6 in s. Enteritidis. The box filled with diagonal lines is the region common to all *Salmonella* serovars adjacent to SPI-6. Open boxes are homologues sequences present in many bacteria species including *E. coli* K-12 and O-157. The thin line is the region adjacent to the *tRNA*-pheV gene in *E.coli* which is absent from *Salmonella*. The bar represents 5 kilobase.

based on the available preliminary serovar Typhimurium whole genome sequence (see Materials and Methods). On the basis of obtained sequence, a 7.4 kb DNA region (designated as *cat2* region) was identified that appeared to be unique for the serovars Enteritidis and Typhimurium as deduced from comparison with the unassembled Typhimurium LT2 and Paratyphi A genome sequences and the available serovar Typhi genome sequence (Fig. 3). The unique *cat2* region was positioned between the Typhi *moaR* gene (2), encoding a positive regulator of the monoamine regulon, and a *tRNA*-pheV gene located adjacent to a putative *yqgA* gene (Fig. 2). This region is located at 68.2 cs on the Typhimurium strain LT2 genome map (27). In serovar Typhi, the region between these two genes was 7.1 kb in size, and contained a homologue of an E7-like colicin immunity gene, and a putative intergrase gene.

Genomic structure of the *cat2*-region

Computer-assisted analysis of the sequence of the *cat2*-region of serovar Enteritidis indicated that the region had a G+C content of 47.2%, which is below the average of 52% for *Salmonella* (11). It contained 7 putative open reading frames (ORFs) (Fig. 2). Database searches using BLAST algorithms (1) indicated that ORF1 and ORF2 were 55% and 80% identical to the *Klebsiella pneumoniae atsB* and *atsA* gene, respectively. These genes are involved in the utilization of inorganic sulfide (23). Interestingly, ORF3 to ORF6 showed considerable similarity both at the nucleotide and predicted protein level (Table 2) with a four-gene operon located in the so-called unstable 102 kb *pgm* locus of *Yersinia pestis* (EMBL/Genbank accession number: AL031866). This locus also included the *cat2* gene (ORF6). For *Y. pestis*, the functions of ORF3-, 4 and 5 proteins have not been determined. However, ORF3 is the equivalent of *stmR* gene in *Salmonella* serovar Typhimurium, which has previously been reported to be specifically transcribed when the bacterium resides in a host cell (21). ORF4 has similarity with the *citE* gene encoding the citrate lyase beta chain. ORF5 has homology to a regulatory protein of unknown function. ORF7 showed 65% positive at the deduced amino acid level with a putative ring-cleaving dioxygenase in *P. aeruginosa*. This gene is located upstream of a *tRNA*-pheV gene, which marks the boundary of this variable region. The characteristics of each ORF are listed in Table 2. A detailed map of the region is shown in Fig. 2.

Southern blot analysis on *Pst*I and *Hin*dIII double digested chromosomal DNA derived from each of the *Salmonella* serovars tested above with a probe specific to ORF6 (*cat2*) showed similar patterns among the 10 *Salmonella* serovars, while again no signal was obtained for the serovars Heidelberg and Paratyphi A (Fig. 1). Dot blots of chromosomal DNA from 12 serovars with 3 probes specific for ORF2, ORF4, and ORF6, respectively, showed similar results. ORF2, ORF4, and ORF6 are present in 10 serovars but absent from the 2 mentioned above (data not shown). These results suggest that the *cat2* region is conserved among the species that were unable to utilize succinate and may have been acquired as a single unit.

The *cat2* region is important for *Salmonella* infection of chicken macrophages

We previously demonstrated that the serovar Enteriditis *cat2* (ORF6) mutant SEM31 is impaired in intracellular survival in chicken macrophages and shows attenuated behaviour *in vivo* (34). In order to investigate whether other genes in the *cat2* region also encode virulence determinants, we inactivated ORF2 and ORF4 by inserting a kanamycin cassette in unique *Pst*I

sites in the same orientation (verified by PCR) as the disrupted ORFs. The resulting mutants, designated Sm1 and Sm2 respectively, exhibited normal growth characteristics in LB medium. Analysis of the behaviour of these mutants in the chicken macrophage HD-11 infection model revealed that both mutants were severely impaired in bacterial survival compared to the parent strain CVI-1. The number of intracellular bacteria recovered from the infected cells was even lower than obtained for the *cat2* mutant SEM31 (Fig. 4). These data strongly suggest that at least three genes located in the *cat2*-region are important for *Salmonella* infection of chicken macrophages.

As Sm1 (ORF2::kan) and Sm2 (ORF4::kan) exhibited the same mutant phenotype as the *cat2* mutant with regard to the infection of macrophages, we also assessed the relationship between impaired intracellular survival and the failure of the bacteria to maintain the stationary phase with succinate as a sole carbon source. As shown in table 2, both Sm1 and Sm2 were unable to grow in M9 minimal media containing succinate as sole carbon source in contrast to the *cat2* mutant. In the presence of glucose, all strains grew equally well. These data indicate that ORF2 and ORF4 are not involved in the regulation of growth on succinate and exert their effect on intracellular survival via a different mechanism.

Fig. 4: Recovery of SPI-6 mutants from the intracellular compartment of HD-11 chicken macrophages. Cells were infected with the parent serovar Enteritidis strain (CVI-1), and mutants with defects in ORF2 (Sm1), ORF4 (Sm2) and ORF6 (SEM31). Data are the mean ± SD of triplicates in one representative experiment.

DISCUSSION

Salmonella pathogenicity islands (SPIs) are large gene cassettes within the *Salmonella* chromosome that confer specific virulence traits and may have been acquired by horizontal gene transfer (20). The *Salmonella cat2* region identified in the present study is composed of 7 ORFs, from which at least three genes promote bacterial survival within chicken macrophages and at least one gene (*cat2*) is important for *Salmonella* virulence in chicken (34). The region appeared to be present in 10 out of 12 *Salmonella* serovars tested, and shared homology with a large region involved in virulence in *Y. pestis*. This together with the low GC content of the region (Table 3) and its location adjacent to a *tRNA*-pheV gene, strongly suggests that the *cat2* region was acquired by horizontal transfer from another microorganism. Although the designation of a chromosomal region as a pathogencity island is somewhat arbitrary, we propose on the basis of its genetic characteristics and its role in virulence to classify the identified *cat2* region as *Salmonella* pathogenicity island 6 (SPI-6).
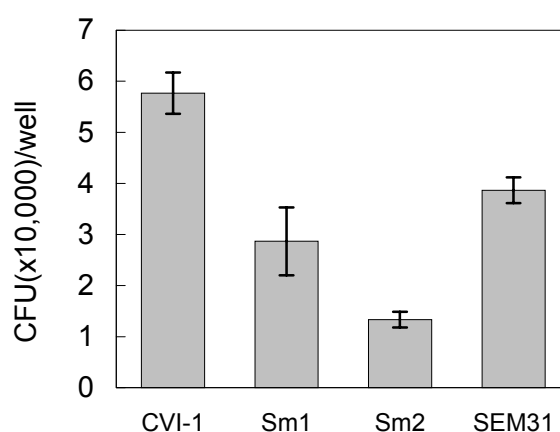
It is assumed that SPIs have evolved at different occasions and may have enabled the pathogen to reach a higher level of host adaptation. SPI-1, which constitutes a complex of more than 40 genes, is required for intestinal infection and encodes a type III secretion apparatus that facilitates translocation of bacterial peptides into the host cell (17, 9). The much smaller SPI-2 to 5 have been suggested to enable the bacteria to pass the lymphoid barrier and disseminate to more distant organs (20). SPI-2 encodes a second type III secretion system that is induced intracellularly (28, 8) and that is required for bacterial survival in macrophage cells (24, 15). Genes located in SPI-3 and SPI-4 also contributes to intra-macrophage survival (5, 32). The recently discovered SPI-5 appears to contribute to enteric rather than to systemic disease (33), although the exact function of the genes in SPI-5 have not been investigated. Our data suggest that SPI-6 belong to the category of SPIs that contribute to intracellular survival in macrophages and that prevent rapid clearance of the bacteria from the liver and spleen (34).

Name	(G+C)%	Related gene	Putative function	Species	Database access ID	E value
ORF 1	50	*atsA*	arylsulfatase regulatory protein	*K. aerogenes*	P20714	3e-113
ORF 2	46	*atsB*	Arylsulfatase precursor	*K. aerogenes*	P20713	0.0
ORF 3	52	*stmR*	transcriptional regulator	s.Typhimurium	AAD54118	2e-157
		ORF 49	transcriptional regulator	*Y. pestis*	CAA21372	5e-120
ORF 4	49	ORF 50	citrate lyase beta chain	*Y. pestis*	CAA21373	9e-94
ORF 5	50	ORF 51	regulatory protein	*Y. pestis*	CAA21374	2e-81
ORF 6	45	*cat2*	4-hydroxybutyrate coA transferase	*C. kluyveri*	P38942	5e-71
		ORF 52	4-hydroxybutyrate coA transferase	*Y. pestis*	CAA21375	0.0
ORF 7	38	-	ring-cleaving dioxygenase	*P. aeruginosa*	AAG04269	2e-38

Table 3: Characteristics of ORFs located in SPI-6. Related genes were identified by searching EMBL/Genbank and the SWISSPROT databases using the blastX algorithm (Altschul, 1997).

SPI-6 was found to be present in 10 *Salmonella* serovars but absent from the serovars Heidelberg, Paratyphi A and Typhi. The latter could not be experimentally confirmed as safety regulations prevented *ex-silico* analysis of the Typhi serovar. The variable presence of SPI-6 among *Salmonella* serovars is not unique and has previously been observed for other SPIs (14, 25). Genome analysis indicated that the serovars Typhi and Paratyphi A that lacked the *cat2*-region, contained 7.1 kb of DNA between the *moaR* and *tRNA*-pheV genes. In this 7.1 kb region, 2 genes were predicted to be present: the E7-like colicin immunity gene and a putative integrase gene located adjacent to the *tRNA*-pheV gene. Whether these genes contribute to bacterial virulence and whether the change of SPI-6 in these serovars limits their pathogenic potential or host range, awaits further investigation.

SPI-6 has probably not been acquired as a single genetic element. Analysis of its GC content reveals that SPI-6 has a mosaic structure (Fig. 2) suggesting that it may have evolved in a multistep process. Genome analysis indicates that a 4 kb region of SPI-6 (ORF3 to 6) is a

homologous to a region in the unstable 102 kb *pgm* locus of *Yersina pestis* (6). The function of the related genes in *Yersina* is unknown but they are located directly adjacent to a region involved in bacterial virulence (7). The other segment of SPI-6 (ORF1 and 2) has no homology to *Y. pestis* genes but contains putative *Klebsiella aerogenes* AtsA and AtsB homologues that function in sulphate metabolism (22). In *Klebsiella*, the *atsAB* (ORF1 and ORF2) genes are regulated by the *moaR* gene (22) which we considered to be located outside SPI-6 as it was present in all *Salmonella* serovars. Here it should be noted that the boundaries of SPI-6 as determined in this work were based on the heterogeneity of the region among *Salmonella* serovars. At this time, we cannot exclude that SPI-6 is larger than the region identified in this work and extends beyond the *moaR* gene. Sequence analysis of the region downstream of *moaR* indicates that approximately 23 kb of DNA is present in all *Salmonella* serovars but absent from *E. coli* (Fig. 3). Thus, a third segment belonging to SPI-6 but common to all *Salmonella* serovars may exist. The mosaic structure of SPI-6 is not inconsistent with its function as a pathogenicity island. For several other SPIs, it has been demonstrated that individual genes or sets of genes have been acquired in different events, even when they act in *Salmonella* as a single functional unit (13)

Pathogenicity islands are often flanked by *tRNA* genes. In *E. coli*, PAI-2, PAI-4 and PAI-5 are located adjacent to the *tRNA*-leuX, *tRNA*-pheV and *tRNA*-pheR genes, respectively (30, 18). In *Salmonella* serovar Typhimurium, SPI-2 is positioned adjacent to the *tRNA*-Val locus (14), and SPI-3 and 5 adjacent to the *SelC* (*tRNA*-Sec) and *SerT* (*tRNA$_1$*-Ser) locus, respectively (9). The SPI-6 discovered in this study was found to be flanked by a *tRNA*-pheV gene. It has been suggested that a bacterial prophage may be responsible for the horizontal transfer of virulence determinants into *Salmonella* serovars (10). One element associated with *tRNA*-pheV site-directed gene transfer, is the CTn*scr94*-like conjugative transposon (16). This transposon specifically recognizes *tRNA*-pheV or *tRNA*-pheU genes to integrate into the host-bacterial chromosome. In this regard, it is interesting to note that the 7.1 kb region in serovar Typhi and serovar Paratyphi A contains an integrase gene, which may indicate the involvement of phages or transposons in the insertion of genes at this site.

Functional analysis of SPI-6 indicated that at least three genes contribute to *Salmonella* virulence. Mutagenesis of ORF2, ORF4 and ORF6 (*cat2*) resulted in impaired infection of chicken macrophages. It is currently unclear how the respective genes exert their effect on survival in the macrophage. The *cat2* gene was confirmed to function in the maintenance of growth arrest under conditions in which succinate was the sole carbon source (34). All serovars that carried the *cat2* gene as indicated by the hybridization experiments, did not grow on succinate, while the few serovars that lacked *cat2* did grow under these conditions. Whether the effect of *cat2* on the growth status of the bacteria is related to its role in facilitating intracellular survival is unknown. Mutation of ORF2 and ORF4 did not influence bacterial growth on succinate, although cellular infection of macrophages was severely impaired. ORF2 and ORF4 show similarity with the *atsB* and *citE* gene that is involved in sulfate and citrate metabolism, respectively. In serovar Typhimurium, the *stmR* (ORF3) gene has recently been reported to be specifically transcribed inside macrophages (21). These findings together with the apparent involvement of *cat2* in succinate utilization and bacterial growth, may indicate that SPI-6 is

involved in creating a phenotype that enables the bacteria to adapt to the altered metabolic requirements in the intracellular environment.

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Chapter 6

Summarizing Discussion

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Summarizing Discussion

Salmonella enterica serovar Enteritidis is one of the major etiologic agents of human food-borne gastrointestinal infections. Efforts to control the number of serovar Enteritidis infections have had a limited success, in part because of the lack of knowledge of the molecular mechanisms that contribute to the spread and development of the infection. In this thesis, novel *Salmonella* virulence determinants were identified through screening of a mutant library of the serovar Enteritidis strain CVI-1 for bacteria that were impaired in their ability to infect chicken macrophages. The method used to generate the library was based on the random transposition of the mini-Tn*10* transposon into the bacterial genome. The HD-11 chicken macrophage cell line was chosen as an infection model instead of the commonly used murine or human cell lines since chicken are the primary host for serovar Enteritidis and consumption of contaminated chicken is a major source of *Salmonella* infection in humans.

Forty-four mutants were identified that were impaired in their ability to enter and/or to survive in the HD-11 cells. Genetic analysis of the mutants showed that most of the affected genes have previously been associated with bacterial virulence in the related serovar Typhimurium. This indicates that both serovars share a large part of their virulence repertoire. Five mutants were found to carry the transposon in genes not previously reported to be involved in virulence. The defects in three of the mutants (the *pbpA2*, *yeg*Q and *cat*2 mutant) could be complemented and were further studied in detail. During these studies, a novel *Salmonella* pathogenicity island was discovered as well as a novel carbon source-dependent type of regulation of the bacterial growth. The latter may be important for the ability of the bacterium to resist oxidative challenge and killing by cationic antimicrobial peptides in an intracellular environment (2).

1. Construction and screening of the mini-Tn*10* library

The transposon derivatives mini-Tn*5* and mini-Tn*10* are widely used tools for random mutagenesis in gram-negative bacteria (6). The *Salmonella* genome consists of about 4.5 million base pairs and is expected to contain approximately 4,000 genes. The Tn*10* mutagenesis library that was generated consisted of 7,680 mutants. It is expected that in this library with a 95% likelihood all genes are disrupted by the transposon, assuming that the transposition was a random event and all mutants are viable. Screening of this library for mutants with an impaired ability to infect and/or survive within chicken macrophages yielded 44 mutants. This number is smaller than the number of virulence genes identified in the related serovar Typhimurium (15). Two reasons can be given for this observation. Firstly, the library was screened for mutants with a defect in short-term bacterial survival inside macrophages, thus excluding genes that play a role in the long-term persistence and propagation of the bacteria within the host cell. Consistent with this hypothesis, approximately half of the genes that were identified have been considered to be involved in motility and attachment, processes that play a role in the early stages of the cellular infection. A second reason for the limited number of virulence genes that was identified might be that mini-Tn*10* had some preference for inserting into the genome at particular positions (hot spots) which then are over-represented in the library (4). An example of the

presumed hot–spot effect might be the finding of four independent mutants in both the *fliD* and *ugd* gene.

2. Types of *Salmonella* mutants found in this study

Cellular infection of macrophages involves three major steps: bacterial attachment to, invasion of, and survival within the host cell. The screening of the mini-Tn*10* mutant library for mutants with an impaired ability to infect and/or survive within chicken macrophages, thus theoretically only yields mutants with defects in the mechanisms that confer these functions. The mutants obtained in this study can be classified according to the scheme in Figure 1. The first group contains mutants in virulence genes that are involved in attachment. These include genes coding for proteins involved in attachment, chemotaxis and motility. A considerable number of these mutants had defects in motility genes, stressing the importance of bacterial motility in *Salmonella* infection. The genes involved in motility of *Salmonella* have been extensively investigated in other bacterial species (14). Therefore, the serovar Enteritidis genes identified in this study were not further investigated.
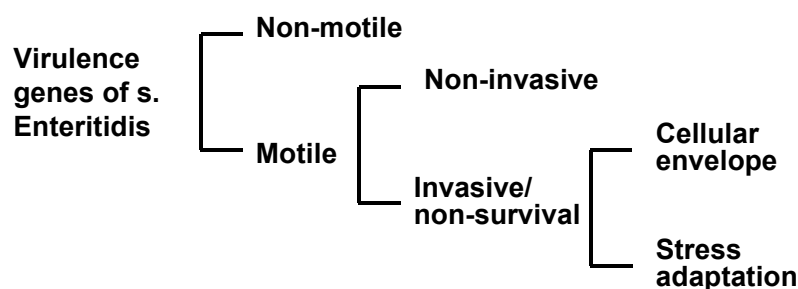

Fig. 1: Classification of the mutants of *serovar Enteritidis* obtained in this study.

A second group of mutants carried defects in genes (*invG, invI* and *spaS*) known to be involved in bacterial invasion of serovar Typhimurium (4). For this group, a larger number of affected genes was expected as for serovar Typhimurium, many genes have been demonstrated to be required for the synthesis of the type III secretion-dependent bacterial invasion machinery (4). This type III secretion apparatus is encoded by SPI-1 which contains 29 ORFs (4), from which a number are required for *Salmonella* invasion in epithelial cells (11). At this time, it is uncertain whether the relative limited number of invasion genes found for serovar Enteritidis implies that the invasion into chicken macrophages is a much less complex process than in other cell types. Thus far, invasion of *Salmonella* into chicken macrophages has only rarely been investigated.

Bacterial survival inside macrophages is an important virulence trait of *Salmonella* (8). LPS is a major constituent of the bacterial outer membrane and is essential for survival of serovar Typhimurium in macrophages (7). LPS biosynthesis is a complex process that involves the sequential addition of monosaccharides to a lipid A backbone, and the transport and appropriate insertion of the glycolipid into the cell wall. A large number of genes have been shown to be involved in the synthesis of Typhimurium LPS. Consistent with this knowledge, a

number of different transposon mutants with defects in LPS biosynthesis genes were found (Chapter 2). Both genes involved in lipid A arabinose modification (*pmrF and pmrB*) and polysaccharide synthesis (*rfaI, rfaJ* and *rfbI*) were identified. No mutants in genes that are essential for the LPS inner core biosynthesis were detected. The absence of mutants with defects in genes involved in the biosynthesis of LPS side-chains may indicate that these residues are not essential for intramacrophage survival under the conditions employed. Because of the considerable knowledge of the role of LPS in *Salmonella* survival within host cells, the serovar Enteritidis genes involved in LPS synthesis were not further studied.

In addition to the LPS genes, a number of motile, non-survival mutants with defects in genes that may be involved in bacterial metabolism and in the starvation-stress response (SSR), were obtained. Among these genes were three regulatory genes (*phoP, oxyR* and *barA*), known to influence virulence in other *Salmonella* serovars (1, 20). Among the remaining 6 mutants, four carried defects in metabolic genes (*ugd, tdh, icd* and *cat2*), one in a gene involved in peptidoglycan synthesis (*pbpA2*), and one in a putative protease precursor (*yegQ*). Three of these genes (*pbpA2, cat2* and *yegQ*) are newly identified *Salmonella* virulence genes and were chosen for further analysis (Chapters 2, 3, and 4). The fact that these genes have not been found previously in serovar Typhimurium may be related to the use of the HD-11 chicken macrophage cell line instead of the frequently used mouse cell line model, and the search for short-term rather than long-term survival deficient mutants. The latter may also explain why in the present study, no mutants in SPI-2 were found. This pathogenicity island has previously been demonstrated to be important for persistence of *Salmonella* in macrophages (18).

The role of the *pbpA2* gene with respect to virulence is not clear. The *pbpA2* mutant was found to be attenuated *in vitro* in chicken macrophages. Because of its homology to penicillin binding proteins, it is possible that the *pbpA2* mutant exhibited an altered peptidoglycan structure. Peptidoglycan plays a role in maintaining the morphology of the bacterial cell and the proper segregation of dividing cells. Furthermore, it has been demonstrated that intracellular *Salmonella* undergoes a change in the cell-wall peptidoglycan structure (19). Thus, it can be imagined that the reduced survival of the *pbpA2* mutant in chicken macrophages is related to the alterations in cell wall structure.

3. The role of the TCA cycle in the virulence of *Salmonella*

The tri-carboxylic acid (TCA) cycle plays an essential role in bacterial metabolism. Under aerobic conditions, this cycle is responsible for the complete oxidation of acetyl-CoA and provides energy to the growing cell. At the same time, intermediates of the TCA cycle are required for the biosynthesis of several amino acids. Under anaerobic conditions, the TCA cycle can no longer provide energy but instead functions in two biosynthetic pathways via succinate-CoA and 2-oxoglutarate (Figure 2) (5). It is not very well understood what the activity of the TCA cycle is when intracellular bacteria grow inside macrophages. Many genes involved in the SSR encode products that are involved in metabolic pathways. These genes can be induced intracellularly i.e. when the bacteria are ingested by macrophages (21) and are often regulated by the σ^s factor and cAMP:CRP (20). Inactivation of these genes causes attenuation of the

bacteria in animals or cultured cells. As shown in Table 1 of Chapter 2, four mutants in metabolic genes were found during the screening of the mini-Tn*10* mutant library. Some of these genes function in the TCA cycle and its related metabolic pathways, as shown in Figure 2. The product of one of these genes, the *cat2* gene, which is located in SPI-6, is the enzyme acetyl-CoA:4-hydroxybutyrate CoA transferase (see also Chapter 3). This enzyme catalyses the
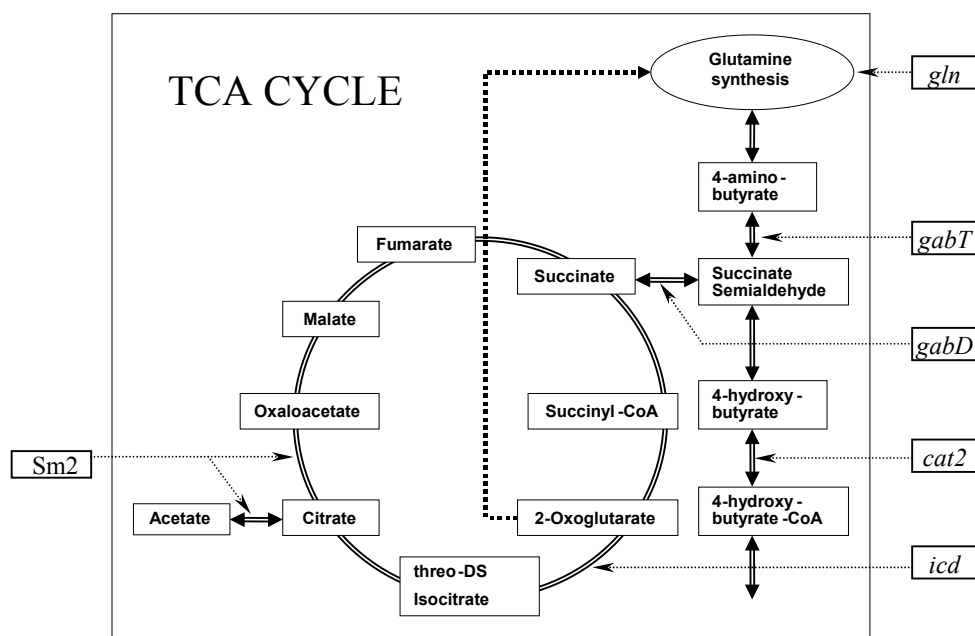

Fig. 2: *Salmonella* virulence genes associated with the TCA cycle and associated metabolic pathways.

conversion of 4-hydroxybutyrate to 4-hydroxybutyrate CoA. Analysis of the *cat2* mutant (SEM31) yielded the surprising finding that this mutant had acquired the ability to utilize citrate and succinate as sole carbon sources. The data obtained in Chapter 3 lead to the speculation that under conditions of nutrient stress (i.e. inside macrophages) *Salmonellae* prefer to utilize distinct carbon sources for other purposes than growth. Experiments in chicken revealed that SEM31 was more rapidly cleared from the liver and spleen than the wild type strain. This attenuated behavior suggests that the enzyme activity associated with Cat2 is particularly relevant for the bacterial survival at the later stages of an infection, which is consistent with the presumed important role of macrophages in the infection of the liver and spleen.

The phenotype of the serovar Enteritidis *cat2* mutant has similarities with that of a serovar Typhimurium mutant carrying a defective *gabD* gene. The GabD protein catalyzes the conversion of succinate into succinate-semialdehyde (Figure 2). Disruption of this gene, which is C-starvation inducible (20), allows the bacteria to utilize 4-aminobutyrate as sole carbon source for growth in contrast to the parental strain. GabD is involved in the metabolism of succinate (Figure 2). Furthermore, it is interesting to note that deletion of the *gln* cluster (Figure 2) reduces survival in macrophages (13), as was observed for the *cat2* mutant. Of additional interest is that mutation of genes encoding the citrate lyase β-chain (*citE*) and the acid-inducible (10) isocitrate dehydrogenase (*icd*) (Figure 2), were also impaired in survival (Chapters 2 and 5). Together, these data suggest a relationship between carbon metabolism, the growth state of

the bacteria and intramacrophage survival of *Salmonella*. In this regard, it may be of interest to mention that bacteria in the stationary growth phase are more resistant to low pH and the oxidative burst killing, utilize nutrients more efficiently, and that succinate protects stationary phase serovar Typhimurium from killing by antimicrobial peptides (2, 10). Thus, maintenance of the stationary phase of growth may be crucial for *Salmonella* to survive intracellularly.

4. SPI-6 and its possible role in virulence

A major achievement in this study was the discovery of *Salmonella* Pathogenicity Island 6 (SPI-6, Chapter 5). SPI-6 consists of 7 open reading frames (ORFs) and is located on the serovar Enteritidis genome adjacent to the *tRNA*-pheV gene (Chapter 5). The first 2 ORFs of SPI-6 are very similar to the *atsAB* genes of *Klebsiella aerogenes* (17). These genes encode an arylsulfatase and arylsufatase activator involved in scavenging sulfur needed for bacterial growth. In the genome of *Salmonella* spp., two types of arylsulfatase genes are present. It is worth emphasizing that the ORF1 and ORF2 of SPI-6 encode a ser-type of arylsulfatase, located in the periplasm, whereas the second set of genes, located elsewhere on the genome, encodes for a cys-type of arylsulfatase, located in the cytoplasm (12). The cys-type of arylsulfatase needs an additional transport system in order to be able to utilize sulfur scavenged from the environment. Therefore, in *Salmonella* serotypes that contain SPI-6 sulfur may be scavenged much more efficiently, which may facilitate intracellular survival .

The ORF3 of SPI-6 is most likely the *stmR* gene, which has also been identified in serovar Typhimurium. It has been shown that the transcription of this gene is induced in *Salmonella* contained in macrophages (16). The corresponding protein belongs to the LysR family of regulators. It has been tested whether the serovar Typhimurium *stmR* gene product is involved in virulence in murine bone marrow derived macrophages and in mice, but no effect on virulence could be demonstrated (16). In this thesis it was demonstrated that SPI-6 is involved in virulence of serovar Enteritidis *in vivo* in chicken and *in vitro*, in chicken macrophages. Therefore, it might very well be that the gene products of SPI-6 are responsible for the host-specific development of systemic disease.

The ORFs 4 to 7 of SPI-6 are homologous to a region in the unstable *pgm* locus of *Yersinia pestis*. On the basis of sequence similarity, it was suggested that the genes encode a citrate lyase-beta chain, a MoaC-like dehydratase, a 4-hydroxybutyrate coenzyme A transferase and possibly a ring-cleaving dioxygenase, respectively (Chapter 5). Although the true function of the respective genes in serovar Enteritidis remains to be established, mutagenesis of ORF4 and ORF6 indicate that at least these SPI-6 genes contribute to *Salmonella* survival inside macrophages (Chapters 2, 3 and 5). Thus, the newly identified SPI-6 is likely involved in the creation of a phenotype that allows the bacteria to better adapt to the intracelluar macrophage environment.

5. Contribution of the newly identified virulence genes to *Salmonella* infection in chicken

Since a group of mini-Tn*10* mutants of serovar Enteritidis was hampered in its survival in chicken macrophage cells (Chapter 1), it was interesting to address the question as to whether these mutants were attenuated *in vivo* in chicken. For this purpose, two mutants were chosen for experimental infections in chicken. The mutants had defects in the *yegQ* gene, which encodes a putative protease, and the *cat2* gene which encodes a 4-hydroxybutyrate coenzyme A transferase. The mutants used in the experimental infections were selected on the following criteria. All mutants carried defects in newly identified *Salmonella* virulence genes and the defects in their ability to infect chicken macrophages could be complemented. Furthermore, the selected mutants belonged to distinct categories, so that their function was not likely related.

The results of the experimental infections showed that all tested mutants were still able to efficiently colonize the chicken gut (Chapter 6). The *yegQ* and *cat2* mutants however, were cleared more rapid from the liver and spleen than the wild type strain. ELISA data indicated that the immune response against the specific LPS was similar for all strains tested (data not shown). This means that the observed differences in clearance could not be attributed to differences in antigenicity. Thus it was concluded that the *yegQ* and *cat2* mutants are attenuated *in vivo*.

6. Future research

The work described in this thesis has led to the discovery of a number of novel virulence factors of serovar Enteritidis. Nevertheless there are many aspects that could be further investigated. First of all, the exact the role of SPI-6 needs further study. For this purpose, an in-depth study of the functions of ORF4, ORF6 and ORF 7 is needed. The gene products of these ORFs should be purified and studied *in vitro*. Next, the alterations in metabolic pathways that likely occur after invasion of macrophages should be determined. Comparison of the wild type and the mutants obtained in this study would facilitate this work. A related question is: "What is the role of succinate in the survival of *Salmonella* in macrophages and what is the role of the stationary phase in survival?". Stationary phase bacteria are better equipped to survive this harmful environment. They can resist the acidic pH, the oxidative burst and survive carbon starvation (9). It would be very interesting to determine whether the newly identified genes are acid-inducible and how the environmental pH is signaled by the intracellular bacteria.

Next the function of the *yegQ* gene product should be further studied. Although in this thesis it is demonstrated that the *yegQ* gene is involved in virulence (Chapter 3), protease activity could not be demonstrated. Since the *yegQ* gene is present in *E. coli* and other, non-pathogenic bacteria, the *yegQ* gene may have multiple functions of which some are related to virulence. To study the function of theYegQ protein, it should be purified and the specificity of its possible proteolytic activity determined. Specific immunostaining of bacteria expressing a *yegQ*-FLAG fusion product inside macrophages with monoclonal antibodies against the FLAG peptide, may indicate the location of the protein during infection. The studies will not only help to understand the function the YegQ protein but may also help to elucidate the function of other members of the U32 peptidase family.

Finally, the mini-Tn*10* mutant library may be a valuable tool in the identification of additional virulence factors. Screening for mutants impaired in long-term survival in macrophages or in the interaction with cells derived from different hosts, may lead to the identification of new, perhaps host specific virulence factors that may be attractive targets for infection intervention or prevention-oriented studies.

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Samenvatting

Infectie met de bacterie *Salmonella enterica* serovar Enteritidis is de afgelopen twee decennia één van de belangrijkste oorzaken van aan voedselvergiftiging gerelateerde darmklachten bij de mens geworden. De belangrijkste bron van infectie is consumptie van besmette pluimveeproducten. Infectie met deze bacterie leidt tot diarree, koorts, spierpijn en algehele malaise. Per jaar is dit lot in Nederland ongeveer 100.000 mensen beschoren. De met enige regelmaat opduikende krantenkoppen "…. Bejaarden dood door Salmonella bacterie", geven aan dat in sommige gevallen deze infectie een fatale afloop kan hebben. Om het aantal gevallen van ziekte bij de mens veroorzaakt door deze bacterie te verminderen, is het van essentieel belang de aanwezigheid van serovar Enteritidis in kippen te weren. Een beter inzicht in de moleculaire biologie van de eigenschappen van serovar Enteritidis die deze bacterie in staat stellen kippen te besmetten is hiervoor noodzakelijk. Met dit doel voor ogen is vier jaar geleden het hiervoor beschreven onderzoek gestart. De nu verkregen gegevens kunnen vervolgens worden gebruikt om adequate maatregelen, die tot doel hebben infectie van kippen door Salmonellae zo effectief mogelijk te voorkomen, te ontwerpen. Zoals gezegd: het doel van het onderzoek beschreven in dit proefschrift was de eigenschappen van serovar Enteritidis die deze bacterie in staat stellen in het maag-darmkanaal van de kip te overleven, te bestuderen. Als aanpak is daartoe gekozen een bank met mutanten van serovar Enteritidis te construeren met behulp van het transposon mini-Tn*10*. Dit transposon integreert op willekeurige plaatsen in de genetische informatie (het DNA) van de bacterie en schakelt daarbij de eigenschap (het gen) die door deze genetische informatie bepaald wordt uit. Vervolgens is de mutantenbank getest op bacteriemutanten die niet langer, of slechter in staat zijn om binnen te dringen in macrofagen van de kip. Dit binnendringen in macrofagen is één van de belangrijkste stadia in het ziekteverwekkende proces. Een aantal van de op deze manier gevonden mutanten is vergeleken met de wild type serovar Enteritidis stam CV-1 in een in vivo infectiemodel in de kip.

In Hoofdstuk 2 is de constructie en het testen van de mutantenbank beschreven. Zevenendertig mutanten, die gestoord zijn in hun vermogen om macrofagen binnen te dringen of in macrofagen te overleven, werden gevonden in de gebruikte test. De eigenschappen die in deze mutantbacteriën afwezig of verminderd zijn, waren bijvoorbeeld de afwezigheid van beweeglijkheid of het vermogen een juiste bacteriecelwand te produceren. De mutanten die een verminderde beweeglijkheid hebben, bleken voornamelijk mutanten te zijn in genetische informatie, nodig voor de biosynthese van flagellen (zweepharen, die de bacterie gebruikt om te zwemmen). De mutanten met een verminderd vermogen tot binnendringen waren grotendeels mutanten in het Type III secretiesysteem dat gelegen is op het zogeheten Salmonella pathogeniteitseiland-1 (SP-1). Een pathogeniteitseiland is een regio binnen de genetische informatie van een bacterie, waar ziekteverwekkende eigenschappen geconcentreerd zijn. In SP-1 zijn die eigenschappen eiwitten die de cellen van een besmet individu worden binnengeloodst met behulp van het Type III secretiesysteem. Mutanten met een verminderd vermogen tot overleving in gastheercellen bleken niet een defect te hebben in een enkel gen, maar onderbroken te zijn in een grote verscheidenheid aan genen, zoals genen betrokken bij regulatie van genexpressie, celwandsynthese of stressrespons. Het feit dat zoveel genetische informatie betrokken is bij de overleving van serovar Enteritidis in gastheercellen geeft aan hoe belangrijk

dit is voor de bacterie. Overeenkomstig de verwachting bleken veel van de gevonden genen ook reeds in het uitgebreid bestudeerde species serovar Typhimurium beschreven te zijn als betrokken bij het ziekteverwekkend vermogen van dit organisme. Een aantal nog niet eerder als virulentiegen beschreven genen is echter ook gevonden onder de 37 mutanten. Drie van deze nieuwe virulentiegenen, *pbpA2* (hoofdstuk 2), *cat*2 (hoofdstuk 3) en *yegQ* (hoofdstuk 4) zijn nader gekarakteriseerd in vitro in HD-11 kippenmacrofagen en in vivo in eendagskuikens. Het *cat2* gen is gelegen in een deel van het chromosoom dat naar alle waarschijnlijkheid een nog niet eerder geïdentificeerd pathogeniteitseiland vertegenwoordigt. Dit SP-6 wordt beschreven in hoofdstuk 5.

In de mutantstam SEM35 is het mini-Tn*10* transposon geïntegreerd in een gen dat overeenkomst vertoont met *pbpA*. Dit gen codeert voor het eiwit PBP2, een penicilline bindend eiwit dat betrokken is bij de synthese van de bacteriële celwand en bij ongevoeligheid van de bacterie voor antimicrobiële peptiden. Het genoom van serovar Enteritidis bevat twee paraloge *pbpA* genen (*pbpA* en *pbpA2*). Deze twee genen hebben waarschijnlijk verschillende functies, daar *pbpA* in tegenstelling tot *pbpA2* niet in staat is het defect in mutant SEM35 te herstellen. Wanneer kippen experimenteel geïnfecteerd worden met SEM35 worden zij zieker en krijgen ernstigere schade aan interne organen dan kippen die geïnfecteerd worden met de wild type CV-1 stam.

In mutant SEM31 is het *cat2* gen onderbroken door het mini-Tn*10* transposon. Het ontstane defect, wat leidt tot minder binnendringend vermogen van de bacterie, kan worden hersteld door toevoeging van een intact *cat2* gen aan SEM31. Dit wijst erop dat het waargenomen phenotype van mutant SEM31 inderdaad het gevolg is van mutatie in het *cat2* gen. Het *cat2* gen codeert voor het enzym 4-hydroxybutyraat co-enzym A transferase dat deel uitmaakt van de butanoate biosynthese route, welke verbonden is met de citroenzuurcyclus. Naast het verlies van het vermogen om in macrofagen te overleven, had SEM31 het vermogen verkregen te groeien in aanwezigheid van barnsteenzuur als enige koolstofbron, iets waartoe de wild type stam CV-1 niet in staat is. Deze waarneming leidde tot de hypothese dat de wild type bacteriën in de stationaire fase van de groei gefixeerd worden door organische zuren als barnsteenzuur. Deze fixatie is een manier om de bacteriën in staat te stellen onder stressomstandigheden tengevolge van hongering te overleven. Deze stress treedt op in de beginfase van een intracellulaire infectie in kippenmacrofagen. In experimentele infecties in kippen veroorzaakte mutant SEM31 minder schade aan weefsels en werd sneller verwijderd uit interne organen dan de overeenkomstige wild type stam.

Het *yegQ* gen is onderbroken in mutant SEM26 en ook deze mutatie kan worden hersteld met behulp van het *yegQ* gen uit de wild type stam. Het YegQ eiwit dat door dit gen gecodeerd wordt, vertoont overeenkomsten met de U32 familie van proteasen. Deze familie van proteasen is ook aanwezig in andere bacteriën dan salmonellae; hun functie is echter grotendeels onbekend. Middels de constructie van een *yegQ* gen, met daaraan gekoppeld het FLAG epitoop, kon worden bepaald dat het YegQ-FLAG eiwit door de bacteriën in het medium wordt uitgescheiden.

Meer uitgebreide studies van het *cat2* gen worden beschreven in hoofdstuk 6. Twaalf verschillende serovars van Salmonella zijn vergeleken met betrekking tot hun vermogen om in media met barnsteenzuur als enige koolstofbron te kunnen groeien. Twee van de twaalf serovars (serovar Heidelberg en serovar Paratyphi) bleken in staat te groeien in media met barnsteenzuur. DNA-DNA hybridisatie-experimenten toonden aan dat in deze twee serovars het *cat2* gen niet aanwezig is. Behalve het *cat2* gen bleek in deze serovars een aangrenzend stuk DNA in het genoom te ontbreken van 7.4 kbp groot. Twee andere open leesramen in dit 7.4 kbp stuk DNA werden eveneens onderbroken door mutaties en de aldus verkregen mutanten bleken evenals SEM31 een verlaagd vermogen te hebben om te overleven in kippenmacrofagen. Dit wijst erop dat de 7.4 kbp regio een functionele eenheid vormt. Alleen mutant SEM31 was echter in staat te groeien op een medium met alleen barnsteenzuur als koolstofbron, hetgeen er op wijst dat de drie open leesramen betrokken zijn bij verschillende metabole processen. Een deel van het 7.4 kbp grote fragment vertoont homologie met een vermoedelijk pathogeniteitseiland in *Y.pestis*. Dit deel heeft een GC gehalte dat lager is dan het gemiddelde in salmonellae en wordt begrensd door een *tRNA*-pheV gen. Al deze gegevens waren aanleiding om de regio waarin het *cat2* gen zich bevindt aan te duiden als een nieuw pathogeniteitseiland in Salmonella (SPI-6).

Het in dit proefschrift beschreven onderzoek draagt bij aan de kennis omtrent de ziekteverwekkende eigenschappen van serovar Enteritidis in kippen. Het feit dat de drie nieuwe virulentiegenen, *pbpA2*, *yegQ* en *cat2* niet eerder ontdekt zijn in andere Salmonella species is waarschijnlijk te wijten aan de keuze voor het gebruik van de kippenmacrofaagcellijn HD-11 in plaats van de gebruikelijke humane - of muizencellijnen, als testsysteem voor intracellulaire infectie. De onderliggende mechanismen van de verminderde overleving in macrofagen zijn een interessant onderwerp voor toekomstig onderzoek dat meer kennis zal opleveren over deze belangrijke fase in het infectieproces. De waarneming dat, bij in vivo experimenten in kippen, twee van de drie mutanten in de nieuw ontdekte virulentiegenen een verminderd ziekteverwekkend vermogen hebben, illustreert het belang van intracellulaire infectie in het ziekteproces.

Summary

In the past two decades, infection with *Salmonella enterica* serovar Enteritidis has become one of the major causes of food-borne gastrointestinal disease in humans. The major source of infection is the consumption of contaminated poultry products. Therefore, the control of *S. enteritidis* in chicken is essential to reduce the incidence of human salmonellosis. Insight in the molecular mechanisms that enable serovar Enteritidis to infect chicken is important to design effective prevention measures. The aim of this thesis is to study the virulence determinants of serovar Enteritidis in the chicken. The approach to reach this goal was to construct a random mutagenesis library of serovar Enteritidis using the transposon mini-Tn*10* and the screening of the library for mutant strains that have impaired cellular infection of chicken macrophages. Mutant strains were compared to the wild type serovar Enteritidis CVI-1 strain in a chicken infection model.

In **Chapter 2** the construction and screening of the mutant library is described. Thirty-seven mutant strains were identified with an impaired ability for cellular infection. Mutants were characterized by their impaired functions, i.e. motility, invasion or survival in the macrophages. The motility impaired mutants were largely affected in flagella genes and the invasion mutants in the Type III secretion system of *Salmonella* pathogenicity Island-1 (SPI-1). Mutants with impaired intracellular survival have mutations in a variety of genes that are involved in gene regulation, cell wall synthesis or stress response. As expected most of the disrupted genes that we found have been described before for the extensively studied serovar Typhimurium. We also identified several novel virulence genes that have not been described before to be involved in virulence. Three of these novel virulence genes, *pbpA2* (**chapter 2**), *cat2* (**chapter 3**) and *yegQ* (**chapter 4**) are further characterized in vitro in HD-11 chicken macrophages and in vivo in day-old chicks. The *cat2* gene is located in a region that is most likely a novel pathogenicity island. This SPI-6 is described in **chapter 5**.

In mutant strain SEM35, the mini-Tn*10* transposon was inserted in a *pbpA* homologue. The *pbpA* gene encodes the protein PBP2, a penicillin binding protein, which is involved in cell wall synthesis and resistance against antimicrobial peptides. The genome of serovar Enteritidis harbors two parologous *pbpA* genes that we designated *pbpA* and *pbpA2*. These genes probably have different functions since in contrast to *pbpA2*, the *pbpA* gene was not able to complement the defect in mutant SEM35.

The *cat2* gene was disrupted in mutant SEM31. The defect in cellular infection of the SEM31 gene could be complemented by the wild type *cat2* gene indicating that *cat2* deficiency was responsible for the mutant phenotype. The *cat2* gene encodes the enzyme 4-hydroxybutyrate coenzyme A transferase that is part of the butanoate pathway that is linked to the citric acid cycle. Besides its inability to survive in macrophages, strain SEM31 was able to grow on succinate as sole carbon source. In contrast, the wild type was unable to grow under the same condition. We speculate that the wild type is arrested in the stationary phase by organic acid such as succinate thus allowing the bacterium to survive under starvation stress that may occur in the early stages of cellular infection in chicken macrophage cells. In the chicken

experiments the mutant SEM31 caused less tissue damage and cleared faster from the internal organs than the wild type.

The *yegQ* gene is disrupted in mutant strain SEM26 and the mutation could be complemented by the wild type *yegQ* gene. The encoded YegQ protein shares similarity with the U32 family of proteases. This family of proteases is present in other bacteria but their function is largely uncharacterized. We constructed a *yegQ* gene with a FLAG epitope and found that the YegQ-FLAG protein was secreted by the bacteria. In the chicken model, mutant SEM26 behaved similarly to SEM31 and was cleared faster from the internal organs than the wild type strain.

More detailed studies on the *cat2* gene are described in **chapter 6**. We compared twelve *Salmonella* serovars for their ability to grow in succinate and found that two serovars (Heidelberg and Paratyphi A) were able to grow on succinate. Hybridization experiments showed that these species lacked the *cat2* gene. We found that a 7.4 kb region flanking the *cat2* gene was absent in these species. Two more open reading frames from the 7.4 kbp region were disrupted and these mutants showed an impaired survival in chicken macrophages similar to SEM31 indicating that the 7.4 kb region is one functional unit. However, only SEM31 was able to grow on succinate indicating that these genes are required for different metabolic processes. In addition, part of the 7.4 kb region showed homology with a putative pathogenicity island of *Y. pestis*, had a lower than average GC content, is flanked by a *tRNA*-pheV. Therefore we designated the *cat2* region as the novel *Salmonella* Pathogenicity Island 6 (SPI-6).

The research described in this thesis has contributed to the knowledge of the virulence of serovar Enteritidis in chicken. The fact that the three novel virulence genes, *pbpA2*, *yegQ* and *cat2*, were not discovered before in other *Salmonella* species probably can be attributed to our choice of the chicken macrophage cell line HD-11 as the target for cellular infection, instead of the commonly used mouse and human cells. The mechanisms that underlay the impaired survival in macrophages are an interesting subject for future research that may tell us more about this important stage of the infection process.

Curriculum Vitae

Yixian ZHAO was born on 26 July 1967 in Harbin, a city in the most northeastern province of China. At the age of 5 he moved with his parents to northwestern China and grew up there. He became deeply fascinated in biology during high school, inspired by his biology teacher, Mr. Chen Xiwu. In the autumn of 1983 he started his undergraduate study in Genetics at the Biology Faculty of Wuhan University, and was awarded the B.Sc. degree in July 1987. After that he worked in the Veterinary Control Institute in Beijing as a researcher. During that time, he was involved in several projects and had different functions including teaching in a professional high school in Yunnan province for one year. In 1995 he came to Utrecht University as a post-graduate trainee for one year, working on the program of replacing *Campylobacter* flagellin antigenic domain with *Salmonella* flagellin g,m-epitope. Then he entered the AIO study on *Salmonella* virulence factors in March 1997, which is described in this thesis.

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