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SURVIVAL STRATEGIES OF *SALMONELLA* ENTERITIDIS TO COPE WITH ANTIBACTERIAL FACTORS IN THE CHICKEN OVIDUCT AND IN EGG WHITE

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Abbreviation list

ABBREVIATION LIST

AvBD	avian beta defensin
BBP	biotin binding protein
BGA	brilliant green agar
BPI	bacterial permeability increasing
BPW	buffered peptone water
CAMP	cationic antimicrobial peptide
CD	cluster of differentation
cDNA	complementary DNA
CDS	coding sequence
cLEAP	chicken liver expressed antimicrobial peptide-2
COEC	chicken oviduct epithelial cells
CXC	chemokine
DMEM	Dulbecco's minimal essential medium
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FCS	fetal calf serum
GAL	gallinacin
gDNA	genomic DNA
HBSS	Hank's buffered salt solution
HMM	high molecular weight
Ig	immunoglobulin
IL	interleukin
iNOS	inducible nitric oxide synthase
IPTG	isopropyl-β-D-thiogalactopyranoside
iv	intravenous
IVET	in vivo expression technology
km	kanamycin
LB	Luria Bertani broth
LBP	lipopolysaccharide binding
LPS	lipopolysaccharide
MALDI-TOF-MS	matrix assisted laser desorption ionization time-of-flight mass spectrometry

MDR	mutidrug resistance
MGCV	MOPS/glucose/casamino acids/vitamins
MOI	multoplicity of infection
MOPS	3(-N-morpholino) propanesulfonate
mRNA	messenger ribonucleic acid
NCP's	national control programs
NF-κB	nuclear factor Kappa Bèta
OAg	O-chain antigen
OC-17	ovocleidin-17
OCX-36	ovocalyxin-36
ORF	open reading frame
OTAP	ovotransferrin antimicrobial peptide
OVAX	ovalbumin-related protein X
OVAY	ovalbumin-related protein Y
PAMP	pathogen associated molecular pattern
PBS	phosphate buffered salin
pi	post infection
PRR	pattern recognition receptor
RNA	ribonucleic acid
RND	resistance nodulation division
ROD	region of difference
SALSA	*Salmonella* serovar microarray
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SNP	Single nucleotide polymorphism
SPI	*Salmonella* Pathogenicity Island
spp	subspecies
str	streptomycin
T3SS	type three secretion system
TGF	transforming growth factor
TIR	Toll/interleukin-1 receptor
TLR	Toll-like receptor
WHO	world health organization

1 General introduction

General introduction

1.1 The *Salmonella* pandemic

Salmonellosis is a worldwide occurring disease caused by bacteria belonging to the genus *Salmonella*. The genus consists of 2 species, *Salmonella bongori* and *enterica*, of which the latter is further subdivided in 6 subspecies (*enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae* and *indica*). Nowadays, approximately 2500 serotypes have been described, based on flagellar (H), capsular (Vi) and somatic (O) antigens. Additionally, some serotypes like *Salmonella* Enteritidis and *Salmonella* Typhimurium are further divided into phagetypes. A distinction between host-restricted, host-adapted and broad host-range serotypes can be made, according to their host specificity and pathogenesis. The former causes a severe systemic disease in a very limited number of related species, while infections with broad host-range *Salmonella* strains can occur in multiple hosts where they cause a self-limiting infection mostly characterized by mild gastro-intestinal symptoms. Depending on the health status of the individual, also these infections can become life-threatening. Human infections with these broad host-range serotypes are often the result of consumption of contaminated food, as these serotypes may reside subclinical inside a wide range of wild and domestic animals. By the end of the 20th century, an increase in human salmonellosis cases has been observed due the rise of *Salmonella enterica* subsp. *enterica* serovar Enteritidis (*Salmonella* Enteritidis). Analysis of the World Health Organization (WHO) *Salmonella* surveillance data showed that by 1987 the number of *Salmonella* Enteritidis infections had increased in 24 out of the 35 countries investigated (Rodrigue *et al.*, 1990). Also in Belgium an increase in human *Salmonella* Enteritidis cases was observed since 1988 with a peak in 1999 (Collard *et al.*, 2008). Epidemiological research of the *Salmonella* Enteritidis infections and outbreaks led to the identification of grade A shell eggs as the major source for this pandemic in many countries (Rodrigue *et al.*, 1990, Patrick Mary E., 2004, St Louis *et al.*, 1988, Coyle *et al.*, 1988). As a result, *Salmonella* control programs were implemented by the European union, starting with a first directive in 1992 (92/117/EEC) (Anonymous, 1992) to monitor and control *Salmonella* in parent breeding flocks. As this directive was insufficient to control *Salmonella* at the level of the laying hen farm Regulation No. 2160/2003 (Anonymous, 2003), aiming to detect and control *Salmonella* at all relevant stages of the production cycle, was issued. Additionally, member states needed to set up national *Salmonella* control programs (NCP's) for which minimal requirements for commercial laying hen flocks were laid down in regulation No. 1091/2005 (Anonymous, 2005). These

included a) that antimicrobials cannot be used to control *Salmonella* b) that member states with a prevalence of *Salmonella* Enteritidis in commercial laying hens higher than 10% are mandatory to vaccinate and c) that live vaccines can only be used during rearing. Finally, Regulation No. 1168/2006 (Anonymous, 2006), which is repealed by Regulation No. 517/2011 (Anonymous, 2011) contains information on the sampling programs for laying hen flocks, while Regulation No. 1237/2007 (Anonymous, 2007) laid down restrictions for the trade of table eggs from flocks infected with *Salmonella* Enteritidis or Typhimurium. The latter states that eggs from *Salmonella* Enteritidis or Typhimurium positive flocks must be banned from the market, unless they are treated in a manner that guarantees that all *Salmonella* bacteria are destroyed. Consequently, a drop in the number of *Salmonella* Enteritidis positive laying hen flocks and in numbers of contaminated eggs was observed. This led to a continuous decline in the number of confirmed human salmonellosis cases since 2004 (EFSA, 2009, EFSA, 2013). The same trend was seen in Belgium, where a drastic decline in the number of salmonellosis cases was observed since 2005 (Collard *et al.*, 2008).

Despite this decline in human cases, salmonellosis still is the second most commonly reported zoonotic disease, following campylobacteriosis. Although eggs are no longer the primary food vehicle causing salmonellosis, it appears that when one considers the risk related to the different sources weighted according to the tonnage of food available for consumption, the risk of *Salmonella* infection still remains the highest when consuming table eggs (EFSA, 2013).

1.2 How *Salmonella* Enteritidis conquered the egg

In order to explain the pandemic caused by *Salmonella* Enteritidis contaminated eggs, it has been suggested that the endemic presence of the avian host-restricted serotype *Salmonella* Gallinarum in poultry has inhibited the colonization of these animals by *Salmonella* Enteritidis. As *Salmonella* Gallinarum was responsible for economic losses in the poultry industry it was eradicated from commercial poultry flocks by the 1970s, while *Salmonella* Enteritidis emerged as a major concern for the food safety by the 1980s. This inverse relationship between the incidence of *Salmonella* Gallinarum infections in chickens and egg contamination by *Salmonella* Enteritidis led to the hypothesis that *Salmonella* Enteritidis filled the ecological niche vacated by the eradication of the avian host-restricted serotype *Salmonella* Gallinarum from poultry (Rabsch *et al.*, 2000).

Since the discovery that *Salmonella* Enteritidis causes human foodborne disease through the consumption of contaminated eggs, several hypotheses have been put forward to explain how

the bacteria might be transmitted to the egg. First of all, it was thought that the external surface of the egg became contaminated during (when the egg passed through an infected cloaca) or after oviposition (after being laid in a contaminated hen house). Nevertheless, extensive cleaning and disinfection measures of un-cracked grade A shell eggs in the United States were unable to stop the *Salmonella* Enteritidis pandemic, suggesting that the bacteria must contaminate the intact egg internally (Braden, 2006, St Louis *et al.*, 1988). This internal contamination could result either from penetration through the egg shell after oviposition (horizontal transmission) or due to incorporation into the forming egg inside the chicken reproductive tract (vertical transmission).

1.2.1 Horizontal transmission

With horizontal transmission the internal egg contents of the completely formed egg becomes contaminated after lay when bacteria penetrate through the egg shell. The potential of *Salmonella* Enteritidis to migrate through the egg shell was demonstrated by several research groups (Miyamoto *et al.*, 1998, Schoeni *et al.*, 1995, De Reu *et al.*, 2006, Messens *et al.*, 2005) and thought to be most efficient immediately after lay when the cuticle is still immature and when a temperature differential is generated (Miyamoto *et al.*, 1998). This would mean that, in order for shell penetration to occur efficiently, the shell must become contaminated during passage through an infected cloaca. Nevertheless, a correlation between fecal carriage of *Salmonella* Enteritidis and contamination of the egg content could not be demonstrated in multiple studies (Gast and Beard, 1990a, Humphrey *et al.*, 1991a, Humphrey, 1994, Gast and Beard, 1990b).

In normal circumstances the chicken reproductive tract is a relatively sterile environment, with the exception of the vagina as *Lactobacillus* and *Bacteroidaceae* spp. were frequently isolated from this part of the reproductive tract (Miyamoto *et al.*, 1998). Despite the fact that the lactobacilli could inhibit the growth of *Salmonella* Enteritidis *in vitro* (Miyamoto *et al.*, 2000, Collie *et al.*, 2006), it has been suggested that a *Salmonella* Enteritidis infection of the cloaca could ascend to the vagina and even the uterus, which in turn may lead to egg surface or egg shell contamination and thus penetration of the egg shell after lay (Miyamoto *et al.*, 1997, Keller *et al.*, 1995). Studies involving intra-vaginal inoculation of laying hens with *Salmonella* Enteritidis resulted in a high proportion of positive eggs without causing a drop in the egg production rate (Miyamoto *et al.*, 1997). Moreover, when different serotypes were intravaginally inoculated and compared, *Salmonella* Enteritidis exhibited a higher potential to

colonize the vaginal tissue (Okamura *et al.*, 2001b, Mizumoto *et al.*, 2005) and to produce *Salmonella* positive eggs (Okamura *et al.*, 2001b). Nevertheless, Okamura *et al.* (2001a) could not link vaginal colonization to contamination on the egg shell. It appears that shell penetration is not a unique trait for the serotype Enteritidis, as other serotypes (Miyamoto *et al.*, 1998, Schoeni *et al.*, 1995) and even unrelated bacteria (De Reu *et al.*, 2006) are able to cross this barrier. Although other *Salmonella* serotypes are present on the egg surface, moslty the serotype Enteritidis can be isolated from the internal content (Humphrey *et al.*, 1991b). This would mean that either egg shell penetration does not take place in practice and that eggs are contaminated during their formation in the reproductive tract or that *Salmonella* Enteritidis has developed survival mechanisms to withstand the antimicrobial components present in the egg shell membranes and egg albumen.

1.2.2 Vertical transmission

Formation of the egg takes place in the chicken reproductive tract, which consists of an ovary and oviduct, the latter made up of 5 segments each fulfilling one specific function. The ovary supports the maturation of the pre-ovulatory follicles, containing the egg yolk. After ovulation the completely maturated follicle is captured by the infundibulum of the oviduct, where after albumen and shell membranes are deposited by the magnum and isthmus part of the oviduct, respectively. Finally, the shell is deposited by the uterus followed by oviposition through the vagina and cloaca. Consequently, depending on the site of reproductive tract colonization, *Salmonella* Enteritidis can be incorporated at different positions in the egg. Colonization of the ovary would lead to incorporation of the bacteria into the yolk, while contamination of the egg white, egg shell membranes or the shell is caused by infection of the magnum, isthmus or uterus respectively (Figure 1.2.2.1).

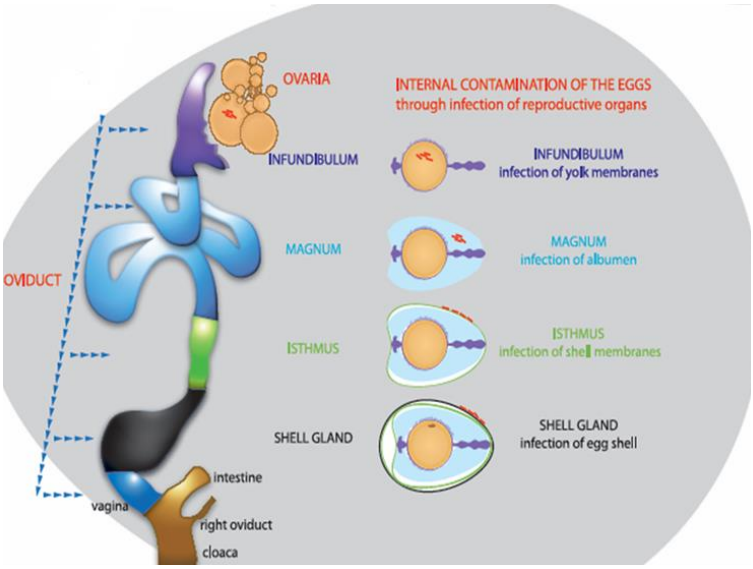

Figure 1.2.2.1: formation of the egg in the reproductive tract and side of egg contamination (adapted from Gantois *et al.* 2009. Mechanisms of egg contamination. FEMS microbiology review: 33, 718-38)

The hypothesis that egg contamination takes place during the formation of the egg in the ovary or oviduct is further strengthened by several observations. *Salmonella* Enteritidis could be detected immunohistochemically (Keller *et al.*, 1995, Hoop and Pospischil, 1993, De Buck *et al.*, 2004a) and isolated postmortem (Hoop and Pospischil, 1993) from the chicken reproductive tract tissues. In addition, bacteria could also be isolated from the reproductive tissue in the absence of intestinal colonization, indicating persistent reproductive tract colonization (Humphrey, 1994, Lister, 1988).

1.2.2.1 Colonization of the ovary

Reports claim that the yolk is the main egg compartment that is positive for *Salmonella* Enteritidis, indicating that the ovary is the main colonization site (Gast and Holt, 2000a, Gast *et al.*, 2002), and this as a consequence of systemic spread from the intestine (Gantois *et al.*, 2009a). It has been reported that *Salmonella* Enteritidis has a higher potential to colonize the ovary in comparison with other serovars, although some studies have demonstrated an equal colonization for *Salmonella* Enteritidis and *Salmonella* Typhimurium (Okamura *et al.*, 2001a, Okamura *et al.*, 2001b, Gantois *et al.*, 2008b, Howard *et al.*, 2005). This is most likely because different strains and different inoculation doses or methods were used in the different studies. Inside the ovary, *Salmonella* Enteritidis appears to be more frequently isolated from the interstitial tissue rather than the yolk itself (Barrow and Lovell, 1991, Gast and Beard, 1990b). It has been demonstrated that *Salmonella* Enteritidis can invade and multiply in the granulosa

cells surrounding the pre-ovulatory follicles (Thiagarajan *et al.*, 1994, Thiagarajan *et al.*, 1996) and that immature follicles are more susceptible (Howard *et al.*, 2005, Methner *et al.*, 1995). On the one hand Methner *et al.* (1995) suggested that invasion of the immature follicle might block their development. On the other hand, others propose that some contaminated granulosa cells might be released together with the yolk mass during ovulation or that *Salmonella* Enteritdis might invade the egg yolk by crossing the perivitelline membrane surrounding the yolk (Thiagarajan *et al.*, 1996). Migration through the perivitelline membrane before ovulation would result in yolk contamination and this would lead to aberrant egg formation. Indeed, the yolk is rich in nutrients, stimulating bacterial growth eventually causing degeneration of the follicle and thus a drop in egg production, which is not observed in laying hens suffering from *Salmonella* Enteritidis infection. Additionally, Gast and Beard (1990b) could not isolate *Salmonella* Enteritidis from yolk contents although whole yolks, containing the perivitelline membrane and some adhering albumen, were contaminated. Consequently, it can be assumed that contamination of the yolk itself occurs only rarely during egg formation, although subsequent penetration through the vitelline membrane might occur after lay (see below).

1.2.2.2 Colonization of the oviduct

In contrast to yolk colonization, multiple studies argue that the upper oviduct (magnum and isthmus) is the main colonization site for *Salmonella* Enteritidis inside the reproductive tract, resulting in contamination of the albumen or egg shell membranes (Shivaprasad *et al.*, 1990, Hoop and Pospischil, 1993, Humphrey *et al.*, 1991b). Colonization of the upper oviduct could result from systemic spread although a descending infection from the ovary might also be possible (Gantois *et al.*, 2009a). *Salmonella* Enteritidis was found on the surface of and within single epithelial cells in the lumen and tubular gland cells of the oviduct (Hoop and Pospischil, 1993, Keller *et al.*, 1995, De Buck *et al.*, 2004a). De Buck *et al.* (2004a) proposed that the isthmus was the main colonization site, while Gast *et al.* (2007b) reported that the infundibulum-magnum junction experienced a higher colonization in comparison to the isthmus-uterus junction. It is possible that *Salmonella* Enteritidis bacteria are persistently carried within the epithelial cells of the magnum and/or isthmus and released from these cells in the lumen at certain time points resulting in contamination of the forming egg. Overall, previously performed *in vivo* studies (Okamura *et al.*, 2001a, Okamura *et al.*, 2001b, Gantois *et al.*, 2008b) comparing the oviduct colonization potential between serotypes, indicate that *Salmonella* Enteritidis is the predominant serotype colonizing this organ. Nevertheless, some contradictory results exist for the serotypes Typhimurium and Heidelberg which, in some

studies, were able to colonize the colonize the oviduct to the same extent as *Salmonella* Enteritidis (Gast *et al.*, 2004, Keller *et al.*, 1997). The discrepancies between the different studies, might be attributed to the strains and inoculation doses or methods used.

1.3 The interaction between Salmonella Enteritidis and the reproductive tract

1.3.1 Antimicrobial defenses of the reproductive tract

It is well known that the local immune system of the reproductive tract plays a major role in the protection of this organ against microbes, including *Salmonella*. Recent studies investigated the organization and function of the innate and adaptive immune system in both the ovary and oviduct.

1.3.1.1 Toll-like receptors and cytokine responses

The innate immunity constitutes the first line of defense and contains cells carrying pattern recognition receptors (PRRs), which recognize the so called pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharides (LPS) or flagellin. Of these PRRs, Toll-like receptors (TLRs) are the best characterized and activation leads to the induction of signaling pathways with subsequent production of cyto- and chemokines to direct and start the innate and adaptive immunity (Kaiser, 2010).

In the chicken TLRs recognizing PAMPs (TLR1-1, 1-2, 2-1, 2-2, 4, 5, and 15) and pathogen nucleic acids (TLR3, 7 and 21) have been identified. To study their role in the local immunity of the reproductive tract, their distribution in the ovary and oviduct together with their expression profile following LPS or *Salmonella* administration has been investigated.

Michailidis *et al.* (2010) reported that mRNA transcripts of all TLR members, except 1-1 and 2-2, could be detected in the ovary of 52 weeks old laying hens. Expression of TLR2, 4 and 15 was also observed in both differentiated and undifferentiated ovarion granulosa cells (Woods *et al.*, 2009), while Subedi *et al.* (2007b) could not detect TLR1-1, 1-2 and 3 in neither the theca or granulosa layer of the ovary. In the vagina, TLR1-1 was the only TLR for which no mRNA could be detected (Michailidis *et al.*, 2011). Additionally, Ozoe *et al.* (Ozoe *et al.*, 2009) demonstrated the presence of 6 TLR mRNA transcripts (TLR1-2, 2, 3, 4, 5 and 7), except TLR1-

1, in all parts of the oviduct. In this study the expression of TLR15 and 21 was not measured and no distinction between TLR2-1 and 2-2 was made.

The influence of *Salmonella* Enteritidis on the expression of these TLRs in the ovary and vagina was investigated in 28- and 104-weeks old birds. In 28-weeks old birds, oral administration of *Salmonella* Enteritidis resulted in the up-regulation of TLR4 and 15, and TLR5 and 15 in respectively the ovary and vagina. In the vagina also a down-regulation of TLR7 was observed. When 104 weeks old birds were used TLR15 and TLR2-1, 4 and 15 were upregulated in the ovary and vagina, respectively. Additionally, in the aged birds a down-regulation of TLR-3 was seen in the ovary (Michailidis *et al.*, 2010, Michailidis *et al.*, 2011). Consequently, these results indicate the importance of LPS and flagellin in bacterial recognition in the reproductive tract as they are recognized by TLR2, 4 (LPS) and TLR5 (flagellin) (Iqbal *et al.*, 2005).

Effect of LPS on TLR expression in the reproductive tract was demonstrated in several studies. To our knowledge no data regarding the influence of flagellin in the reproductive tract are available. Intravenous injection of LPS resulted in the up-regulation of TLR4 mRNA in both the theca and granulosa cells of F_3 follicles of laying hens with temporal differences, while no changes in the expression level of TLR2 could be observed (Subedi *et al.*, 2007b). Woods *et al.* (2009) demonstrated that expression of TLR2 and TLR4 in granulosa cells is dependent upon the maturation status of the follicle, ranging from an accelerated response in the follicle which is next to ovulate to an LPS tolerance response in the prechierarchal follicles in order to prevent excessive inflammation. A role for TLR4 in the oviduct immunity was also demonstrated by Ozoe *et al.* (2009), who showed an increase in the expression of this receptor in the vagina after both intravenous and intravaginal LPS injection in 350 days old hens.

The stimulation of TLR4 by LPS probably also induced the expression of IL-1β and IL-6 in both the ovary, uterus and vagina (Ozoe *et al.*, 2009, Subedi *et al.*, 2007b, Nii *et al.*, 2011, Abdelsalam *et al.*, 2012). In the uterus and vagina also the chemokine CXCLi2 (IL-8) was increased after intravenous administration of LPS (Nii *et al.*, 2011), but this molecule was not studied in the ovary. Finally, a study performed by Li *et al.* (2009a) demonstrated that *Salmonella* Enteritidis elicited the expression of chemokines CXCLi1 (K60), CXCLi2 (IL-8), CCLi3 (K203) and CCLi4 (MIP-1β) together with that of the pro-inflammatory mediator inducible nitric oxide synthase (iNOS) in chicken oviduct epithelial cells. Also the transcription of the anti-inflammatory cytokine IL-10 was induced, while that of the transforming growth factor (TGF)-β3 was repressed. Activation of TLRs and subsequent production of cyto- and

chemokines by *Salmonella* or LPS in the reproductive tract may lead to the influx of granulocytes, macrophages and lymphocytes (Ozoe *et al.*, 2009, Withanage *et al.*, 2003). Additionally, also an increased expression of antimicrobial components might be induced by these TLRs as IL-1β caused an increase in the expression of avian beta-defensin 12 (AvBD12) (Abdelsalam *et al.*, 2012).

Taking everything into consideration, it can be hypothesized that when *Salmonella* Enteritidis colonizes the reproductive tissue, several TLRs are triggered. The recognition of *Salmonella* Enteritidis by these receptors might stimulate the expression of several cytokines, chemokines, cationic antimicrobial peptides (CAMPs) and finally the influx of immune cells.

1.3.1.2 Antimicrobial agents

As chicken heterophilic granulocytes (avian counterpart of neutrophils) lack myeloperoxidase and have minimal ability to generate an oxidative burst during phagocytosis, cationic antimicrobials are probably the principal antimicrobial effectors of these cells (Evans *et al.*, 1995). In addition, numerous antimicrobial peptides are secreted by the epithelial cells lining the oviduct.

1.3.1.2.1 Avian beta-defensins

Among the antimicrobial agents produced by the reproductive tract, the defensin family has been recognized as one of the key mediators of the innate immunity. Defensins constitute a large family of small, cationic peptides characterized by the presence of 6-8 cysteine residues. Based on the spacing pattern of the cysteins, the defensins can be divided into five groups; plant, invertebrate, α-, β-, and θ-defensins, but only the family of β-defensins or gallinacins is found in chickens (Kaiser, 2010, Anastasiadou *et al.*, 2013). The availability of the chicken genome led to the identification of new β-defensins/gallinacins by two different research groups that used a different numbering system (Lynn *et al.*, 2004, Xiao *et al.*, 2004). To avoid confusion a new standard nomenclature was proposed by Lynn *et al.* (2007) and their name was changed to avian beta-defensins (AvBD) of which 14 (AvBD1-14) have been identified in the chicken (Table 1.3.1.2.1.1).

Table 1.3.1.2.1.1: avian β-defensin numbering system (adapted from Lynn et a;. 2007. Avian beta-defensin nomenclature: A community proposed update: 100, 86-89.

New gene/protein name	Nomenclature by Lynn *et al.* (2004)	Nomenclature by Xiao *et al.* (2004)
Avian beta-defensin 1 (AvBD1)	Gallinacin 1 (GAL1)	Gallinacin 1 (GAL1)
Avian beta-defensin 2 (AvBD2)	Gallinacin 2 (GAL2)	Gallinacin 2 (GAL2)
Avian beta-defensin 3 (AvBD3)	Gallinacin 3 (GAL3)	Gallinacin 3 (GAL3)
Avian beta-defensin 4 (AvBD4)	Gallinacin 7 prepropeptide (GAL7)	Beta defensin 4 (GAL4)
Avian beta-defensin 5 (AvBD5)	Gallinacin 9 prepropeptide (GAL9)	Beta defensin 5 (GAL5)
Avian beta-defensin 6 (AvBD6)	Gallinacin 4 prepropeptide (GAL4)	Beta defensin 6 (GAL6)
Avian beta-defensin 7 (AvBD7)	Gallinacin 5 prepropeptide (GAL5)	Beta defensin 7 (GAL7)
Avian beta-defensin 8 (AvBD8)	Gallinacin 12 prepropeptide (GAL12)	Beta defensin 8 (GAL8)
Avian beta-defensin 9 (AvBD9)	Gallinacin 6 prepropeptide (GAL6)	Beta defensin 9 (GAL9)
Avian beta-defensin 10 (AvBD10)	Gallinacin 8 prepropeptide (GAL8)	Beta defensin 10 (GAL10)
Avian beta-defensin 11 (AvBD11)		Beta defensin 11 (GAL11) /Gallicin 11
Avian beta-defensin 12 (AvBD12)	Gallinacin 10 prepropeptide (GAL10)	Beta defensin 12 (GAL12)
Avian beta-defensin 13 (AvBD13)	Gallinacin 11 prepropeptide (GAL11)	Beta defensin 13 (GAL13)
Avian beta-defensin 14 (AvBD14)		

Recently, a new family of AvBD, the ovodefensins, was introduced. The proteins belonging to this family own the conserved cysteine arrangement of the AvBD, although the space between the cysteine residues is different. In chickens, only 1 member of this family, gallin, has been identified so far but three forms are encoded on chromosome 3 (Gong *et al.*, 2010).

Although the exact mechanism of action of the AvBD is not completely known, it is proposed that these cationic peptides interact electrostatically with the negative charges on the bacterial membrane. Consequently, these interactions might lead to disruption of the membrane potential followed by an increased membrane permeability and eventually bacterial cell death. Moreover, it is thought that these peptides can be transported to intracellular sites where they might interfere with DNA, RNA and protein synthesis. Additionally, AvBDs can also act as chemoattractants for other immune cells (Sugiarto and Yu, 2004, van Dijk *et al.*, 2008).

Antimicrobial activities of AvBD have been demonstrated against both Gram-positive and Gram-negative bacteria, including *Salmonella* Enteritidis. Chicken heterophil peptide 1 (CHP1) or AvBD1 was effective against *Salmonella* Enteritidis and Typhimurium (Evans *et al.*, 1995). A lethal effect against *Salmonella* Typhimurium was achieved by AvBD13 (GAL11) at a concentration of 500 µg/ml (Higgs *et al.*, 2005), while a concentration of 128 µg/ml of AvBD9 (GAL6) was insufficient to completely kill *Salmonella* Typhimurium. A 49% growth inhibition could be observed for the latter when a high salt concentration was added (van Dijk *et al.*, 2007). Also AvBD4, 5 and 6 (GAL7, 9 and 4) exhibited antimicrobial activity against both *Salmonella* Enteritidis and Typhimurium. *Salmonella spp*. are known to resist cationic peptides by reducing

the anionic charges on their membrane. Consequently, a higher activity was observed for AvBD5 (GAL9) probably because this defensin has a reduced cationic charge making it more capable to interact with the modified membrane of *Salmonella* (Milona *et al.*, 2007). Both *Salmonella* Enteritidis and Typhimurium were also susceptible to the antimicrobial activity of AvBD11 (Hervé-Grepinet *et al.*, 2010). A strong antimicrobial activity was demonstrated for AvBD 5 and 10 (GAL9 and 8) against *Escherichia coli* (*E. coli*) but their potential to kill *Salmonella* was not investigated in this study (Ma *et al.*, 2008). Finally, the ovodefensin, gallin, was capable of inhibiting *E. coli*, although no antibacterial activity could be detected against *Salmonella* Enteritidis or Typhimurium (Gong *et al.*, 2010, Hervé-Grepinet *et al.* 2014).

Recent studies also focused on the expression of defensins in the reproductive tract. In the ovary the expression of 11 of these AvBD, namely AvBD1, 3, 4, 5, 7, 8, 9, 10, 11, 12 and 14 was detected. No mRNA transcripts could be detected for AvBD6 and 13, while AvBD2 was expressed at almost undetectable levels (Michailidis *et al.*, 2012). The absence of AvBD2 and 6 are in contrast with a previous study (Subedi *et al.*, 2007a) in which expression of AvBD2 and 6 (GAL2 and 6) was demonstrated in the ovarian stroma. Additionally these authors also reported the expression of AvBD 1, 2, 7, 8, 10 and 12 (GAL1, 2, 7, 8, 10 and 12) in the theca layer and AvBD1, 8, 10 and 12 (GAL1, 8, 10 and 12) in the granulosa layer of F_3 follicles. Another study located the expression of AvBD6 and 5 (GAL4 and 9) in the ovary but failed to detect AvBD4 (GAL7) (Milona *et al.*, 2007). The protein product of AvBD8, 10 and 12 was later demonstrated by Western blot and immunohistochemistry in both the theca and granulosa layer (Abdelsalam *et al.*, 2010).

10 AvBD were shown to be expressed in all parts of the oviduct (AvBD1, 2, 3, 4, 5, 8, 9, 10, 11 and 12), while AvBD7 showed only faint bands in the isthmus, uterus and vagina. As in the ovary, transcripts of AvBD6 and 13 were not observed and AvBD14 was not studied (Mageed *et al.*, 2008). The expression of AvBD3 and 4 and the absence of AvBD6 in the vagina were in contrast with Anastasiadou *et al.* (2013) who demonstrated that AvBD6 was expressed in the vagina of 52 week old birds, while AvBD4 mRNA was not detected and expression of AvBD3 occurred at almost undetectable levels. A discrepancy for the expression of AvBD6, 8 and 13 was also seen in the primary tubular gland cells of the oviduct, as it appeared that in these cells transcription of AvBD6 and 13 occurred. Nevertheless, the expression of AvBD6 and 8 was inconsistent in these cells (Ebers *et al.*, 2009). Additionally, transcription of AvBD14 was observed in the vagina and in primary epithelial cells of the isthmus (Ebers *et al.*, 2009, Anastasiadou *et al.*, 2013). Protein products of AvBD3, 11 and 12 could be demonstrated in the

surface epithelium all over the oviduct and AvBD3 was also found inside the tubular gland cells of the isthmus and magnum (Abdel Mageed *et al.*, 2009). Gong *et al.* (2010) demonstrated that all three forms of the ovodefensin, gallin, were expressed in the magnum, while form 2 and 3 were also transcribed in the isthmus and uterus.

All together, it can be assumed that AvBD are transcribed in the reproductive tract and that their expression level influenced by LPS or *Salmonella* Enteritidis. Consequently, several studies, listed below, focused on the expression of these defensins in both the ovary and oviduct. Although some minor differences between the studies exist, they are most likely caused by differences in RNA source (tissue versus cells) and the use of different breeds of laying hens.

Furthermore, a role for AvBDs in the local reproductive tract immunity could also be suggested by the up-regulation of their expression after LPS or *Salmonella* Enteritidis administration. Intravenous (iv) injection of 1 mg LPS/kg body weight (BW) led to an increase in the expression of AvBD1, 7 and 12 (GAL1, 7 and 12) in the theca layer of the ovary after 3 hours, which remained high during the course of the experiment. However, when the LPS dose was doubled (2 mg/BW) only an up-regulation of AvBD8 (GAL8) was observed. At the same time AvBD1 and 12 (GAL1 and 12) had the tendency to decline in the granulosa layer (Subedi *et al.*, 2007a). A recent study also demonstrated the up-regulation of AvBD10 in the theca layer (Abdelsalam *et al.*, 2012). In the vagina this iv-injection caused an up-regulation of AvBD3, 5, 10, 11 and 12 (GAL3, 5, 10, 11 and 12) with temporal difference, probably because they are produced by different cell types which differ in their recognition and response time (Abdel Mageed *et al.*, 2009). When LPS was administered on 24 hour pre-cultured vaginal epithelial cells an increased expression of AvBD1 and 2 (GAL1 and 2) was observed next to that of AvBD3 (GAL-3) (Yoshimura *et al.*, 2006b). This discrepancy in AvBD1 and 2 (GAL1 and 2) expression between the *in vitro* and *in vivo* studies is probably due to a better LPS stimulation in the *in vitro* study. Additionally, iv-injection with LPS enhanced the density of AvBD3 and 11 immunoreactive products in the uterus (Mageed *et al.*, 2011).
In addition to LPS administration, also oral administration of *Salmonella* Enteritidis resulted in the up-regulation of a subset of AvBDs (AvBD4, 5, 7, 11 and 12) in the ovary of 28-weeks old laying hens. On the contrary, a down-regulation of AvBD14 was observed (Michailidis *et al.*, 2012). In the vagina, this oral administration led to an up-regulation of AvBD5, 7, 10, 11, 12 and 14 (Anastasiadou *et al.*, 2013). In primary epithelial cells of the isthmus an up-regulation of only AvBD2 and 6 was detected, while AvBD4, 9, 10 and 11 were suppressed (Ebers *et al.*, 2009).

1.3.1.2.2 Other antimicrobial agents

In addition to the AvBDs the innate immune system utilizes a broad range of factors to protect itself against potential pathogens.

Silphaduang *et al.* (2006) demonstrated the antimicrobial action of histones H1 and H2B in the chicken ovary (H1 and H2B) and oviduct (H1). Despite their antimicrobial activity against *Bacillus subtilis* (*B. subtilis*) and *E. coli*, they appear to be inactive against *Salmonella* Enteritidis. Although histones are principal structural proteins of eukaryotic chromosomes, a role in the innate defense against microorganisms could be suggested, as histones of other species, or their derivates, have shown the potential to induce membrane permeabilization (Koo *et al.*, 2008) or nucleic acid binding (Park *et al.*, 1998).

Michailidis *et al.* (2010) demonstrated the expression of the chicken liver expressed antimicrobial peptide-2 (cLEAP-2) in the ovary, but not in the oviduct, of the chicken. These authors also revealed a significant up-regulation in the transcription of this gene in the ovary after oral gavage with *Salmonella* Enteritidis. Nevertheless, cLEAP-2 was ineffective against *Salmonella* Enteritidis while it could easily kill the *Salmonella* Typhimurium SL1344 wild type strain through permeabilization of the outer membrane (Townes *et al.*, 2009, Townes *et al.*, 2004).

Also peroxidase activity may participate in the host innate defense system, as the reaction products may cause oxidative stress and thus harbor antimicrobial activity. In the chicken reproductive tract peroxidase activity was found especially in the vaginal surface epithelium, but not in the other segments except for some positive cells in the isthmus. Peroxidase activity can be found in biological secretions such as milk and saliva due to the presence of lactoperoxidase in mammary glands and salivary glands in mammals. Although the peroxidase present in the chicken reproductive tract is not identified yet, it can be suggested that the peroxidase activity of the vaginal epithelial cells contributes to the killing of microbes ascending from the cloaca, while the peroxidase activity synthesized in the isthmus, might be transmitted to the eggshell membranes (Yoshimura *et al.*, 2006a).

More antimicrobial components are produced along the reproductive tract and incorporated at different compartments of the egg. These proteins are originally purified from and more extensively studied inside the egg and therefore discussed below.

1.3.1.3 Cells of the innate and adaptive immune system

Next to antimicrobial agents, the number of immune cells present in an organ can be an indication of the immune potential of an organ. Consequently, different studies focused on the distribution of immune cells in the reproductive tissue and their role in *Salmonella* infection, which revealed that both a cell-mediated as a humoral response are induced after *Salmonella* Enteritidis administration. Immunohistochemical analysis of the oviduct showed the presence of macrophages, lymphocytes and immunoglobulins. Macrophages are observed in the mucosa of all segments of the postnatal oviduct (Khan *et al.*, 1998) and are located in the mucosal epithelium and stroma in 175 days old laying hens (Zheng and Yoshimura, 1999). The influx of both B- and T-lymphocytes starts at 5 weeks of age and a peak is reached at 15 weeks in the glandular parts of the oviduct (magnum, isthmus and uterus) for both cell types. Also in the infundibulum, B-lymphocytes peaked at 15 weeks while in the vagina peaks are reached at 19 and 21 weeks of age for T- and B-lymphocytes, respectively. Plasma cells are first seen at 11 weeks and a peak is observed at 32 weeks where after they declined, except in the vagina (Khan *et al.*, 1996, Khan *et al.*, 1997). During the laying period, CD4+ and CD8+ T-cells predominate in the vagina and less in the isthmus and magnum (Withanage *et al.*, 1997). On the contrary, Ig+ cells are found throughout the oviduct but mainly in the magnum, isthmus and uterus (Kimijima *et al.*, 1990, Withanage *et al.*, 1997). IgA, IgM and IgY are also detected in the mucosal epithelium (Zheng 1997), but IgYγ-chain mRNA can only be found in the plasma-like cells in the stroma and not in the mucosal epithelium. The authors suggest that IgY is produced by the plasma-like cells and released in the mucosal tissue where after it is taken up by epithelial cells and subsequently secreted in the lumen (Zheng *et al.*, 2000).

Infection with *Salmonella* Enteritidis causes a transient induction of the immune system in both the ovary and oviduct. Hoop and Pospischil (1993) observed a slight inflammatory response with heterophil infiltration in naturally infected hens. Intravenous infection results in an initial macrophage decline, which later on recovers to normal pre-inoculum levels. T-cells reach a peak 10 days post infection (pi), while B-cells peak at day 14 pi. Numerous CD4+ and CD8+ cell aggregates of various sizes are observed and *Salmonella* Enteritidis specific antibodies are secreted in both serum and oviduct luminal compartments. IgG and IgM peak at 2 weeks pi, while IgA declines rapidly. The increase of T and B-cells together with immunoglobulins correlates with a decline in the *Salmonella* Enteritidis recovery rate from the oviduct and ovary at day 14. Nevertheless, although the immune cells return to their normal levels by day 21 pi, there is no complete clearance of *Salmonella* Enteritidis from the ovary and oviduct. Once

reproductive organ infection is established, *Salmonella* persists for a long time with intermittent shedding (Withanage *et al.*, 1999, Withanage *et al.*, 1998).

1.3.2 Virulence factors of *Salmonella* Enteritidis involved in colonization of the reproductive tract

Spread of *Salmonella* Enteritidis to the reproductive tract most likely occurs by taking advantage of the macrophages, in which the bacteria survive easily through the use of the Type III Secretion System (T3SS) encoded on the *Salmonella* Pathogenicity Island (SPI) 2. The role of this island in the systemic spread of *Salmonella* Enteritidis to the reproductive tract has been demonstrated by the use of an *ssrA* mutant, which exhibits a lower potential to colonize the reproductive tract due a diminished systemic spread as a consequence of a reduced intra-macrophage survival (Bohez *et al.*, 2008). Once inside the reproductive tract, *Salmonella* Enteritidis might invade and reside within the epithelial cells of these organs. The potential to invade and reside within primary chicken oviduct epithelial cells (COEC) was demonstrated by Li *et al.* (2009b) and was depending on components of the T3SS encoded by both SPI1 (invasion) and SPI2 (intracellular survival). Additionally, it has been suggested that the T3SS-2 effector *pipB* might inhibit the expression of AvBD9, 10 and 11 in *Salmonella* infected COEC (Ebers *et al.*, 2009). *Salmonella* Enteritidis does not seem to be superior in the invasion and proliferation within COEC when compared with other serovars (Gantois *et al.*, 2008b), probably because functional SPIs1 and 2 are found within almost all serovars. A comparison between the genome of *Salmonella* Enteritidis, Typhimurium and Gallinarum led to the identification of so called Regions of Difference (ROD), which were only present in *Salmonella* Enteritidis and Gallinarum (Thomson *et al.*, 2008). It could thus be that these gene clusters made it possible for *Salmonella* Enteritidis and Gallinarum to colonize the reproductive tract more efficiently as compared to other serovars. Consequently, Coward *et al.* (2012) investigated the role of 5 of these islands in reproductive tract colonization for *Salmonella* Enteritidis but could not confirm their involvement. In order to search for genes that are especially expressed during reproductive tract colonization an *'in vivo'* expression technology (IVET) screening was performed. Most of the genes identified in this study are involved in the biosynthesis of amino and nucleic acids, the carbohydrate and energy metabolism and the cell membrane and cell wall integrity. Also stress-related, motility, regulatory, virulence plasmid and genes with unknown function were picked up (Gantois *et al.*, 2008a). The best studied *Salmonella* Enteritidis virulence factor is LPS, as this outer membrane component might promote reproductive tract

colonization by two mechanisms. First of all, it appears that the LPS O-antigen chain length influences the reproductive tract colonization potential of *Salmonella* Enteritidis. The O-antigen chain is composed of O-antigen subunits (OAgs) and synthesized in a stepwise manner in which the O-antigen polymerase (*wzy*) catalyses the concatenation between the different polysaccharide subunits. Additionally, O-antigen chain length is determined by the *wzz* and *febE* genes, respectively leading to Long-OAgs (16-35 subunits) and very-long OAgs (> 100 subunits). Coward *et al.* (2013) demonstrated that both a *wzy* and *febE* deletion strain, exhibiting respectively only one O-antigen (OAg) repeat and no very long OAgs, are less efficient in the colonization of the ovary and oviduct. On the contrary, the *wzz* disruption, devoid of long OAgs, didn't exhibit any defect and had colonization levels similar to that of the wild type. The latter was also observed by Parker *et al.* (2002) who showed that a *wzz* deletion strain was even more virulent than the wild type strain, as more heterophilic granulomas were produced in comparison with the wild type. The latter was probably caused by a higher concentration of very-long OAgs found on this mutant (Coward *et al.* 2013). Secondly, *Salmonella spp*. are known to resist cationic antimicrobial peptide activity by reducing the negative charge of their outer membranes including LPS (Townes *et al.*, 2004). It could thus be hypothesized that a decreased immune response caused by the production of HMM LPS and the lack of antimicrobial peptide action might result in incomplete clearance of *Salmonella* Enteritidis from the reproductive tract. Finally, also fimbriae seem to play an important role in the colonization of the reproductive tract. Li *et al.* (2003) describe the presence of binding sites for SEF21 (type 1) fimbriated *Salmonella* Enteritidis strains in the oviduct. It is also demonstrated that type 1 fimbriae bind to isthmus secretions, which may lead to an efficient transport of the bacteria to the shell membranes of the forming egg (De Buck *et al.*, 2003). These data were further strengthened by the observation that a type 1 fimbrial mutant (*ΔfimD*) has a reduced potential to contaminate eggs (De Buck *et al.*, 2004c).

Additionally, another fimbrial operon, SEF14 seems to be involved in the attachment of *Salmonella* Enteritidis to ovarian granulosa cells, as pre-incubation of these cells with purified SEF14 reduced the attachment of *Salmonella* Enteritidis to the cells (Thiagarajan *et al.*, 1996). It was also shown that a low level expression of the SEF14 gene, *sefD*, by *Salmonella* Enteritidis might mitigate the host response and thus facilitate the infection pathway which leads to egg contamination (Morales *et al.*, 2012).

Finally, some *Salmonella* Enteritidis strains are resistant to oxidative stress induced by hydrogen peroxide (Shah *et al.*, 2012a)

1.4 The interaction between *Salmonella* Enteritidis and the egg

It is clear that the region of colonization in the reproductive tract determines the site of incorporation into the forming egg. Infection of the ovary would lead to incorporation of *Salmonella* Enteritidis into the yolk, while persistence in the magnum, isthmus or uterus gives rise to contamination of the egg white, shell membranes or egg shell respectively.

1.4.1 Contamination of the forming egg

Colonization of the ovary would lead to incorporation into the yolk, but studies report that *Salmonella* Enteritidis is more often associated with the vitelline membrane rather than with the interior yolk contents and that subsequent penetration through the vitelline membrane might occur (Gast and Beard, 1990b, Gast and Holt, 2001). Penetration through this membrane was however not observed at the chicken body temperature of 42°C, suggesting that transfer to the yolk before oviposition is unlikely to occur (Guan *et al.*, 2006). Penetration is more frequently observed at 25 to 30°C (Gast *et al.*, 2005, Gast *et al.*, 2007a) and it is suggested that entry into the yolk is more likely to occur over time when albumen viscosity and vitelline membrane integrity decline, thus at later time points post-lay (Gast *et al.*, 2005).

In addition to transovarian transmission, it is also demonstrated that *Salmonella* Enteritidis can persist in the oviduct and that the bacteria are carried along with the albumen or the egg shell membranes into the forming egg (Humphrey *et al.*, 1991b, Shivaprasad *et al.*, 1990, Hoop and Pospischil, 1993). Interestingly, a higher percentage of forming eggs is positive for *Salmonella* Enteritidis in comparison with laid eggs (Keller *et al.*, 1995, Barrow and Lovell, 1991). The ovum spends about 26 hours in the oviduct of which 5 hours in the magnum and isthmus and 21 hours in the uterus at the chicken body temperature of 42°C. The importance of this high temperature was demonstrated by Guan *et al.* (2006) who showed that *Salmonella* Enteritidis could resist the antimicrobial properties of the albumen at 42°C for 24 hours but not for 96 hours. In the meantime, the bacteria could easily be recovered from this matrix after 120 hours at 37°C. It could thus be hypothesized that the combination of heat stress and the presence of antimicrobial components, present especially in the egg white, control *Salmonella* contamination during further formation of the egg. The recovery of *Salmonella* Enteritidis from laid eggs might thus mean that the serotype has acquired the potential to resist the hostile environment of the egg albumen at the chicken body temperature. This was supported by the

fact that also *Salmonella* Typhimurium could be isolated from the forming eggs, although this serotype could no be recovered from laid eggs (Keller *et al.*, 1997). These results are in line with a recently performed large scale egg white survival assay, demonstrating superior survival of *Salmonella* Enteritidis in egg white for 24 hours at 42°C compared to other serovars (De Vylder *et al.*, 2013). An enhanced survival of *Salmonella* Enteritidis compared to Typhimurium in egg white was also observed at 37°C (Clavijo *et al.*, 2006). These results were not confirmed by Gantois *et al.* (Gantois *et al.*, 2008b), who demonstrated that although the serotypes Virchow and Hadar are much more susceptible to the egg albumen, Typhimurium and Heidelberg were also capable of surviving in egg white at 42°C. Also Guan *et al.* (2006) could not show a difference between *Salmonella* Enteritidis and Typhimurium at 42°C.

1.4.2 Antimicrobial defenses of the chicken egg

In addition to a high body temperature, the chicken provides the forming egg with a variety of antimicrobial components to protect the developing embryo from microbial aggressors. Recent proteomic research has revealed that antimicrobial agents are present in all compartments of the hen egg, while the use of bioinformatics led to the identification of an arsenal of potential antimicrobial proteins. Nevertheless, here we will focus on those molecules for which the antimicrobial activity has been demonstrated.

1.4.2.1 Antimicrobial components and antibodies

Although many of the AvBD are expressed in the chicken all over the chicken reproductive tract, only 5 have been demonstrated in the egg: AvBD3 (egg shell), AvBD9 (egg yolk and shell), AvBD10 (eggshell), AvBD11 (egg white and shell) and AvBD12 (eggshell) (Abdel Mageed *et al.*, 2009, Jonchere *et al.*, 2010, Mann, 2007, D'Ambrosio *et al.*, 2008, Mann *et al.*, 2006, Mann, 2008). Additionally, also the chicken ovodefensin, gallin, was isolated from egg white (Gong *et al.*, 2010). The innate antimicrobial activity inside the egg is further strengthened by the presence of histones in all compartments of the egg (D'Ambrosio *et al.*, 2008, Mann, 2007, Mann, 2008, Mann *et al.*, 2006, Mann and Mann, 2008). Antimicrobial activity against *B. subtilis*, *Staphylococcus aureus (S. aureus)*, *E. coli* and *Pseudomonas aeruginosa* (*P.aeruginosa*) has been demonstrated for the eggshell matrix protein ovocalyxin-36 (OCX-36) (Gautron *et al.*, 2011). The protein sequence of OCX-36 exhibits a significant similarity with the lipopolysaccharide binding (LBP)/Bacterial Permeability increasing (BPI) and the BPI-related palate, lung and nasal epithelium clone protein (Plunc) family proteins, who themselves belong to the superfamily of proteins known to be key components of the innate

immune system (Gautron *et al.*, 2007). While BPI proteins bind to the lipid A component of LPS and transfer bacteria to the LPS receptor (Hailman *et al.*, 1994), BPI proteins also bind LPS but permeabilize the cytoplasmic membrane causing a decrease in the electrochemical gradient (Dann and Eckmann, 2007). In the meantime, three additional BPI/LBP-like proteins have been demonstrated in the egg: TENP, BPI-like-2 and similar-to-BPI protein. Another component of the egg defense system which is related to the function of the immune system is ovocleidin-17 (OC-17). OC-17 shares significant identity with the C-type lectin-like proteins, that recognize specific carbohydrate structures on pathogens and thus aid in directing the adaptive immune response by inducing cytokine responses and T-cell polarization (den Dunnen *et al.*, 2010). OC-17 has shown to bind bacterial polysaccharides and exhibits a bactericidal activity against *B. subtilis* and *S. aureus* but not *E.coli.* (Wellman-Labadie *et al.*, 2008). Additionally, a glycopeptide derived from ovomucin was unable to bind *Salmonella* Enteritidis or Typhimurium although it could interact with *E. coli* O157:H7 and thus probably protects against infection with this foodborne pathogen (Kobayashi *et al.*, 2004). Finally, all three types of immunoglobulins, have been identified in both the egg yolk and egg white. IgA, IgM and IgY are released into the oviduct from the infundibulum, magnum and uterus (Kimijima *et al.*, 1990), while IgY in the yolk is incorporated from the blood stream. Eggs from chickens infected with *Salmonella* have been shown to contain *Salmonella* specific antibodies, but these might have no influence on the replication of *Salmonella* in the yolk (Takase *et al.*, 1999), although specific IgYs isolated from yolks of immunized laying hens were capable of interfering with the growth capacity of *Salmonella* Enteritidis and Typhimurium in liquid medium (Chalghoumi *et al.*, 2009).

1.4.2.2 Cell wall and nucleic acid degrading components

Lysozyme is an N-acetyl-muramidase that hydrolyses the β-(1,4) glycosidic bond between N-acetylmuramic acid and N-acetylglucosamine in the bacterial peptidoglycan layer. In Gram-negative bacteria this layer is protected by the outer membrane and lysozyme mediated hydrolysis of peptidoglycan is inhibited by the binding of lysozyme to LPS (Ohno and Morrison, 1989). Nonetheless, Pellegrini *et al.* (1997) demonstrated the bactericidal activity of lysozyme derived peptide 98-112 against both Gram-negative and Gram-positive bacteria independent from its muramidase activity. The peptide, organized as a helix-loop-helix structural motif, was able to penetrate the Gram-negative outer membrane by a self-promoted uptake pathway in order to form multimeric pores in the cytoplasmic membrane (Ibrahim *et al.*, 2001). These results were later confirmed by Mine et al (2004), who isolated a peptide ranging from residues

98 to 108 from lysozyme capable of causing membrane damage in *E. coli*. Additionally, these authors also purified the 15-21 peptide exerting antimicrobial activity against *S. aureus* but not *E. coli*. Furthermore, both endo- and exonuclease activity were demonstrated in egg white. These nuclease activities are likely mediated by proteins and possibly gained access to the DNA through the pores formed by other antimicrobial components (Lu *et al.*, 2003).

1.4.2.3 Chelators

Nutrients are essential for the developing embryo. Consequently, they are bound by chelators preventing them to be used for bacterial growth. The best known chelator interfering with bacterial growth is ovotransferrin. Ovotransferrin is capable of binding two Fe^{3+} ions per molecule and thus deprives the bacteria from iron that is necessary for their growth (Garibaldi, 1960). The iron chelating antimicrobial activities of ovotransferrin have been demonstrated against a variety of bacteria, including *Salmonella* Enteritidis (Chart and Rowe, 1993). Baron *et al.* (1997) showed that iron deficiency was the major mechanism implicated in the inhibition of *Salmonella* Enteritidis growth in egg white. When *Salmonella* Enteritidis was incubated in egg white at 37°C the concentration of the bacteria steadily decreased, while iron supplementation resulted in extensive growth. Besides these iron chelating capacities, ovotransferrin seems to possess an antimicrobial activity not related to iron. At a high pH and under reducing conditions, ovotransferrin undergoes autocleavage allowing the release of functional domains (Ibrahim *et al.*, 2006). One of the peptides generated by autocleavage is the ovotransferrin antimicrobial peptide 92 (OTAP-92). OTAP-92 was capable of killing both Gram-negative and positive bacteria and the former probably by crossing the outer membrane and damaging the biological function of the inner membrane.

In addition to ovotransferrin the egg also contains avidin, the riboflavin-binding protein, the plasma retinol binding protein and the vitamin D binding protein to provide the embryo with biotin, riboflavin, retinol and vitamin D, respectively. The interaction of these chelators with *Salmonella* growth is unclear.

1.4.2.4 Protease inhibitors

Pathogenic bacteria secrete proteases to hydrolyse host proteins in order to inactivate them or to use them as nutrients. Subsequently, host organisms are equipped with several protease inhibitors to inactivate these bacterial secreted proteases to protect themselves. The majority of these protease inhibitors in egg white belong to the serine protease inhibitor superfamily.

Although ovalbumin is structurally related to this family, it does not seem to possess any inhibitory activity on proteases, but proteolytic digestion of ovalbumin by trypsin and chymotrypsin yields several peptide fragments with antimicrobial activity against Gram-positive and Gram-negative bacteria (Pellegrini *et al.*, 2004). In addition, two ovalbumin paralogs hβe been identified; ovalbumin-related protein X (OVAX) and Y (OVAY). OVAX was able to inhibit *Salmonella* Enteritidis although it was not able to inhibit trypsin, plasmin or cathepsin G (Rehault-Godbert *et al.*, 2013). Ovoinhibitor was capable of inhibiting trypsin, subtilisin and chymotrypsin while it displayed antimicrobial activity against *Bacillus thuringiensis*, a strain possessing 5 serine proteases (Bourin *et al.*, 2011). Growth of *Bacillus subtilis* was inhibited by ovocalyxin-32, a carboxypeptidase inhibitor limiting bacterial colonization on the egg surface (Xing *et al.*, 2007).

In addition to serine protease inhibitors, also the cysteine protease inhibitor, cystatin, was capable of inhibiting both Gram-positive and negative bacteria, although a higher concentration was needed to inhibit *Salmonella* Enteritidis in comparison with *E. coli* (Wesierska *et al.*, 2005).

1.4.3 Virulence factors allowing *Salmonella* Enteritidis to survive inside the egg

Genes encoding proteins involved in structure and function of the cell wall were frequently identified as involved in egg albumen resistance at 37°C (Clavijo *et al.*, 2006). These results are in line with an IVET study in which genes involved in cell membrane and cell wall integrity were picked up as being highly expressed in the chicken reproductive tract and in eggs (Gantois *et al.*, 2008a). It appears that the majority of the egg contaminating *Salmonella* Enteritidis strains are characterized by their ability to produce HMM LPS structures (Guard-Petter *et al.*, 1997). The importance of a well-defined LPS structure was demonstrated by Gantois *et al.* (2009b) who reported that a *Salmonella* Enteritidis putative dehydrogenase, *rfbH*, mutant could not survive the incubation in egg white at 42°C. Later Coward *et al.* (2013) demonstrated that both the *wzy* and *febE* genes are important for the persistence of *Salmonella* Enteritidis in egg white at the chicken body temperature. Moreover, the LPS-lysozyme interaction is more rapidly established and more complexes are formed at higher temperatures (Ohno *et al.*, 1991). It could thus be hypothesized that the production of long O-antigen chains might increase the resistance of *Salmonella* Enteritidis to egg white antimicrobial factors. A striking feature of many antimicrobial components is that they are small cationic peptides binding the negative charges in the cell membrane eventually resulting in permeabilization of the bacterial membranes. It

could thus be hypothesized that *Salmonella* Enteritidis efficiently modifies its LPS structure by reducing its negative charge to prevent the binding of these cationic molecules. Permeabilization of the outer membrane would also lead to a higher accessibility to the outer membrane for lysozyme. *Salmonella* Enteritidis, however has developed a strategy to escape this threat by the synthesis of a lysozyme inhibitor (PliC) (Callewaert *et al.*, 2008), but the relevance of this gene for the survival in forming eggs has not yet been defined. In addition to the membrane damage caused by egg white also the DNA damage is counteracted, the latter by the putative DNA repair enzyme *yafD* and the exonuclease III *xthA* at 37°C (Lu *et al.*, 2003). Finally, iron chelation by ovotransferrin is a key antimicrobial activity of the egg. *Salmonella* Enteritidis is capable of growing in an iron-restricted environment due to the secretion of the siderophores enterobactin and salmochelin (Chart and Rowe, 1993, Crouch *et al.*, 2008). The role of these siderophores in the persistence of *Salmonella* Enteritidis in egg albumen was confirmed by the fact that mutants in genes involved in synthesis and transport of enterobactin were no longer capable of surviving in this matrix at 37°C (Kang *et al.*, 2006).

1.5 *Salmonella* Enteritidis behavior in the egg post lay

1.5.1 Behavior in eggs post-lay

As discussed above eggs are most likely contaminated in the albumen or on the vitelline membrane. The fact that naturally contaminated eggs only contain a low number of bacteria (Humphrey *et al.*, 1989) together with the observation that multiplication of *Salmonella* Enteritidis in eggs is delayed until a few days after lay, suggests that the albumen is more likely to be contaminated as deposition near the vitelline membrane might favor excessive proliferation (Gast and Holt, 2000b, Fleischman *et al.*, 2003, Murase *et al.*, 2006, Murase *et al.*, 2005) compared to the albumen (Chen *et al.*, 2005, Gast and Holt, 2000b). Nevertheless, it appears that multiplication inside the egg is influenced by the age of the egg, as the number of *Salmonella* cells isolated from eggs of naturally infected hens increases with time after deposition (Humphrey *et al.*, 1991b, Humphrey and Whitehead, 1993). It was also demonstrated that storage had little impact on the inhibiting activities of the albumen but rather on the integrity of the vitelline membrane (Humphrey and Whitehead, 1993, Chen *et al.*, 2005). Consequently, it could be hypothesized that this loss of membrane integrity might lead to leakage of nutrients into the albumen which in turn would trigger chemotaxis of *Salmonella*

towards and penetration through the vitelline membrane eventually resulting in multiplication in the yolk (Cogan *et al.*, 2004). These results could not be confirmed by Messens *et al.* (2004), who could not observe leakage of nutrients from the yolk after 3 weeks of storage. Addionally, this study also suggested that *Salmonella* Enteritidis could survive more easily in albumen of freshly laid eggs than in that of stored eggs. It was assumed that the enhanced growth in this fresh albumen is caused because its lower pH in comparison with albumen from stored eggs.

1.5.2 *Salmonella* Enteritidis virulence factors in eggs post lay

It is clear that factors necessary for the survival in the forming egg also determine the behavior of *Salmonella* Enteritidis in the albumen of the laid egg. For example, Gantois *et al.* (2009b) reported that after 8 days of incubation at 20°C a 10^5 fold growth difference was observed between the *rfbH* mutant and the wild type. The importance of LPS was later confirmed because a putative rhamnosyltransferase (*rfbN*) mutant was less capable of surviving in egg white when incubated at 25°C for 24 hours (Shah *et al.* 2012b). The transposon mutagenesis screening performed by Shah *et al.* (2012b) also identified the ribosomal protein methyltransferase *ksgA*, the SPI-4 *siiE* gene, the SPI-14 *SEN0803* gene, three genes belonging to the RODs (SEN1152, SEN1393, SEN1966) and 2 genes involved in motility (*fliH* and *fliB*), as necessary for survival of *Salmonella* Enteritidis in egg white at 25°C. The role of flagella was already demonstrated as the non-flagellated serotypes Pullorum and Gallinarum and the aflagellated *Salmonella* Enteritidis mutant *ΔfliC* were incapable of growing in eggs (Cogan *et al.*, 2004). Subsequently, in order to multiply and penetrate through the vitelline membrane, *Salmonella* Enteritidis must attach to this membrane. Curli fimbriae (SEF17) may be necessary for this interaction as a curli-fimbria deficient strain (*ΔagfA*) is impaired in its potential to invade and multiply inside the yolk (Cogan *et al.*, 2004).

1.6 Summary of *Salmonella* Enteritidis virulence factors needed to colonize the reproductive tract and contaminate the egg

It is generally accepted that the *Salmonella* pathogenicity islands mediate the systemic spread of *Salmonella* Enteritidis to the reproductive tract. Once inside the reproductive tract it can be assumed that these islands establish the invasion and survival of the bacteria in the upper

oviduct epithelial cells. As soon as a stable colonization of the reproductive tract is reached, type 1 fimbriae enable the adhesion of *Salmonella* Enteritidis to the oviduct secretions leading to incorporation of the bacteria into the egg white of the forming egg. As egg white is synthesized to protect the developing embryo from infections, it contains a myriad of antimicrobial components such as, lysozyme, ovotransferrin, defensins and other CAMPs. After incorporation into the egg white of the forming egg it takes on average about 24 hours before the egg is laid. In other words, in order to contaminate the egg post lay, *Salmonella* Enteritidis has to survive the antimicrobial environment of the egg white at the chicken body temperature of 42°C for 24 hours. Consequently, previous research has shown that *Salmonella* Enteritidis activates several defense mechanisms to protect itself from this environment. A first line of defense is achieved by a structural rearrangement of the LPS moiety leading to a reduction of the overall negative LPS charge and the increase in the LPS OAgs chain length making the bacterial membrane unreachable for the antimicrobial peptides. Additionally, DNA actions of damaging components are balanced by a DNA repair mechanisms, while nutrient starvation and the lysozyme activity are counteracted by the secretion of siderophores and lysozyme inhibitors. Consequently, *Salmonella* Enteritidis can survive the hostile environment of the egg white of the forming egg leading to contamination of the egg after ovipostion and subsequent contagion of the human foodchain.

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2 Scientific aims

Scientific aims

Egg contamination by *Salmonella* Enteritidis is one of the most important causes of human-foodborne gastro-enteritis throughout the world. As *Salmonella* Enteritidis has an increased potential to persist inside the upper reproductive tract compared to most other serovars, it is now generally accepted that contamination of the egg by *Salmonella* Enteritidis occurs through colonization of the reproductive tract with subsequent incorporation into the forming egg. Until recently, only limited information regarding the *Salmonella* Enteritidis virulence factors involved in this process were available. Fortunately, several high throughput methods have been developed to simplify the quest to search for genes that are important for colonization and pathogenesis. One of these techniques, the *in vivo* expression technology or IVET screening, was previously used to identify genes that are activated inside the reproductive tract but not under standard laboratory conditions, which suggests a potential role for these genes in the colonization of this organ (Gantois *et al.*, 2008a). As both the universal stress proteins (*usp*) A and B were identified during this screening, the first aim of this thesis was to confirm the role of these genes in reproductive tract colonization by the use of defined deletion mutants **(chapter 3.1)**. In order to further extend our knowledge on how *Salmonella* Enteritidis is able to persist inside the reproductive tract, a genome-wide microarray-based transposon library was used to identify mutants that are not capable to colonize the reproductive tract tissue **(chapter 3.2)**.

It is clear that once *Salmonella* Enteritidis has conquered the upper reproductive tract, it can be incorporated into the egg white of the forming egg. The observation that *Salmonella* Enteritidis could be isolated from both forming and laid eggs, while *Salmonella* Typhimurium could only be isolated from forming eggs (Keller *et al.*, 1997), led to the hypothesis that *Salmonella* Enteritidis has developed an enhanced survival potential against the hostile environment of the egg white at the chicken body temperature of 42°C in comparison to other serotypes. The superiority of *Salmonella* Enteritidis to persist inside this matrix was recently confirmed in a large scale experiment with different *Salmonella* strains (De Vylder *et al.*, 2013). As until now only limited information regarding the survival of *Salmonella* Enteritidis in egg white at 42°C is available, we aimed to identify genes that are up-regulated after contact with egg white **(chapter 3.3)** and genes that are necessary for the survival during incubation in egg white at 42°C **(chapter 3.4)**.

3 Experimental studies

Objective chapter 3.1

The universal stress proteins A and B were previously identified during an *in vivo* expression technology screening, in which their promoters were activated after contact with egg white but not under standard laboratory conditions. It could thus be suggested that both these genes might have a role in the establishment of oviduct colonization and egg contamination by *Salmonella* Enteritidis.

Subsequently, the first chapter of this thesis was dedicated to confirm the role of these two genes in oviduct colonization and egg contamination.

3.1 *Salmonella* Enteritidis universal stress protein (*usp*) gene expression is stimulated by egg white and supports oviduct colonization and egg contamination in laying hens

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Salmonella Enteritidis universal stress protein (*usp*) gene expression is stimulated by egg white and supports oviduct colonization and egg contamination in laying hens

Abstract

Salmonella enterica subspecies *enterica* serovar Enteritidis has caused a worldwide egg-associated pandemic since the mid 1980s. The exact mechanisms causing this egg tropism are still largely unknown, and only a few *Salmonella* genes have been implicated in the interaction with the oviduct or eggs. An *in vivo* expression technology screening performed previously, identified the *uspA* and *uspB* genes as being highly expressed in the chicken oviduct and in eggs. Here, we demonstrate that *uspA* and *uspB* gene expression is indeed induced after contact with egg white. Intra-oviduct inoculation of *Salmonella* Enteritidis *uspB* and *uspBA* mutant strains showed that the mutants had a decreased ability to colonize the magnum and isthmus of the oviduct, the organs that produce the egg white and eggshell membranes, respectively, at 7 days post-inoculation. Intravenous challenge showed that a *Salmonella* Enteritidis *uspBA* mutant strain had a decreased ability to contaminate eggs. Analogous to the function of universal stress proteins A and B in other bacterial species, we hypothesize that the *Salmonella uspA* and *uspB* genes are involved in long term persistence of *Salmonella* Enteritidis in harmful environments, such as in the oviduct and eggs, by conferring resistance against compounds that damage the bacterial cell membrane and DNA.

Introduction

Hen eggs contaminated with *Salmonella enterica* subspecies *enterica* serovar Enteritidis (*Salmonella* Enteritidis) have been a major source of human food-borne salmonellosis over the last 25 years (Patrick Mary E., 2004, Braden, 2006, EFSA, 2007). Although eggs can be contaminated on the shell, it is increasingly accepted that *Salmonella* Enteritidis caused a pandemic as a result of internal egg contamination (Gantois *et al.*, 2009a). Indeed, it has been shown that *Salmonella* Enteritidis is capable of colonizing the oviduct tissue (Okamura *et al.*, 2001a, Okamura *et al.*, 2001b, Gantois *et al.*, 2008b) and is able to survive the antimicrobial action of egg white (Gantois *et al.*, 2008b). Recently, data has been generated on the molecular mechanisms of oviduct colonization and egg white resistance. Using an *in vivo* expression technology (IVET) approach, genes involved in cell wall integrity, regulation of fimbrial operons, stress responses and motility were identified as highly expressed in the oviduct tissue (Gantois *et al.*, 2008a). This expression screening method identified two universal stress protein genes (*uspA* and *uspB*) as being highly expressed in the oviduct tissue and in eggs. UspA is a cytoplasmic autophosphorylating serine/threonine phosphoprotein that can be found in a variety of organisms, including bacteria, archaea, fungi, protozoa and plants (Kvint *et al.*, 2003). UspB, on the other hand, is anchored in the cytoplasmic membrane and is probably not as widely distributed as UspA (Farewell *et al.*, 1998). Although it has been reported previously that these genes are expressed as a consequence of environmental stress conditions and seem to contribute to stress resistance (Kvint *et al.*, 2003, Persson *et al.*, 2007, Liu *et al.*, 2007), very limited information is available on the functional role of *Salmonella usp* genes. Because the *Salmonella* Enteritidis *uspA* and *uspB* genes are highly expressed in the oviduct tissue, the current study was designed to evaluate the role of these genes in oviduct colonization and egg contamination.

Materials and Methods

Bacterial strains

The isogenic streptomycin resistant variant of *Salmonella enterica* serotype Enteritidis phage type 4 strain 147 was used in this study (147^{str}). This strain was originally isolated from egg contents. The virulence of this strain has been tested in laying hens and a strong capacity to colonize the reproductive organs has been demonstrated (Methner *et al.*, 1995). *Salmonella* Enteritidis 147^{str} *uspA*, *uspB* and *uspBA* deletion mutants were constructed using the one-step inactivation method, with a linear PCR product, as described by Datsenko and Wanner (2000).

A kanamycin resistance cassette, flanked by FRT-sites, was amplified from the pKD4 helper plasmid using P-primers (Table 3.1.1) that had a 50bp extension at the 5' side of the pKD4 specific primers, homologous with the flanking region of the target gene. The resulting PCR product was used for recombination on the *Salmonella* Enteritidis 147^{str} chromosome using the pKD20 helper plasmid encoding the λ Red system, promoting recombination between the native and PCR adjusted antibiotic resistance cassette. Recombinant clones were selected on kanamycin containing plates and replacement of the target gene by the resistance cassette was confirmed by PCR. The deletion was P22-transduced into a new 147^{str} background (Gemski and Stocker, 1967) and the antibiotic resistance cassette was eliminated using the pCP20 helper plasmid, encoding the FLP-recombinase mediating recombination between the FRT-sites flanking the kanamycin resistance cassette (Datsenko and Wanner, 2000). The targeted genes were completely deleted from start to stop codon, as confirmed by sequencing analysis.

Table 3.1.1: Primers used to create deletion mutants

Primer	Sequence
***uspA*-P1**	5'-AAACGCCAGTAGCTCAATGGTCATCGACAACTTATGGAAGGAGTAACACTTGTGTAGGCTGGAGCTGCTTC-3'
***uspA*-P2**	5'-ATTGACTATAGACCAGACGCGGTCTTAGCCGCCAGCCGGCACGGCAAGTACATATGAATATCCTCCTTAG-3'
***uspB*-P1**	5'-GGAGGCTTATCTAATACGAGCGGGTCAGGAAACTGGCCCGCTTTTTTTATTGTGTAGGCTGGAGCTGCTTC-3'
***uspB*-P2**	5'-GCGGTAGTCCCGGAGAGGAACTCCGTGGGCGGGTCGCCGGGGAGGAGATCATATGAATATCCTCCTTAG-3'

Measuring uspA and uspB expression

Measurement of *uspA* and *uspB* expression was achieved using *Salmonella* Enteritidis 147^{str} carrying a plasmid (pCS26) containing a transcriptional fusion between the promoters of *uspA* or *uspB* and the reporter genes *luxCDABE* (*Salmonella* Enteritidis 147^{str} *uspA-luxCDABE* and *uspB-luxCDABE*) (Van Immerseel *et al.*, 2004). The pCS26 plasmid is a low copy number derivative from pZS. A FluoroScan Ascent luminometer (Labsystems, Helsinki, Finland) was used to quantify light production (luminescence) as a marker of *uspA* and *uspB* expression. Bacterial cultures of *Salmonella* Enteritidis 147^{str} *uspA-luxCDABE* and *uspB-luxCDABE* were grown overnight in Luria Bertani (LB) broth (Sigma, St Louis, MO, USA) at 37°C and diluted 1/100 in 200 µl phosphate buffered saline (PBS) or 200 µl PBS supplemented with 25% sterile stirred egg white (obtained from eggs derived from hens that were not vaccinated against *Salmonella*) in 96-well microplates. These 96-well plates were incubated at 37°C and light production was measured every 15 min for 22 h.

Intra-oviductal infection trial

The experimental protocol was approved by the ethical committee of the Faculty of Veterinary Medicine, Ghent University. Twelve 21-week-old commercial *Salmonella*-free chickens (as demonstrated by serology and bacteriology) were premedicated intramuscularly with buprenorphine hydrochloride at 0.05 mg/kg (Temgesic, Schering-Plough, New Jersey, US) and atropine at 0.05 mg/kg. Anaesthesia was induced by the administration of isoflurane (Schering-Plough, New Jersey, US) through a face mask. Following intubation with a 3.0 uncuffed tracheal tube (Hudson RCI, Temecula, California, US), a continuous oxygen flow of 1.5 to 2.0 L/min was administered carrying 1.5 to 3 % isoflurane. The birds were covered with a sterile surgical blanket and defeathered on the abdominal surface. After disinfection of the incision area with a povidone iodine solution (Braunol, B. Braunol Medical, Prague, Czech Republic), the abdomen was opened through a midline incision and the oviduct segments were carefully exposed. Overnight cultures of *Salmonella* Enteritidis 147^{str} wild type, *ΔuspA*, *ΔuspB* and *ΔuspBA* were diluted in PBS to obtain bacterial suspensions of 1 x 10^8 cfu/ml. For each strain, 3 birds were infected. The oviducts were inoculated with 2 mL of the bacterial suspension at the isthmus-magnum transition zone using a 27 gauge needle. After inoculation, the oviducts were reintroduced into the abdomen and the abdominal wall was sutured. After recovery from anaesthesia, the birds were placed in separate cages on wood shavings. The animals had unrestricted access to drinking water and feed. One week after infection, the hens were euthanized by intravenous injection of embutramid (T61, Intervet, Belgium). Samples of spleen, oviduct and ovary were taken for bacteriological analysis.

Intravenous infection trial

The experimental protocol was approved by the ethical committee of the Faculty of Veterinary Medicine, Ghent University. Forty White Leghorn *Salmonella*-free laying hens were housed on the floor (provided with wood shavings) from the age of 18 weeks until the end of the experiment. On the day of arrival, it was confirmed that the hens were *Salmonella*-negative using bacteriological culture of cloacal swabs and an ELISA for the detection of anti-*Salmonella* antibodies in serum (Desmidt *et al.*, 1996). The animals had unrestricted access to drinking water and feed. At the age of 24 weeks, 2 groups of 20 laying hens were intravenously inoculated with a 1 ml volume of culture containing 5 x 10^6 cfu of the *Salmonella* Enteritidis 147^{str} wild type or the corresponding *uspBA* deletion mutant strain. Three weeks after infection (pi) all birds were euthanized by intravenous injection with embutramid. Samples of spleen,

oviduct and ovary were taken for bacteriological culture. Throughout the experiment, eggs were collected and cultured for the detection of *Salmonella*.

Bacteriological culture

Samples of spleen, liver, oviduct and ovary were weighed. Spleen, liver and ovary samples were homogenized with a stomacher in a 10-fold volume of buffered peptone water (BPW) (Oxoid, Basingstoke, Hampshire, UK). The oviduct samples were sliced into very small pieces and subsequently homogenized with a stomacher. Ten-fold dilutions were inoculated onto brilliant green agar (BGA) (Lab M Limited, Bury, Lancashire, UK) containing 100 µg streptomycin/ml to determine the number of *Salmonella*/g of tissue. Samples that were negative after direct plating were pre-enriched overnight in BPW at 37°C and afterwards enriched in tetrathionate brilliant green broth (1/10) (Merck, Darmstadt, Germany) by overnight incubation at 37°C. A loopful of this culture was then streaked onto BGA containing 100 µg streptomycin/ml, and the agar incubated overnight at 37°C.

Upon collection, eggs were placed in a bath of iodine solution to decontaminate the shell. After 5 min, the eggs were transferred to 70% ethanol. The eggs were then broken aseptically and the content of 1 egg was collected in a sterile plastic bag. The contents were homogenized with a stomacher for 2 min and 40 ml of BPW was added to the content of each egg. These suspensions were then incubated at 37°C for 48 h. Further enrichment in tetrathionate brilliant green broth was performed overnight at 37°C. To detect *Salmonella*, a loopful of this culture was plated onto BGA containing 100 µg streptomycin/ml.

Results

UspA and uspB expression is induced after contact with egg white

Luminescence of *Salmonella* Enteritidis 147^{str} carrying the pCS26 plasmid, containing a fusion of the promoter of *uspA* or *uspB* with *luxCDABE*, was increased after contact with 25% egg white in PBS, relative to unsupplemented PBS (Fig. 1). Maximal expression was seen at about 12 h post-inoculation (Figure 3.1.1). Similar data were obtained using 10% and 50% solutions of egg white, while a 5% egg white solution induced a limited increase in both *uspA* and *uspB* expression (data not shown). Addition of egg white to LB medium also induced an increase in *uspA* and *uspB* expression (data not shown), although the differences were smaller because of the higher level background expression of both genes in unsupplemented LB medium.

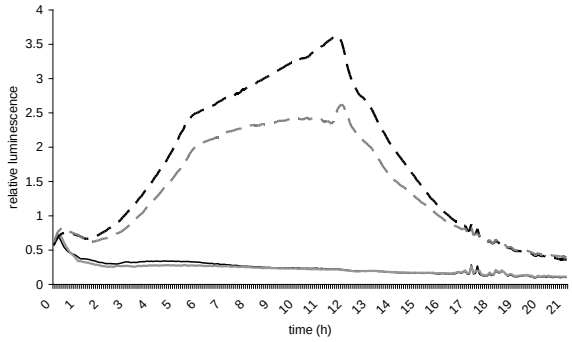

Figure 3.1.1: Luminescence of *Salmonella* Enteritidis 147^{str} carrying a plasmid containing a fusion of the promoter of *uspA* or *uspB* with luxCDABE. Luminescence was recorded over 22 hours after inoculation of *Salmonella* Enteritidis *uspA-luxCDABE* (black) and *Salmonella* Enteritidis *uspB-luxCDABE* (grey) into PBS either containing (dashed) or not containing 25% egg white (solid).

Reproductive tract colonization and egg contamination after intra-oviductal infection is decreased in usp mutants

Seven days after inoculation of *Salmonella* Enteritidis 147^{str} and *ΔuspA*, *ΔuspB* and *ΔuspBA* mutants into the oviduct, the mutants had a decreased level of colonization compared to the wild type in all segments of the reproductive tract except the vagina and infundibulum. There were significantly lower concentrations of the *ΔuspB* and *ΔuspBA* mutants in the isthmus and the magnum (Table 3.1.2). No differences were detected between treatment groups in caecal or spleen colonization.

Table 3.1.2: Concentration of *Salmonella* Enteritidis *ΔuspA*, *ΔuspB*, *ΔuspBA* and wild type in various organs 7 days after intra-oviductal infection.

Tissue	WT	*ΔuspA*	*ΔuspB*	*ΔuspBA*
Spleen	3.6 ± 1.3	3.7 ± 0.7	2.5 ± 0.5	3.9 ± 0.1
Caeca	4.4 ± 2.3	5.3 ± 1.2	3.7 ± 2.2	4.5 ± 0.9
Vagina	3.7 ± 1.2	4.5 ± 0.7	3.9 ± 0.9	3.4 ± 0.5
Uterus	7.0 ± 0.8	5.7 ± 1.6	5.2 ± 1.1	5.3 ± 1.2
Isthmus	7.9 ± 0.9	5.9 ± 0.8	5.0 ± 0.8*	4.5 ± 0.8*
Magnum	6.7 ± 0.3	6.2 ± 0.4	4.7 ± 0.2*	5.0 ± 0.1*
Infundibulum	4.3 ± 0.7	5.6 ± 0.6	4.2 ± 0.5	4.9 ± 0.3
Ovary	7.4 ± 0.2	5.9 ± 1.4	5.5 ± 2.3	5.7 ± 1.3

The mean log value (cfu/gr tissue) ± standard deviations are shown.
* Significant difference ($P<0.05$) between birds inoculated with *ΔuspA, ΔuspB, ΔuspBA* strains and birds inoculated with wild type.

Reproductive tract colonization and egg contamination after intravenous infection is decreased in usp mutants

The proportion of hens laying was 36% and 32% in the first week after infection, 35% and 31% in the second week and 70% and 66% in the third week for the birds infected with the wild type

and the *ΔuspBA* strain, respectively. Fifteen of the 197 eggs (7.76%) gathered throughout the experiment from the group infected with the wild type strain were positive, while only 3 of 170 eggs (1.76%) from the *ΔuspBA* inoculated group were *Salmonella* positive (Table 3.1.3). Colonization of the reproductive tract by the *ΔuspBA* mutant was decreased compared to the wild type strain, but no differences were detected in spleen or liver colonization (Table 3). The number of *Salmonella* positive caecal samples was significantly lower for the *ΔuspBA* strain compared to the wild type strain.

Table 3.1.3: Proportion of *Salmonella* positive organ samples and eggs 3 weeks after intravenous inoculation of 24-week old laying hens with wild type *Salmonella* Enteritidis 147^{str} strain or a *uspBA* deletion mutant.

	Caeca	Liver	Spleen	Oviduct	Ovary	Eggs
WT	10/20	13/20	20/20	8/20	11/20	15/197 (7.8%)
ΔuspBA	4/20	9/20	18/20	3/20	4/20	3/170 (1.8%)
P-value	0.047*	0.2	0.15	0.077	0.022*	0.010*

* Values significantly different from each other within the same column (P<0.05)

Discussion

As *Salmonella* Enteritidis is able to colonize the chicken reproductive tract and to survive in egg white (Gantois *et al.*, 2008b) , *in vivo* expression technology (IVET) screening was used to identify genes that are highly expressed in the oviduct and in eggs (Gantois *et al.*, 2008a). The study demonstrated that promoters of the *uspA* and *uspB* genes were activated during colonization of the oviduct and in eggs, suggesting a role for these genes in oviduct colonization and egg contamination. In the study reported here, we showed that egg white activates transcription from the *uspA* and *uspB* promoters. Moreover, we showed that a *uspBA* mutant was less able to colonize the oviduct and to contaminate eggs. Transcription of universal stress proteins is induced during inhibition of growth by a variety of stress conditions, including nutrient starvation, heat, oxidative stress and osmotic shock (Nystrom and Neidhardt, 1992, Farewell *et al.*, 1998). Thus it could be that the stressful environment of the reproductive tract and the egg white activates transcription of both genes. In addition to the chicken body temperature of 42°C, the limited availability of nutrients in the oviduct, and the high pH, limited iron availibility and antibacterial agents encountered in the oviduct mucus and the egg white may be responsible for this activation.

Mutants in which *uspB* and both *uspA* and *uspB* are deleted have a decreased capacity to colonize the chicken reproductive tract and to contaminate eggs, although they grow normally in LB at 42°C. Indeed, after intra-oviductal infection, the *uspB* and *uspBA* mutants were significantly decreased in their ability to colonize the isthmus and magnum of the oviduct. A similar phenotype was observed after intravenous infection of chickens with the *uspBA* mutant, with the *uspBA* mutant less able to colonize the chicken reproductive tract compared to the wild type. In addition, eggs of chickens infected with the *uspBA* mutant were significantly less likely to be contaminated than those of chickens infected with the wild type.

The universal stress proteins A and B are proposed to have a role in establishing a persistent colonization in stressful and harmful environments and are thought to play a role in bacterial virulence. Indeed, a *Salmonella* Typhimurium *uspA* mutant was less virulent when it was given orally to BALB/c mice at low doses (10^6cfu/ml) (Liu *et al.*, 2007). Universal stress proteins have been shown to be necessary for the persistence of bacteria during periods in which growth is limited. Previous studies have shown that *E. coli* (Nystrom and Neidhardt, 1994, Nystrom and Neidhardt, 1993, Nachin *et al.*, 2005) and *Salmonella* Typhimurium (Liu *et al.*, 2007) strains carrying a deletion of the *uspA* gene have an impaired ability to survive for prolonged periods of time during stasis caused by different stressors, including nutrient starvation, temperature changes and oxidative stress. Limited access to oxygen results in the upregulation of Rv2623, a conserved hypothetical protein containing 2 universal stress protein domains, in *Mycobacterium bovis* (Boon *et al.*, 2001). A similar phenomenon has been observed in *Pseudomonas aeruginosa*, with Usp-like proteins (PA3309 and PA4352) upregulated during pyruvate fermentation (Schreiber *et al.*, 2006). Mutations in these genes decrease the survival of the pathogen under anaerobic stress conditions (Schreiber *et al.*, 2006, Boes *et al.*, 2006). It can thus be hypothesized that prolonged persistence of *Salmonella* Enteritidis in the reproductive tract and the egg might require the induction of universal stress proteins, as the bacteria encounter a myriad of other stressors, including high temperature, high pH and many antimicrobial substances. Expression of these genes during colonization of the oviduct would facilitate the survival of the bacteria in these hostile environments. Consequently, after colonization of the oviduct, the bacteria could easily be incorporated into the forming egg, where expression of the genes might mediate survival during a period of growth arrest in the egg white.

Although the molecular mechanisms explaining the protective capacity of the UspA and UspB proteins under stress conditions are still largely unknown, a role in alteration of the membrane

composition during stationary phase, leading to increased resistance to environmental stressors, has been suggested. In *E. coli uspA* has been shown to be regulated by FadR, a global regulator of fatty acid synthesis and degradation. FadR is a mediator of cell membrane composition and integrity (Farewell *et al.*, 1996). *E. coli uspA* mutants, have increased sensitivity to weak acids and exhibit increased permeability of the cell membrane (Farewell *et al.*, 1996), and *uspB* mutants are more sensitive to 10% ethanol in the stationary phase, indicating that *uspB* might have a role in sensing and/or mediating alterations in membrane composition during the stationary phase (Farewell *et al.*, 1998).

It is also speculated that *uspA* in *E. coli* is involved in protection against DNA damage or in repair of the non-replicative chromosome during stasis, because *uspA* expression is in part controlled by RecA, part of the SOS response, which is activated upon DNA damage. Furthermore, *uspA* mutants are much more sensitive to UV irradiation and mitomycin C exposure (Diez *et al.*, 2000). Sensitivity to mitomycin C is also seen in *uspB* mutants, which thus may be involved in recombination-dependent repair of DNA in *E. coli* (Persson *et al.*, 2010). Thus, universal stress proteins may be highly expressed in the chicken's oviduct and eggs as a consequence of stressors that cause DNA damage. The reproductive tract and eggs restrict bacterial growth and contain high concentrations of agents that can damage the membrane and DNA, including lysozyme (Board, 1969) and endo- and exonucleases (Lu *et al.*, 2003). Induction of these genes would thus be necessary to cope with these stressors and could enable the bacteria to survive in these stressful environments for a prolonged period of time.

Conclusion

In conclusion, it was demonstrated that expression of the universal stress proteins A and B of *Salmonella* Enteritidis is induced in the hostile environments of the reproductive tract and egg white. In addition we found that *uspB* and *uspBA* mutants have a decreased ability to colonize the chicken reproductive tract and contaminate eggs. This suggests that these genes confer resistance to the variety of membrane and DNA damaging components in the oviduct and the egg. Additionally, we hypothesize that the universal stress proteins might be required for the long term survival of *Salmonella* Enteritidis in the chicken reproductive tract and in eggs.

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Objective chapter 3.2

Althought the IVET-screening performed by Gantois *et al.* (2008) already identified some genes important for oviduct colonization, it must be mentioned that this approach doesn't provide any information on whether the identified genes result in a virulence defect when mutated. Consequently, a transposon mutagenesis method was used during the second chapter of this thesis to identify genes involved in oviduct colonization.

3.2 Microarray-based detection of *Salmonella* Enteritidis genes involved in chicken reproductive tract colonization

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Microarray-based detection of *Salmonella* Enteritidis genes involved in chicken reproductive tract colonization

Abstract

Salmonella Enteritidis has developed the potential to contaminate table eggs internally, by colonization of the chicken reproductive tract and internalization in the forming egg. The serotype Enteritidis has developed mechanisms to reside inside the oviduct more successfully than other serotypes. Until now, the strategy exploited by *Salmonella* Enteritidis to do so remains largely unknown. For that reason, a microarray-based transposon library screen was used to identify genes which are essential for the persistence of *Salmonella* Enteritidis inside primary chicken oviduct cells *'in vitro'* and inside the reproductive tract *'in vivo'*. In total 81 genes, with a potential role in persistence in both the oviduct cells and the oviduct tissue, were identified. Major groups of importance include the *Salmonella* Pathogenicity Islands 1 and 2, genes involved in stress responses, cell wall and LPS structure and the Region Of Difference (ROD) genomic islands 9, 21 and 40.

Introduction

Table eggs and derived products were traced back as the primary food vehicle responsible for a *Salmonella* Enteritidis pandemic that started in the late 80's (EFSA, 2012, Braden, 2006). It is still incompletely understood why this particular *Salmonella* serotype is more successful than other serotypes in colonizing the chicken egg. Indeed, many other serotypes can be present in the environment of the laying hen, but *Salmonella* Enteritidis is the main serotype associated with eggs (EFSA, 2007). Intensive cleaning and disinfection of the eggshell surface failed to eliminate the serotype from eggs, indicating that it must be present in the internal egg content (Braden, 2006). Most likely, the bacteria are incorporated in the egg during its formation in the chicken reproductive tract (Keller *et al.*, 1995, Miyamoto *et al.*, 1997, Okamura *et al.*, 2001a, Gantois *et al.*, 2009a). Reproductive tract colonization capacities of different serotypes have been compared in different studies and show that *Salmonella* Enteritidis is superior in reproductive organ colonization compared to other serotypes, with the exception of *Salmonella* Typhimurium (Gantois *et al.*, 2008b, Keller *et al.*, 1997, Raspoet *et al.*, 2011).

A previously performed '*in vivo*' expression technology (IVET) screening identified genes involved in amino acid and nucleic acid metabolism, motility, cell wall integrity and stress responses as being expressed by *Salmonella* Enteritidis inside the reproductive tract (Gantois *et al.*, 2008a). A role for type 1 fimbriae (De Buck *et al.*, 2004c), the LPS structure (Coward *et al.*, 2013) and stress responses (Raspoet *et al.*, 2011) in reproductive tract colonization has already been confirmed by the use of defined deletion mutants. Knock-out strains can provide important information on the relevance of single genes in the pathogenesis of egg infections, but a whole genome approach that identifies all mutated genes that are important in this process is hitherto lacking. In this study, we identified genes that are required for persistence of *Salmonella* Enteritidis inside the reproductive tract using a genome-wide microarray-based transposon library that selects mutations that are causing decreased colonization of the oviduct. On the one hand our results confirm those found by other studies as genes encoding cell wall components and proteins involved in stress responses were identified. On the other hand, we demonstrate a potential role for the *Salmonella* pathogenicity islands (SPI), phage-associated proteins together with unique regions present in *Salmonella* Enteritidis but not in *Salmonella* Typhimurium, the so called regions of difference (ROD).

Materials and methods

Bacterial strains, growth conditions and construction of transposon library

For the construction of the transposon library, an isogenic streptomycin resistant variant of the *Salmonella* Enteritidis 147 strain (147^{str}) was used. The library was made according to the method previously described (Badarinarayana *et al.*, 2001, Lawley *et al.*, 2006, Chan *et al.*, 2005). In a first step, the pJA1 plasmid was transferred to the *Escherichia coli* (*E. coli*) SM10 λ*pir* strain and maintained under ampicillin (100 µg/ml) (Sigma-Aldrich, St. Louis, MO, USA) selective pressure. The pJA1 suicide vector contains IS10 inverted repeats flanking a kanamycin resistance cassette with an adjacent, outward-directed T7 transcriptional promoter. In addition, the plasmid harbors a mini-Tn10 transposase under control of an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible $LacI^q/P_{tac}$ promoter and RP4 mob region for conjugation. A standard overnight conjugation reaction, in the presence of IPTG (Sigma-Aldrich, St. Louis, MO, USA) but without antibiotics, was performed between the *E. coli* SM10 λ*pir* and the *Salmonella* Enteritidis 147^{str} strain. Under influence of IPTG the transposable element, containing a kanamycin resistance gene and T7 promoter, was excised from the pJA1 plasmid and integrated randomly into the genome of *Salmonella* Enteritidis 147^{str}. The next day, all colonies were scraped from the plate into 10mM $MgSO_4$ (Sigma-Aldrich, St. Louis, MO, USA) and 10-fold dilutions were streaked onto LB agar plates containing 200 µg/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA) and 30 µg/ml kanamycin (Sigma-Aldrich, St. Louis, MO, USA) to select for successfully transposed *Salmonella* Enteritidis 147^{str} mutants. A mixture of about 50.000 mutants were collected and stored in 15% glycerol (Sigma-Aldrich, St. Louis, MO, USA) solution at -80°C.

Isolation of oviduct tubular gland cells

Primary tubular gland cells of the chicken reproductive tract were isolated according to the protocol of Jung-Testas *et al.* (Jung-Testas *et al.*, 1986), with slight modification. A daily subcutaneous injection of 1mg estradiol-benzoate (Sigma-Aldrich, St-Louis, USA) dissolved in sesame oil was given to seven days old chicks for 10 consecutive days. Three weeks later the chicks received a second round of injection for 7 days with the same amount of estradiol-benzoate. The next day, chicks were euthanized with an intravenous embutramid injection (T61; Intervet, Belgium). Part of the oviduct, ranging from the beginning of the magnum till the end of the isthmus was aseptically removed, opened longitudinally and rinsed three times in Hank's

balanced salt solution (HBSS; Gibco, Invitrogen, Auckland, New Zealand). The oviduct segment was cut into small pieces and allowed to dissociate for 30 min at 37 °C in minimal essential medium (DMEM; Gibco, Invitrogen, Auckland, New Zealand) containing collagenase (1 mg/ml) (Sigma-Aldrich, St-Louis, USA) supplemented with 50 µg/ml penicillin-streptomycin (Gibco, Invitrogen, Auckland, New Zealand) The tissue suspension was centrifuged for 5 min at 1200 rpm at 37 °C, supernatant was removed and tissue was further trypsinized (0.25% trypsin-3 mM EDTA in DMEM) for 5 min under agitation at 37 °C. Fetal calf serum (FCS; Gibco, Invitrogen, Auckland, New Zealand), was added to neutralize the trypsin and cells were centrifuged at 1200rpm for 5 min at 37°C. Next, cells were treated with lysis buffer (0.87% NH_4Cl and 0.1% $NaHCO_3$ in HBSS) to eliminate red blood cells. The resulting cell suspension was filtered, using a Ø70 µm cell strainer, and then centrifuged at 1200 rpm. Consequently, the cells were washed twice with DMEM containing 10% FCS. The cells were seeded at 1.10^6 cells/ml in cell culture flasks in DMEM supplemented with 15% FCS, insulin (0.12 IU/ml) (Sigma-Aldrich, St-Louis, USA), fibronectin (1µg/ml) (Sigma-Aldrich, St. Louis, MO, USA), β-estradiol (50 nM) (Sigma-Aldrich-St-Louis, USA), penicillin-streptomycin (50µg/ml) and fungizone (amphotericin B, 250 ng/ml) (Gibco, Invitrogen, Auckland, New Zealand). Flasks were placed in a cell incubator at 37 °C with 5% CO_2 for 2h. During this period fibroblasts attached to the cell culture flask while the oviduct cells remained in suspension. Non-adhering oviduct cells were removed and seeded in tissue culture 24-well plates at 1 x 10^6 cells/ml. Two days post-isolation, the wells were evaluated for confluent growth and used for *in vitro* experiments. The experimental protocol was approved by the ethical committee of the Faculty of Veterinary Medicine, Ghent University.

Transposon library selection on oviduct cells 'in vitro' and oviduct loops 'in vivo'.

Identification of Salmonella Enteritidis genes important to persist inside primary tubular gland cells of the oviduct in vitro

The *Salmonella* Enteritidis transposon library (initial library) was grown for 7h at 37 °C in Luria broth (LB; Sigma-Aldrich, St. Louis, MO, USA) with agitation in the presence of streptomycin (200 µg/ml) and kanamycin (30 µg/ml). The bacterial suspension was centrifuged, resuspended in cell medium without antibiotics, fungizone and FCS and added to the cells at a concentration of 1.10^7 cfu/ml (multiplicity of infection 10 (MOI10)). The plates were centrifuged for 10 min at 1500 rpm to obtain optimal contact of the bacteria with the cell layer. The cells were

incubated for 1h at 37 °C, rinsed 3 times with HBSS and cell medium containing gentamycin (100 µg/ml) (Gibco, Invitrogen, Auckland, New Zealand) was added. After 1h the gentamycin concentration was lowered to 30 µg/ml and cells were incubated further for another 14h. Plates were rinsed 3 times with HBSS and cells were lysed using triton 1% (Sigma-Aldrich, St-Louis, USA). Plates were placed on a MTS 2/4 digital microtiter plate shaker for 10 minutes at maximum speed. Afterwards, HBSS was added and intracellular bacteria were collected. Harvested intracellular bacteria (output library) were grown in LB-medium with streptomycin and kanamycin for 7 hours, used for a second round of invasion. By performing multiple passages important mutants are more properly selected as their absence in the output library compared to the initial library becomes more pronounced. In total, 3 subsequent passages were run and the experiment was performed with 5 independent replicates.

Identification of Salmonella Enteritidis genes important for colonization of the oviduct in vivo

For the *in vivo* identification of genes, an intra-oviduct inoculation approach was chosen, because in this way identified genes cause a defect in oviduct colonization, when deleted. When oral or intravenous inoculation methods are used, some genes might be detected because they cause a defect in gut colonization or in systemic spread when deleted.

The experimental protocol for this procedure was approved by the ethical committee of the Faculty of Veterinary Medicine, Ghent University. Three, 21-week-old commercial laying hens were pre-medicated intramuscularly with buprenorphine hydrochloride at 0.05 mg/kg (Temgesic, Schering-Plough, New Jersey, US) and atropine at 0.05 mg/kg. Anaesthesia was induced by the administration of isoflurane (Schering-Plough, New Jersey, US) through a face mask. Following intubation with a 3.0 uncuffed tracheal tube (Hudson RCI, Temecula, California, US), a continuous oxygen flow of 1.5 to 2.0 L/min was administered carrying 1.5 to 3% isoflurane. The birds were covered with a sterile surgical blanket and defeathered on the abdominal surface. After disinfection of the incision area with a povidone iodine solution (Braunol, B. Braunol Medical, Prague, Czech Republic), the abdomen was opened through a midline incision and the oviduct segments were carefully exposed. A 7h old culture of the *Salmonella* Enteritidis transposon library was centrifuged and diluted in HBSS until 10^7 cfu/ml were obtained. The oviducts were inoculated with 1 ml of the bacterial suspension at the isthmus-magnum transition zone using a 27 gauge needle. After inoculation, the oviduct was reintroduced into the abdomen and the abdominal wall was sutured. After recovery from

anaesthesia, the birds were placed in separate cages on wood shavings. The animals had unrestricted access to drinking water and feed. 2 days after infection, the hens were euthanized by intravenous injection of embutramid (T61, Intervet, Belgium). Magnum and isthmus were aseptically removed and opened longitudinally. Oviducts were rinsed 3 times in HBSS supplemented with 100 µg/ml gentamycin to kill extracellular bacteria. Tubular gland cells were isolated by the protocol of Jung-Testas *et al.* (Jung-Testas *et al.*, 1986) as previously described but with an additional 50 µg/ml of gentamycin in all enzyme solutions and without penicillin and streptomycin until the cells were lysed with 1% triton for 10 min.

DNA isolation and RNA transcription of transposon library

In order to perform microarray hybridization, genomic DNA (gDNA) from the initial library and gDNA from the *'in vitro'* and *'in vivo'* selections were purified using the phenol/chloroform extraction method. 10 µg of the gDNA was digested with HinP1I (New England Biolabs, Ipswich, MA, USA) and 2 µg was ligated to the Y-linkers described by Tavazoie and Church (Tavazoie and Church, 1998). Using a PCR with a transposon and linker specific primer (Badarinarayana *et al.*, 2001), the transposon containing fragments were amplified. 1 µg of PCR product was used in an *'in vitro*' transcription reaction from the T7-promoter in the transposon adjacent region. The MEGAscript T7 transcription kit (Ambion, Austin, Texas, USA) was used for this purpose according to the manufacturer's protocol, except that all reaction volumes and reagents were doubled. RNA was purified by lithium chloride precipitation as described within the MEGAscript kit manual.

Microarray procedure

RNA recovered from the initial and selected transposon libraries was reverse transcribed into cDNA and subsequently Cy5-labeled using random hexamer primers and a Klenow fragment. cDNA was hybridized on a whole-genome *Salmonella* Typhimurium/Enteritidis SALSA cDNA microarray, carrying 5877 coding sequences (CDS), together with Cy3-labeled *Salmonella* Enteritidis genomic reference DNA (Hautefort *et al.*, 2008). Two microarray chips were hybridized for each biological replicate. Slides were scanned on an Axon 4000A scanner (Axon Instruments, Foster City, CA, USA). Spots showing a reference signal lower than the background plus 2 standard deviations or obvious blemishes were excluded from subsequent analysis. Local background was subtracted from spot signals and Cy fluorescence ratios were calculated using GenePix version 1.4 software (Agilent, Santa Clara, CA, USA). To

compensate for unequal dye incorporation or any effect of the amount of template, data centering was performed by bringing the median natural logarithm of the ratios for each group of spots printed by the same pin to zero. Data that passed the quality controls were analyzed using Genespring version GX7.3 software (Agilent, Santa Clara, CA, USA). Signal values of the output library were normalized against those of the initial library and used to identify mutants for which the gene value had at least a 2-fold decrease (fold difference < 0.5) after the selection procedure compared to the initial library grown in LB. Significance of the centered data, at $p \leq 0.001$ for *'in vitro'* tests and $p \leq 0.05$ for *'in vivo'* tests, was determined using a parametric-based statistical test adjusting the individual p-value with the Benjamini and Hochberg false discovery rate multiple test correction. Microarray protocols are described in detail at http://www.ifr.ac.uk/safety/microarrays/protocols.html. As the microarray is mainly annotated for *Salmonella* Typhimurium, gene sequences were used in a BLAST search to look for their *Salmonella* Enteritidis (SEN) homologue.

Results and discussion

To obtain information about the oviduct colonization mechanisms of *Salmonella* Enteritidis, a genome-wide microarray-based transposon library screening was performed. The idea behind this technique is that mutants harboring deletions in genes that are potentially important in oviduct colonization are no longer capable to persist or multiply inside the oviduct cells. Consequently, their numbers will be decreased compared to mutants in which genes that are not essential for oviduct colonization are mutated. Recovery of the mutants followed by DNA isolation and RNA synthesis from the T7-promoter (located on the inserted transposon) with subsequent microarray hybridization thus gives information about the mutants that are eliminated during the selection procedure, i.e. intracellularly in oviduct cells or in oviduct tissue. Using this technique the *in vitro* and *in vivo* screening respectively identified 241 and 323 genes being potentially important for oviduct colonization. 81 of these genes were identified to be important in both the *'in vitro'* screening as in the *'in vivo'* trial and thus considered to be the most important ones (figure 3.2.1). These genes are categorized according to their function in table 3.2.1 and the potential role in oviduct colonization for the more relevant genes is discussed below.

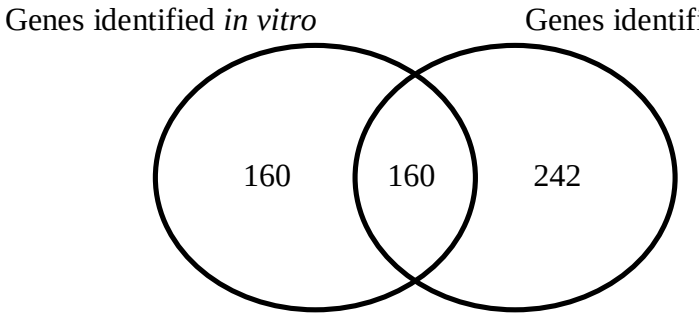

Figure 3.2.1: Venndiagram of important genes for oviduct colonization, identified during the in vitro and in vivo screening

Table 3.2.1: Genes important for persistence inside the reproductive tract, identified during both the *'in vitro'* and *'in vivo'* screening.

Gene Group	SEN Name/locus tag	Gene product
***Salmonella* Pathogenicity islands**		
SPI-1		
	hilA	invasion protein regulator
	invI	secretory protein
	prgH	pathogenicity 1 island effector protein
	prgK	pathogenicity 1 island effector protein
SPI-2		
	ssaB	putative pathogenicity island 2 secreted
	ssaC	putative outer membrane secretory protein
	ssaJ	putative pathogenicity island lipoprotein
	ssaM	putative pathogenicity island protein
	ssaN	putative type III secretion ATP synthase
	ssaT	putative type III secretion protein
	ssaU	putative type III secretion protein
	sscB	putative pathogenicity island protein
	SEN1968	phage integrase (remnant)
SPI-3		
	marT	putative transcriptional regulator
Regions of difference		
ROD9		
	SEN1013B	Hypothetical protein
	SEN1013A	Hypothetical protein
	SEN1009	Hypothetical protein
	SEN1001	Putative DNA binding protein
ROD21		
	SEN1993	Putative DNA binding protein (histone-like protein hlpII)
	SEN1990	Putative DNA binding protein
	SEN1988	Hypothetical protein
	SEN1985	Hypothetical protein
	SEN1984	Exported protein
	SEN1981	Hypothetical protein
	SEN1976	Putative type IV prepilin protein (remnant)
	SEN1974	Hypothetical protein
ROD40		
	SEN4291	Putative type I restricted-modification system
	SEN4290	Putative type I restricted-modification system
Phage-related		
	SEN0921(ϕSE10)	prophage encoded virulence protein

	SEN1144 (ϕSE12)	Putative bacteriophage holin
	SEN1165 (ϕSE12A)	putative phage terminase (remnant)
	SEN1167 (mig-3) (ϕSE12A)	Phage tail fiber protein (remnant)
	SEN1390 (ϕSE14)	Putative membrane protein
Virulence plasmid		
	repA2	DNA replication protein
	pSENV_079	Hypothetical protein
	traA	Conjugational transfer pilin subunit traA
LPS composition and fimbriae		
	rfaB	lipopolysaccharide 1,6-galactosyltransferase
	rfbI	glycosyl transferase
	rfbN	putative rhamnosyltransferase
	rfbP	undecaprenyl-phosphate
	rfbS	Paratose synthase
	rfbV	O-antigen transporter
	rfbX	O-chain glycosyltransferase
	Udg	UDP-glucose 6-dehydrogenase
	pegC	Putative outer membrane usher protein
	ampD	Anhydro-N-acetylmuramyl-tripeptide
	envF	Lipoprotein
SPI-17		
	SEN2380	Putative transposase
DNA biosynthesis and stress reponses		
	pyrD	dihydroorotate dehydrogenase
	ogt	O6-methylguanine-DNA-alkyltransferase
	hslV	heat shock protein
	htrA	protease DO precursor; heat shock protein
	yciG	Conserved hypothetical protein
Other genes		
	btuF	cobalamin periplasmic binding protein
	cdaR	carbohydrate diacid regulator
	cspB	cold shock protein
	glnH	glutamine-binding periplasmic protein precursor
	SEN0168	4-hydroxythreonine-4-phosphate dehydrogenase
	SEN0328	Hypothetical protein
	SEN0708	LysR-family transcriptional regulator
	SEN0744A	Putative cytoplasmic protein
	SEN0853B	Conserved hypothetical protein
	SEN1419	Putative amino acid transporter permease protein
	SEN1418	ABC transporter ATP-binding subunit
	SEN1335	Putative inner membrane protein
	SEN1204	Putative membrane protein
	SEN1916	Putative membrane protein
	SEN2003	Putative DNA binding protein
	SEN2190	Putative hydrolase
	SEN2218	Putative membrane protein
	SEN2256	Putative transmembrane transport protein
	SEN2489	Putative transposase
	SEN2601	Hypothetical protein
	SEN4651	Putative fimbrial protein
	sppA	protease IV
	stfA	Major fimbrial subunit stfa (putative fimbrial subunit)
	yaaU	Putative metabolite transport protein
	ydiM	Putative MFS family transport protein
	ydeE	Putative membrane protein
	ynaJ	Putative membrane protein
	ynfC	Exported protein

Pathogenicity islands

Genes belonging to the *Salmonella* pathogenicity islands (SPI) 1, 2 and 3 were identified as important for the persistence of *Salmonella* Enteritidis inside the reproductive tract. SPI-1 encodes for a type three secretion system (T3SS) enabling the uptake of the bacteria inside non-phagocytic cells. The importance of SPI-1 genes in the invasion and the survival of *Salmonella* Enteritidis in chicken oviduct epithelial cells was already demonstrated by Li *et al.* (Li *et al.*, 2009b). These authors showed that mutations in the effector proteins *sipB*, *sipA*, *sopB* and *sopE2* led a decrease in the invasion of oviduct cells, while those in *sipB* and *sipA* also were less able to survive inside these cells. Intracellular survival/proliferation is a function mainly attributed to SPI-2 (Hautefort *et al.*, 2008, Hensel *et al.*, 1997). It was already demonstrated that a *Salmonella* Enteritidis mutant in *ssrA*, the key regulator of SPI-2, had a lower potential to colonize the reproductive tract and this probably due to a diminished systemic spread (Bohez *et al.*, 2008). Here we show that in addition to systemic spread to the reproductive tract, SPI-2 might also be involved in the persistence of *Salmonella* Enteritidis inside the reproductive tract and the oviduct epithelial cells. In addition to SPI-1 and SPI-2, also one gene, *marT*, belonging to SPI-3, was shown to be of importance for oviduct colonization. Tukel *et al.* (Tukel *et al.*, 2007) showed that *marT* is involved in the transcriptional activation of *misL*, a fibronectin binding protein increasing the invasiveness into epithelial cells.

Regions Of Difference

Genes belonging to regions of difference (ROD) 9, 21 and 40 were identified in our study to be involved in chicken reproductive tract colonization. RODs are genomic islands that were shown to be present in the genome of *Salmonella* Enteritidis but not in Typhimurium (Thomson *et al.*, 2008).

ROD9 is a truncated form of SPI-19 found in *Salmonella* Gallinarum, where it encodes a type VI secretion system (T6SS) indispensable for efficient colonization of the gut, spleen and liver in young chickens (Blondel *et al.*, 2010). SPI-19 is necessary for survival of *Salmonella* Gallinarum inside avian macrophages, without contributing to cytotoxicity and cell death (Blondel *et al.*, 2013). As SPI-19 of *Salmonella* Enteritidis lost many of the T6SS essential components it was suggested that this system is not functional within this serotype (Blondel *et al.*, 2009). Transfer of the complete *Salmonella* Gallinarum SPI-19 cluster to Enteritidis resulted in an increased ileum, liver and spleen colonization 1 day post-infection despite a

colonization defect after 3 days (Blondel *et al.*, 2010). Others hypothesized that some genes of ROD9 might be retained in *Salmonella* Enteritidis to enhance its colonization potential, as the SEN1001 gene of the *Salmonella* Enteritidis ROD9 cluster, appeared to be essential for colonization of mice and survival inside murine macrophages (Silva *et al.*, 2012). Finally, a *Salmonella* Enteritidis ROD9 deletion mutant was not defective in reproductive tract colonization (Coward *et al.*, 2012).

ROD21 is present in *Salmonella* Enteritidis, Gallinarum and Dublin but absent in Typhimurium (Porwollik *et al.*, 2005, Thomson *et al.*, 2008). The cluster contains a Toll/interleukin-1 receptor (TIR) domain containing protein SEN1975 or TlpA that might suppress NF-κB induction while it promotes host cell apoptosis (Silva *et al.*, 2012, Quiroz *et al.*, 2011, Newman *et al.*, 2006). A *tlpA* mutant was defective in intracellular survival inside human THP1 macrophages and mice infected with the *tlpA* mutant survived the challenge better as compared to the wild type (Newman *et al.*, 2006). ROD21 also comprises 2 putative type IV pilin proteins (SEN1976 and SEN1978), which might be linked to several functions including motility, adhesion, biofilm and bacterial aggregate formation, but also invasion (Quiroz *et al.*, 2011, Silva *et al.*, 2012). Also 2 conjugational transfer proteins (SEN1979 and SEN1980) and a histone-like nucleoid-structuring (H-NS) regulator (SEN1993) are located on ROD21, of which the latter was shown to be a homologue of the uropathogenic *E. coli hnsT* gene, which promotes the expression of virulence genes (Quiroz *et al.*, 2011). Nevertheless, a role for ROD21 in reproductive tract colonization could not be demonstrated as a ROD21 deletion mutant had the same colonization levels in this organ as the wild type after oral inoculation (Coward *et al.*, 2012).

The ROD40 locus encodes for a type I restriction/modification system, which degrades foreign DNA (Thomson *et al.*, 2008), but the role of ROD40 in reproductive tract colonization still remains to be investigated.

Phage-associated genes

Five *Salmonella* Enteritidis genes belonging to ϕSE10, ϕSE12, ϕSE12A or ϕSE14 phages have been identified during our screening to be important for oviduct colonization. Shah *et al.* (Shah *et al.*, 2012b) showed that the SEN1393 gene of ϕSE14 is involved in the invasion of *Salmonella* Enteritidis inside Caco-2 and LMH chicken liver cells. Additionally, this mutant was attenuated for survival inside chicken macrophages and showed a significantly reduced growth in egg albumen compared to the wild type. In contrast a ϕSE14 deletion strain colonized

the liver and spleen of BALB/c mice as well as the wild type strain 2 days post-infection (Santiviago *et al.*, 2010). No studies have investigated the role of these phage-associated genes in oviduct colonization yet.

Virulence plasmid

Some serotypes of *Salmonella,* including Enteritidis, harbor serotype specific plasmids (SSP), encoding genes necessary for replication (*repA* and *repA2*) and conjugation (the often degraded tra operon), and carrying virulence genes such as the *Salmonella* plasmid virulence (*spv*) operon and the *pef* and *fae* fimbrial operons (Rotger and Casadesus, 1999, Feng *et al.*, 2012). Our screening suggests that *repA2, traA* and the hypothetical protein (pSENV_079) are important for *Salmonella* Enteritidis colonization of the chicken reproductive tract.

LPS composition and fimbriae

A previously performed IVET-screening already showed that genes involved in cell membrane and cell wall integrity were highly expressed during colonization of the reproductive tract by *Salmonella* Enteritidis (Gantois *et al.*, 2008a). Seven of the genes identified in our study are involved in the composition of the bacterial membrane. Of the cell wall structural proteins, 7 are involved in LPS biosynthesis (*rfaB, rfbI, rfbN rfbP, rfbS, rfbX and rfbV*). The importance of LPS biosynthesis in oviduct colonization was already demonstrated by Coward *et al.* (2013). These authors showed that both the *wzy* (exhibiting only 1 O-antigen repeat) and the *febE* (defective in the production of very long O-antigen repeats with more than 100 subunits) deletion strains are less efficient in the colonization of the ovary and oviduct in comparison to a wild type strain. Also, one gene of SPI-17 was found to be involved in persistence of *Salmonella* Enteritidis inside the reproductive tract in our study. SPI-17 of *Salmonella* Enteritidis is a degenerate prophage encoding for *gtrA* and *gtrB*, which are involved in LPS O-antigen modification in *Shigella flexneri* (Thomson *et al.*, 2008, Vernikos and Parkhill, 2006, Guan *et al.*, 1999). Additionally the UDP-glucose 6-dehydrogenase (*ugd, udg, pagA* or *pmrE*), shown to modify the lipid A component of LPS (Gunn *et al.*, 1998), was identified in our study as involved in oviduct colonization. Finally, a gene of the peptidoglycan recycling system (*ampD*) and an envelope lipoprotein (*envF*) were identified but their role in reproductive tract colonization is not clear.

Although type 1 (*SEF21*) fimbriae are necessary for adhesion to the isthmus secretions (De Buck *et al.*, 2003), we here demonstrate a potential role for the *peg* fimbrial operon (*pegABCD*) in oviduct colonization as a *pegC* mutant was identified during the transposon library screening. This operon is only found in *Salmonella* Enteritidis, Gallinarum and Paratyphi B. Transcription of this operon was induced during reproductive tract colonization (Gantois *et al.*, 2008a) and a *Salmonella* Enteritidis *pegD* mutant had a decreased egg white survival capacity compared to the wild type (Shah *et al.*, 2012b).

DNA biosynthesis/repair and stress responses

The importance of nucleic acid biosynthesis and stress responses for the colonization of the reproductive tract by *Salmonella* Enteritidis was already shown by Gantois *et al.* (2008a). They showed that the expression of many genes of the nucleic acid biosynthesis pathways were up-regulated in the oviduct. In addition, a deletion of the *purA* gene, involved in the pyrimidine metabolism, resulted in a reduced colonization potential of the chicken reproductive tract in comparison to spleen colonization(Gantois *et al.*, 2008a). In the current study, another gene of the pyrimidine metabolism pathway (*pyrD*), and the O6-methylguanine-DNA-transferase (*ogt*), necessary for the repair of DNA alkylation damage, were identified. The *ogt* gene is regulated by the alternative sigma factor σ^S (RpoS) and is involved in the survival of bacteria under starvation or stress conditions (Ibanez-Ruiz *et al.*, 2000) which can be encountered in the reproductive tract. One of the stress conditions encountered during colonization of the reproductive tract is the high chicken body temperature of 42°C. Consequently it is not surprising that in our study two heat inducible genes (*hslV* and *htrA*) were identified. HslV is the proteolytic subunit of the hslVU complex, while HtrA is a periplasmic serine protease. Both genes are involved in the elimination of unfolded and damaged proteins, typically present in bacteria that encounter unfavorable conditions. Finally, *YciG* of the *yciGFE-katN* operon is transcribed as a polycistronic message and harbors a putative RpoS-dependent promoter upstream of *yciG*, directing the transcription of genes essential for the general stress response (Robbe-Saule *et al.*, 2001).

Other genes

In addition to the above mentioned genes, many other genes with various functions have been found, but their role in reproductive tract colonization needs further investigation.

Conclusion

In order to identify genes that are necessary for the persistence of *Salmonella* Enteritidis inside the chicken reproductive tract, a genome-wide transposon screening was performed. Taken everything into consideration we could hypothesize that *Salmonella* Enteritidis might invade and survive inside the chicken oviduct epithelial cells by use of its pathogenicity islands. To further cope with the antimicrobial factors present inside these cells or the reproductive tract, the bacteria might alter its membrane composition, enhance its DNA repair strategies and activate some stress management strategies to cope with the high chicken body temperature. Finally, this study also suggests a potential role for the virulence plasmid and RODs9, 21 and 40. Although the role of these RODs could not be confirmed by other research groups, who used defined single island deletion mutants to study their function in reproductive tract colonization, further research is necessary to determine their specific function as redundancy between these islands may exist.

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Objective chapters 3.3 and 3.4

As *Salmonella* Enteritidis is not only superior in the colonization of the chicken reproductive tract, but also in the survival in egg white during egg formation, both the IVET and transposon mutagenesis screening were used to identify genes necessary for survival in this matrix.

3.3 The *Salmonella* Enteritidis TolC outer membrane channel is involved in egg white survival due to an essential role in counteracting ovotransferrin-mediated iron depletion

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In preparation

The *Salmonella* Enteritidis TolC outer membrane channel is involved in egg white survival due to an essential role in counteracting ovotransferrin-mediated iron depletion

Abstract

Salmonella Enteritidis has developed the potential to contaminate eggs by surviving in the antimicrobial environment of the hen's egg white. This has led to food-borne salmonellosis infections in humans due to the consumption of contaminated eggs and egg-derived products. The molecular mechanisms of *Salmonella* Enteritidis egg white survival are not fully understood. Using *in vivo* expression technology and promoter-reporter fusions we showed that the promoter of the *tolC* gene, encoding the TolC outer membrane channel, is activated by egg white at the chicken body temperature. A *Salmonella* Enteritidis *tolC* deletion strain was no longer capable of surviving in egg white. Using chromatographic separation techniques and subsequent testing of antimicrobial activities, ovotransferrin was identified as the egg white antimicrobial factor inhibiting the *tolC* deletion strain. We give evidence that the antimicrobial action is due to the iron depriving characteristics of ovotransferrin and not because of the ovotransferrin derived antimicrobial peptide OTAP-92. A *Salmonella* Enteritidis resistance-nodulation-division pump mutant has an identical phenotype as the *tolC* deletion strain, indicating exported molecules, supposedly siderophores, to be involved in resistance against ovotransferrin mediated iron deprivation.

Introduction

Eggs are crucial for the chicken reproductive cycle and must support the development of the chicken embryo for 21 days. Consequently, eggs are equiped with all the essential nutrients for this maturation process, on top of physical and chemical defense mechanisms that limit microbial invasion and multiplication (Jonchere *et al.*, 2010). Nevertheless, *Salmonella enterica* serotype Enteritidis has developed the potential to efficiently contaminate eggs, and thus the ability to cause human foodborne infections due to the consumption of contaminated chicken eggs or egg products. It has been hypothesized that the main contamination route for *Salmonella* Enteritidis is through colonization of the chicken reproductive tract (Lister, 1988, Keller *et al.*, 1995, Gast and Holt, 2000a). It is assumed that the upper oviduct is the main colonization site and that most *Salmonella* Enteritidis bacteria are deposited with the egg albumen or egg shell membranes (Gast and Beard, 1990b, Humphrey *et al.*, 1991b, Keller *et al.*, 1995, Miyamoto *et al.*, 1997, De Buck *et al.*, 2004b). Once deposited inside the egg white, the bacteria have to cope with numerous antimicrobial components, including lysozyme, ovotransferrin, defensins and cystatin (Qiu *et al.*, 2012) for about 20 to 26 hours, during which time the shell is formed around the egg white in the uterus, and this at the hen's body temperature of 42°C. *Salmonella* Enteritidis strains are superior to other *Salmonella* serotypes in tolerating these conditions (De Vylder *et al.*, 2013, Keller *et al.*, 1997). Survival mechanisms exploited by *Salmonella* Enteritidis are still not completely understood, but roles have been attributed to the lipopolysaccharides (LPS) (Gantois *et al.*, 2009b), lysozyme inhibitors (Callewaert *et al.*, 2008) and protein and DNA damage repair mechanisms (Lu *et al.*, 2003, Clavijo *et al.*, 2006). To elucidate the survival strategies of *Salmonella* Enteritidis in egg white, an '*in vivo'* expression technology (IVET)-screening was performed and the promoter of the *tolC* gene was found to be highly expressed in egg white, as opposed to standard '*in vitro'* culture conditions, suggesting a potential role for this gene in egg white survival. TolC is an outer membrane channel, involved in siderophore export (Bleuel *et al.*, 2005) and is part of the multidrug resistance (MDR) pumps. MDR-pumps confer antibiotic resistance but also have a role in bacterial pathogenicity as they export host-derived antimicrobial agents and thus allow the bacteria to colonize and survive in certain hostile host niches (Piddock, 2006). Until now, 9 of these pumps have been identified in *Salmonella.* Two of these pumps belong to the major facilitator (MFS) (EmrAB and MdfA), 1 to the multidrug and toxic compound extrusion (MATE) (MdtK), 1 to the ATP-binding cassette efflux (ABC) (MacAB) and 5 to the resistance-nodulation-division (RND) (AcrAB, AcrD, AcrEF, MdtABC, MdsABC) transporter family

(Nishino *et al.*, 2006). Two pumps (MdfA and MdtK) simply span the cytoplasmic membrane, while the other 7 transporters are multicomponent systems spanning both the inner and outer membrane. Except for MdsAB, which is capable of using MdsC, all multicomponent system pumps require TolC as outer membrane channel for their function (Horiyama *et al.*, 2010). Although TolC has been shown to be involved in survival in harmful environments (Baucheron *et al.*, 2005), there is no information on whether TolC is either or not essential for survival in egg white, and if so, on the mechanism of TolC-mediated egg white survival. Therefore, we further investigated the role of TolC in egg white survival by *Salmonella* Enteritidis. We demonstrated that the *tolC* promoter is activated after contact with egg white and that a *tolC* deletion strain has decreased survival capacities in this matrix. We also showed that ovotransferrin is the antimicrobial factor activating the *tolC* promoter and inhibiting growth of the *tolC* deletion strain by iron restriction. TolC-dependent RND-pumps were shown to be involved in counteracting ovotransferrin-mediated antibacterial effects. Finally we provide evidence that the previously identified ovotransferrin derived antimicrobial peptide OTAP-92 is not involved in the ovotransferrin mediated inhibition of a *tolC* mutant.

Materials and methods

In vivo expression technology (IVET)-selection in egg white at the chicken body temperature of 42°C

To elucidate the egg white survival strategies of *Salmonella* Enteritidis at the chicken body temperature, a previously constructed *ΔpurA*/pIVET1 library was used (Gantois *et al.*, 2008a, Gantois *et al.*, 2009b). IVET is a so called promoter trap technology designed to monitor global transcriptional activation in a certain ecological niche. Briefly, random genomic fragments (1-4kb) of the *Salmonella* Enteritidis 147 genome were cloned into the pIVET1 suicide vector, in front of the *purA/lacZY* fusion construct. These constructs were then transferred by conjugation into a *Salmonella* Enteritidis 147 *purA* deficient strain, were homologous recombination at the chromosomal fragment occurred. As the auxotrophe *purA* deletion strain is no longer capable of surviving in purine deficient environments e.g. egg white (Gantois *et al.*, 2009b), bacteria harboring an active promoter in front of the *purA/lacZY* fusion construct are positively selected in this ecological niche due to transcription of the *purA* gen. As most virulence genes are only expressed under specific conditions and not standard laboratory circumstances, recovered bacteria are subjected to a second selection procedure based on *lacZY* expression on lactose

containing MacConkey agar plates. Consequently, this technology was used to identify *Salmonella* Enteritidis virulence factors necessary for the survival in egg white at the chicken body temperature. This library was grown for 4 hours in Luria broth (LB) (Sigma-Aldrich, St Louis, MO, USA) supplemented with streptomycin (100 µg/ml) (Sigma-Aldrich), carbenicillin (50µg/ml) (Sigma-Aldrich), 1.35% adenine (Sigma-Aldrich) and 0.337% thiamine (Sigma-Aldrich). Bacterial cell counts were determined by serial dilutions on LB agar plates. The library was stored at 4°C until the next day and further diluted until 1 x 10^7 cfu/ml in Hank's buffered salt solution (HBSS) (Life Technologies, Bleiswijk, The Netherlands). The shells surface of freshly (< 24 hours) laid eggs was decontaminated in lugol solution (Sigma-Aldrich), and immersed in disolol (Sigma-Aldrich). Egg albumen was aseptically separated from egg yolk, collected in a sterile recipient and mixed for 5 min using a magnetic stirrer. To obtain a final concentration of approximately 10^6 cfu/ml egg albumen 200µl of the library was diluted into 1800µl egg albumen. Suspensions were incubated at 42°C for 24 hours, diluted and plated onto lactose containing MacConkey agar plates supplemented with streptomycin, carbenicillin, adenine and thiamine. Recovered bacteria were scraped from the plates and pooled for a second round of egg white infections to enrich for bacterial cells with high *purA* expression and thus cells in which an egg white activated promoter was present upstream of the *purA/lacZY* fusion. After the second round of egg infections, colonies lacking *lacZY* expression on MacConkey agar plates were picked up and used for identification of the sequences cloned in front of the *purA/lacZY* fusion construct by the PCR-based method of Kwon and Ricke (Kwon and Ricke, 2000). Finally, obtained sequences were used in a BLAST analysis against the *Salmonella enterica* serovar Enteritidis genome of strain P125109. Twelve independent experiments were performed in this way.

Salmonella Enteritidis tolC and RND-4 deletion mutants and tolC complement construction

Because the *ΔpurA*/pIVET1 library was constructed in an isogenic streptomycin resistant variant of the *Salmonella enterica* serotype Enteritidis phage type 4 strain 147 (147^{str}), originally isolated from egg content (Methner *et al.*, 1995), this strain was also used for the construction of *Salmonella* Enteritidis 147^{str} *ΔtolC* and *ΔRND-4* (*acrAB*, *acrD*, *acrEF* and *mdtABC* were deleted one by one) strains according to the one-step inactivation method previously described by Datsenko and Wanner (2000). A kanamycin resistance cassette, flanked by FRT-sites, was amplified from the pKD4 helper plasmid using P-primers (Table 3.3.1) that had a 50bp extension at the 5' side of the pKD4 specific primers, homologous with

the flanking region of the target gene. The resulting PCR product was used for recombination on the *Salmonella* Enteritidis 147^{str} chromosome using the pKD20 helper plasmid encoding the λ Red system, promoting recombination between the native and PCR adjusted antibiotic resistance cassette. Recombinant clones were selected on kanamycin containing plates and replacement of the target gene by the resistance cassette was confirmed by PCR. The deletion was P22-transduced into a new 147^{str} background (Gemski and Stocker, 1967) and the antibiotic resistance cassette was eliminated using the pCP20 helper plasmid, encoding the FLP-recombinase mediating recombination between the FRT-sites flanking the kanamycin resistance cassette (Datsenko and Wanner, 2000). The targeted genes were completely deleted from start to stop codon, as confirmed by sequencing analysis. To complement the *tolC* gene, a *BamHI-XbaI tolC* fragment was amplified from the *Salmonella* Enteritidis 147^{str} wild type genome and ligated into the *BamHI-XbaI* digested pBBR1MCS-2 plasmid, a broad-host-range cloning vector containing kanamycin resistance that allows constitutive gene expression of the complemented gene from the *lacZ* promoter (Kovach *et al.*, 1995). The resulting recombinant plasmid was transferred into the *Salmonella* Enteritidis *ΔtolC* strain (*ΔtolC*/p*tolC*) with subsequent selection on LB plates containing kanamycin (100 µg/ml). Obtained colonies were validated by PCR using standard M13 primers for which sequences were present on the plasmid. To analyze effects of the complementation plasmid on the phenotype, the empty complementation plasmid was electroporated in the *ΔtolC* strain, constructing the *Salmonella* Enteritidis 147 strain *ΔtolC* /p*BBR1MCS-2.*

Measuring tolC promoter expression

The *tolC* promoter was amplified from the *Salmonella* Enteritidis 147^{str} wild type genome using L-primers (Table 3.3.1) integrating BamHI and Xho restriction sites at the PCR fragment. The resulting promoter fragment was ligated into the pCS26 plasmid, creating a fusion construct between the *tolC* promoter and the *luxCDABE* genes. Plasmids were electroporated into the *Salmonella* Enteritidis 147^{str} wild type strain and transformed colonies were selected on agar plates containing kanamycin (100µg/ml). Integration of the promoter into the pCS26 plasmid was verified by PCR and the obtained strain was designated as *Salmonella* Enteritidis 147^{str} *tolC-luxCDABE.* The strain was grown overnight in LB medium, centrifuged and resuspended in sodium phosphate buffer (0.1 M; pH8) and then diluted 1/20 in either sodium phosphate buffer (0.1 M; pH8), sterile stirred egg white or 20 mg/ml ovotransferrin (Sigma-Aldrich) dissolved in the sodium phosphate buffer. 200 µl of these suspensions were brought into a 96-

well microplate and incubated at 37°C in a FluoroScan Ascent luminometer (Labsystems, Helsinki, Finland) to measure light production, and thus *tolC* promoter expression, every 15 min, for 24 h. Obtained values were reduced with the background noise as seen for the *Salmonella* Enteritidis 147 *luxCDABE* strain, harboring the empty pCS26 plasmid. Three independent experiments, with three repeats per test, were performed.

Table 3.3.1. Primers used in this study

Primer	Sequence
For gene deletion	
***tolC*-P1**	5'-TTTTTACAAATTGATCAGCGCTAAATACTGCTTCACAACAAGGAATGCAATGTGTAGGCTGGAGCTGCTTC-3'
***tolC*-P2**	5'-AGACCTACAAGGGCACAGGTCTGATAAGCGCAGCGCCAGCGAATAACTTACATATGAATATCCTCCTTAG-3'
***acrAB* P1**	5'-ATTTTTGCGTAAAAAAGGCCGCTTGCGCGGCCTTATCAACAGTGAGCAAATGTGTAGGCTGGAGCTGCTTC-3'
***acrAB* P2**	5'-AGGACCTTTGACCATTGACCAATTTGAAATCGGACACTCGAGGTTTACATCATATGAATATCCTCCTTAG-3'
***acrD* P1**	5'-GAAGCAGTTCAAATCTATAACGATATGTAGAAACACGAGGTTCCCCTTTATGTGTAGGCTGGAGCTGCTTC-3'
***acrD* P2**	5'-GAAAGTCGCCTTTTTTGTGCCCGACACCTCGTATCAGGCTGGCCGGGAGCCATATGAATATCCTCCTTAG-3'
***acrEF*-P1**	5'-CTTACCGTTATCCGGTGAATAACGAGCTTTCGGTTTTTTAAGGAACAGTATGTGTAGGCTGGAGCTGCTTC-3'
***acrEF*-P2**	5'-TAAAAGACGTACCGTTTAAAAAGGCGTCCGAAGACGCCTCTGTTTACCGGCATATGAATATCCTCCTTAG-3'
***mdtABC*-P1**	5'-GAATAATCCCGACCGTGTTCCATAATTCCTAGGATGAGAAACTTATACCGTGTGTAGGCTGGAGCTGCTTC-3'
***mdtABC*-P2**	5'-GCCAATTGCCACAATCCAGAGTTGCCAGCGGGTATTGTCAGGAAGTTCTGCATATGAATATCCTCCTTAG-3'
For complementation	
***tolC*-C1**	5'-TAGCGGATCCAACAAGGAATGCAAATGAAGAA-3'
***tolC*-C2**	5'-GTACTCTAGATCAATGCCGGAATGGATTGC-3'
For LuxCDABE-fusion	
***tolC*-L1**	5'-ATGCCTCGAGTGTGCTGCCCTGCTAGCAAT-3'
***tolC*-L2**	5'-TCAGGGATCCTTAGTGTGCTGTAACCCCGAC-3'

Egg white survival of Salmonella Enteritidis ΔtolC

Egg white survival assay

Bacterial cell counts of overnight cultures of the *Salmonella* Enteritidis 147^{str} wild type, *ΔtolC, ΔtolC*/p*BBR1MCS-2* and *ΔtolC*/p*tolC* strains were determined by serial dilutions on LB agar plates. Cultures were stored at 4°C until the next day and further diluted to 1 x 10^5 cfu/ml in sodium phosphate buffer (0.1 M; pH8). 100 µl of the bacterial suspensions were added to 900 µl of egg albumen, prepared as previously described, to obtain a final concentration of approximately 10^4 cfu/ml egg albumen. Inoculated egg albumen suspensions were incubated at 42°C for 24 hours, where after the number of surviving bacteria was determined by plating 200 µl of serial dilutions on antibiotic supplemented LB agar plates. Four independent experiments with each three repeats were performed. To evaluate the viability of the mutants at 42°C without egg white all strains were incubated for 24 hours at 42°C in tryptic soy broth (TSB) (Oxoid, Basingstoke, UK).

Agar spot assay

20 µl drops of sterile stirred egg white were spotted onto agar plates on which bacterial cultures of the *Salmonella* Enteritidis wild type 147^{str}, *ΔtolC*, *ΔtolC*/p*BBR1MCS-2, ΔtolC*/p*tolC* and *ΔRND-4* strains, with a density of 0.5 McFarland, were confluently streaked. Drops were allowed to dry and plates were incubated at 37°C so that growth or inhibition could be investigated the next day.

Identification of egg white component with antimicrobial activity against Salmonella Enteritidis ΔtolC

Mucin free egg white was prepared according to the method described by Guérin-Dubiard *et al.* (Guerin-Dubiard *et al.*, 2005), with minor modifications. Briefly, egg white was aseptically separated from egg yolk and diluted ¼ in distilled water. The pH was adjusted to pH6 and the egg white was stirred overnight at 4°C. The precipitated mucins were removed the next day by centrifugation at 3000 g, at 4°C for 5 min. The supernatant was collected and diluted ½ with sodium phosphate buffer (0.1M; pH6). The mixture was brought onto a SP-sepharose cation exchanger (GE healthcare Biosciences AB, Uppsala, Sweden), on which stepwise elution was performed using increasing concentrations of NaCl (up to 1 M). All obtained fractions were concentrated using a vivaspin 6 centrifugal concentrator (3kDa; Sartorius Stedim Biotech GmbH, Göttingen, Germany) and used in the previously described agar spot assay. Fractions that had an inhibitory effect on the Δ*tolC* deletion strain were pooled and used for subsequent size exclusion chromatography on a SD75 column (GE healthcare Biosciences AB, Uppsala, Sweden). 1 ml fractions were collected, concentrated and tested for their inhibitory activity in the agar spot assay. Fractions with antimicrobial activity against the Δ*tolC* deletion strain were brought on SDS-PAGE 12% gels. Proteins were visualized using Coomassie Brilliant Blue staining (Sigma-Aldrich, St Louis, USA). Bands were cut out of the gel and subjected to in-gel protein digestion with trypsin (Devreese *et al.* 2002) followed by mass spectrometric characterization. After mixing of 1 µl of the digestion mixture with 10 µl a-cyano siniapinic acid (5 mg/ml), 1 µl was spotted onto the target plate and analyzed with the 4800 plus MALDI TOF/TOF Analyzer (Applied Biosystems, Foster City, CA). A NCBI BLAST-search was done with the obtained amino acid sequences (http://blast.ncbi.nlm.nih.gov/Blast/). Identified proteins were purchased from Sigma-Aldrich and used in the agar spot assay at different concentrations (5, 10, 25, 50, 100 and 200mg/ml).

Mechanism of ovotransferrin-mediated antibacterial activity against Salmonella Enteritidis ΔtolC

Iron restriction

Ovotransferrin was identified to be the antimicrobial egg white component active against *Salmonella* Enteritidis *ΔtolC*. To study whether ovotransferrin-mediated iron deprivation was involved, the low-phosphate and low-iron 3-(N-morpholino)propanesulfonate (MOPS)/glucose/casamino acids/vitamins (MGCV) medium was prepared as described by Orchad *et al.* (Orchard *et al.*, 2012). A concentration of ovotransferrin, similar to that found in egg white (20 mg/ml) (Sellier *et al.*, 2007) was dissolved in this medium A) without $FeCl_3$, B) with 2.4 µM $FeCl_3$ for low-iron conditions, C) 500 µM $FeCl_3$ for ovotransferrin saturating conditions and D) 1000 µM $FeCl_3$ for very high iron conditions exceeding ovotransferrin saturation. 20 µl drops of these suspensions were used in the agar spot assay with the *Salmonella* Enteritidis wild type, *ΔtolC*, *ΔtolC*/p*BBR1MCS-2* and *ΔtolC*/p*tolC*. To investigate the role of multidrug efflux pumps in this process, also the *Δ*RND-4 strain was tested in the agar spot assay.

Antibacterial activity of OTAP-92

It has been demonstrated already that OTAP-92 is an antibacterial peptide derived from ovotransferrin. Ovotransferrin antibacterial peptide (OTAP-92) was generated by specific cleavage at aspartyl residues in a diluted acid procedure and chromatographically purified as described earlier (Ibrahim *et al.*, 2000). Briefly, the hydrolysate was injected into a fast-protein liquid chromatography system (BioLogic LP; Bio-Rad, Tokyo, Japan), with a prepacked Sephadex G-50 column, equilibrated and eluted with pyridine-acetate buffer (pH 5.5). The resultant fraction, containing the peptide, was subjected to reversed-phase HPLC, using $5C_{18}$-AR-II column and a linear gradient elution of 1–50% acetonitrile over 100 min. Peptide elution was monitored at 215 nm. The peptide was vacuum-dried and resuspended in distilled water before analysis. The identity of OTAP-92 peptide was confirmed by MALDI–TOF–MS analysis (Voyager DE-PRO; PE-Applied Biosystems, Foster City, CA, USA) operated in positive ion mode. OTAP-92 was dissolved in water to a final concentration of 450 µg/ml and used in the agar spot assay with the *Salmonella* Enteritidis wild type, *ΔtolC, ΔtolC*/p*BBR1MCS-2, ΔtolC*/*ptolC* and *ΔRND-4* strains.

Statistical analysis

Data were analyzed with GraphPad Prism, version 5, using a Mann Whitney Test to compare the means of the log10 values between wild type, *ΔtolC ΔtolC*/p*BBR1MCS-2*, and *ΔtolC*/p*tolC* in the egg white survival assays.

Results

IVET selection in egg white at the chicken body temperature

To search for gene promoters that are activated in egg white at the chicken body temperature and thus potentially involved in the survival of *Salmonella* Enteritidis in egg white, an IVET-screening was performed. This experiment showed that promoters of 62 different genes (table 3.3.2) were activated after contact with egg white at 42°C, but not under standard *'in vitro'* culture conditions. One of the promoters identified was the promoter of the *tolC* gene, which encodes for an outer membrane channel that is part of the multidrug efflux pump systems.

Table 3.3.2: Genes induced by egg white at 42°C

Gene Group	Gene name	Gene product
Metabolic genes		
	bcsB	Cellulose biosynthesis protein subunit B
	dedA/truA	DedA protein (dsg-1 protein)/ tRNA pseudouridine synthase A
	deoA	thymidine phosphorylase
	dnaX	DNA polymerase III subunits gamma and tau
	frr	Ribosome recycling factor
	gcl	glyoxylate carboligase
	glyS	glycine-tRNA synthetase, beta subunit
	gyrA	DNA gyrase subunit A
	hisH/hisB/hisC	His operon
	hypA	hydrogenase nickel incorporation protein
	hypf	hydrogenase maturation protein
	leuS	leucyl-tRNA synthetase
	manY	phosphotransferase enzyme II, C component
	mreB	rod shape-determining protein
	nuoC	NADH dehydrogenase I chain C; chain D
	plsC	1-acyl-glycerol-3-phosphate acyltransferase
	prpC	methylcitrate synthase
	ptsA	phosphoenolpyruvate-protein phosphotransferase
	pyrG	CTP synthase
	rnr	Ribonuclease R
	rpoZ	DNA-directed RNA polymerase omega chain
	selA	L-seryl-tRNA(Ser) selenium transferase
	thiJ	4-methyl-5(b-hydroxyethyl)-thiazole monophosphate biosynthesis protein
	ubiE	Biquinone/menaquinone biosynthesis methyltransferase
	ung	Uracil-DNA glycosylase
	yfcX/yfcY	Putative fatty acid oxidation complex alpha subunit/putative 3-ketoacyl-CoA thiolase
Regulatory genes		
	baeR	putative two-component system response regulator

	malT	MalT regulatory protein
	SEN2865	Probable amino acid transporter protein
	tolC	Outer membrane protein TolC precursor
	tsx	Nucleoside-specific channel-forming protein tsx precursor
	ttk	Putative TetR-family transcriptional regulator
Cell membrane		
	bigA	putative surface-exposed virulence protein
	hflK	HflK protein
	murA	UDP-N-acetylglucosamine
	rfaD	ADP-L-Glycero-D-mannoheptose-6-epimerase
Transport		
	caiT	probable carnitine transporter
	citA	citrate-proton symporter
	gltP	proton glutamate symport protein
	mtr	probable amino acid permease
	ybdA	Hypothetical membrane protein p43
	yejF	Putative ABC-transporter ATP-binding protein
	yhhJ	Putative ABC-2 type superfamily transport protein
	yicJ	Sodium:galactoside family symporter
fimbriae		
	fimA	type-1 fimbrial protein, a chain precursor
Motility		
	fliG	flagellar motor switch protein
	motA/motB	motility protein A/motility protein B
Stress		
	yrfI	Heat shock protein
Pathogenicity islands		
	mgtC	Mg(2+) transport ATPase protein C
Plasmid related		
	parA	Plasmid partition protein A
	ppdB/ppdA	prepilin peptidase dependent protein B/A precursor
Unknown function		
	elaB	conserved hypothetical protein
	rmbA	conserved hypothetical protein
	yadQ/yadR	Putative membrane protein/conserved hypothetical protein
	ycbB	Putative exported protein
	ycbL	Conserved hypothetical protein
	yegU	Putative hydrolase
	ygdI	Possible lipoprotein
	ygdL	Conserved hypothetical protein
	yhcK	Putative GntR-family transcriptional regulator
	yihW	Putative DeoR-family transcriptional regulator
	yraL	Conserved hypothetical protein

tolC promoter expression is increased after contact with egg white.

To confirm the activation of the promoter of the *tolC* gene in egg white, a luminescence measurement of the *Salmonella* Enteritidis 147^{str} *tolC-luxCDABE* strain was performed. This clearly showed that egg white indeed functions as a trigger for activation of the *tolC* promoter. The *tolC* promoter became activated almost immediately after contact with egg white. Maximal expression was reached after 1 h and decreased substantially from that moment until control levels (sodium phosphate buffer; 0.1 M; pH8) were reached at 10 h of incubation. Reported values represent 1 experiment, but replicas displayed similar results (Figure 3.3.1).

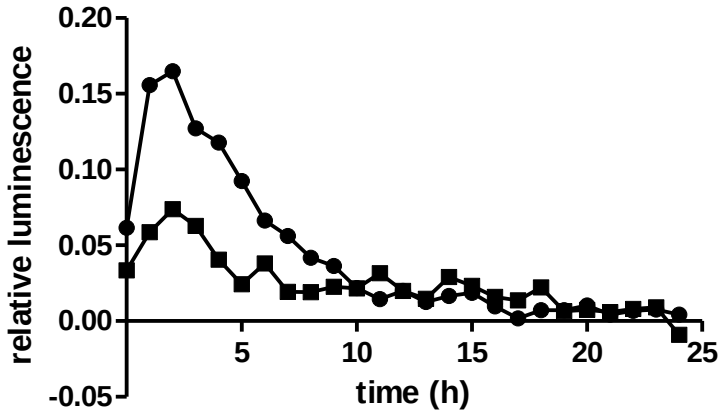

Figure 3.3.1: Luminescence measurement of *Salmonella* Enteritidis 147^{str} *tolC-luxCDABE* in egg white (spheres) and sodium phosphate buffer (squares) at 37°C. The result represents the value of one of three independent experiments which each showed the same trend.

A Salmonella Enteritidis tolC deletion mutant is no longer capable of surviving in egg white

To study the role of the *Salmonella* Enteritidis *tolC* gene in survival in egg white, a deletion mutant and its complement were constructed and incubated for 24 hours in sterile stirred egg white at 42°C. Bacterial cell counts of the *Salmonella* Enteritidis 147^{str} *ΔtolC* and *ΔtolC*/p*BBR1MCS-2* were significantly decreased when compared to the bacterial cell counts of the wild type strain. The wild type phenotype was restored by complementation of the *tolC* gene, as the *ΔtolC*/p*ΔtolC* strain exhibited a similar survival potential to that of the wild type strain (Figure 3.3.2A). These data indicate that a *tolC* deletion strain is much more sensitive to the antimicrobial action of egg white and show the importance of TolC for survival of *Salmonella* Enteritidis in egg white at 42°C. These data were also confirmed by the agar spot assay as a clear growth inhibition zone was detected at the zones where the egg white was spotted on a confluent layer of the *Salmonella* Enteritidis 147str *ΔtolC* strain, but not for the wild type or complemented strain (Figure 3.3.2B).

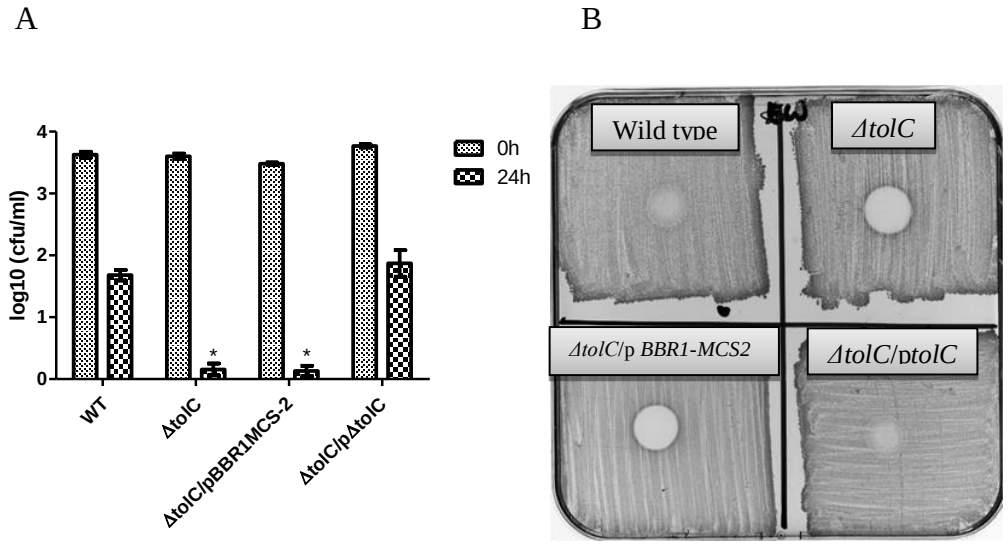

Figure 3.3.2: Survival of *Salmonella* Enteritidis wild type, *ΔtolC* and strain *Δtol*/p*tolC* at 42°C in egg white. A) The results represent the means of four independent experiments conducted in triplicate ± standard errors of the mean. * P < 0.05 of mutant compared to wild type or complement. B) Inhibition of *ΔtolC* due to egg white drops in the agar spot assay.

Ovotransferrin is the egg white antibacterial component that is antibacterial for a tolC deletion strain

To identify the egg white component with antimicrobial activity against a *tolC* deletion strain, chromatographic separation of egg white proteins was performed. Following cation exchange and size exclusion chromatography, testing of fractions in agar spot assays and analyzing the composition of the antibacterial fractions, two egg white proteins (ovalbumin and ovotransferrin) were identified using MALDI-TOF. Both proteins (Sigma-Aldrich) were used in the agar spot assay at different concentrations. After overnight incubation at 42°C a clear growth inhibition zone was observed at spots where ovotransferrin was spotted on the plate streaked with the *tolC* deletion strain, from a concentration of 10mg/ml onwards, while ovalbumin had no antibacterial activity. As for egg white, the phenotype was completely restored in the complemented mutant. Because the concentration of ovotransferrin in egg white is estimated to be 20mg/ml (Sellier *et al.*, 2007),and because this concentration gave a clear inhibition zone in the agar spot assay against the *tolC* deletion strain, this concentration was used in all further experiments (Figure 4 (see 3.6)).

tolC promoter expression is increased after contact with ovotransferrin

To evaluate whether ovotransferrin was responsible for the increase in *tolC* promoter activation when *Salmonella* was incubated in egg white, *tolC* promoter activation was measured after

contact with ovotransferrin using the *Salmonella* Enteritidis *tolC-luxCDABE* strain. As shown in figure 1B, 20 mg/ml ovotransferrin induced *tolC* promoter activity immediately after contact with ovotransferrin. As these results are similar to those observed in egg white we could postulate that ovotransferrin is (one of) the egg white component(s) inducing *tolC* promoter activation in egg white. Reported values represent 1 experiment, but replicas displayed similar results (Figure 3.3.3)

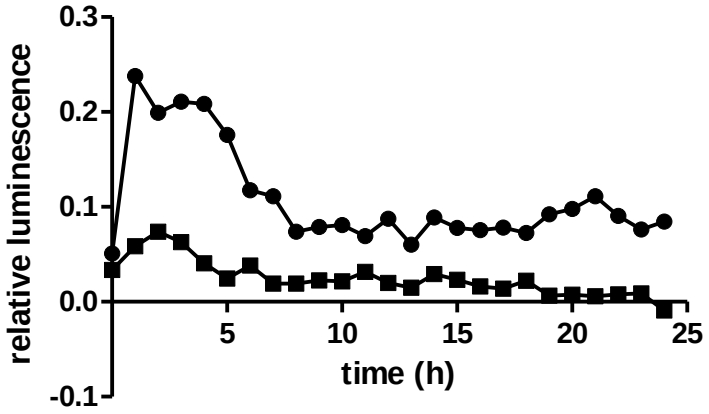

Figure 3.3.3: Luminescence measurement of *Salmonella* Enteritidis 147^{str} *tolC-luxCDABE* in ovotransferrin (spheres) and sodium phosphate buffer (squares) at 37°C. The result represents the value of one of three independent experiments which each showed the same trend.

Ovotransferrin inhibits tolC and RND deletion mutants by restriction of iron

Ovotransferrin is an iron chelator binding free iron and thus limiting bacterial cell growth. To investigate the role of iron deprivation in inhibition of a *tolC* mutant by ovotransferrin, an agar spot assay in the absence or presence of different iron concentrations was performed. For this purpose ovotransferrin was dissolved in low phosphate medium as phosphate may precipitate iron and thus interfere with the results (Orchard *et al.*, 2012). The *tolC* mutant was capable of growing in the ovotransferrin drops once ovotransferrin was completely saturated by iron and excess free iron was present, indicating that ovotransferrin mediates *Salmonella* Enteritidis Δ*tolC* inhibition through iron restriction (Figure 3.3.4).

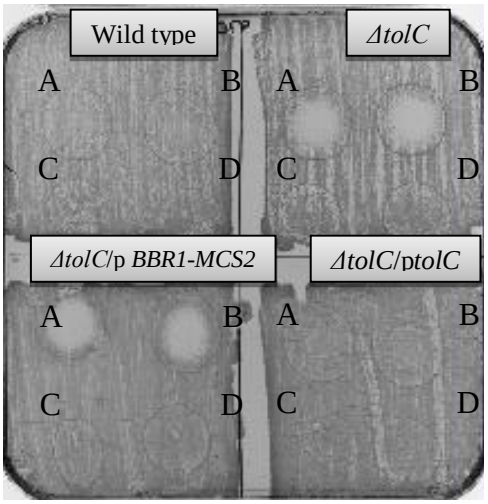

Figure 3.3.4: inhibition of *Salmonella* Enteritidis wild type, *ΔtolC* and strain *Δtol*/p*tolC* by A) ovotransferrin without $FeCl_3$ B) ovotransferrin with 2.4 µM $FeCl_3$ C) ovotransferrin with 500 µM $FeCl_3$ and D) ovotransferrin with 1000 µM $FeCl_3$.

Also the *ΔRND-4* was tested in this assay. Although the mutant was inhibited by ovotransferrin in the absence of iron or in the presence of a minimal amount, the *ΔRND-4* strain could grow once iron concentrations were high enough to saturate ovotransferrin (data not shown).

Survival in OTAP-92

Although the best known antimicrobial function of ovotransferrin is the restriction of iron, also other antibacterial activities have already been demonstrated. It has been shown previously that ovotransferrin derived antimicrobial peptide OTAP-92 is capable of killing an *Escherichia coli* and a *Staphylococcus aureus* strain (Ibrahim *et al.*, 1998). Nevertheless, OTAP-92 was not capable of inhibiting the *Salmonella* Enteritidis *tolC* deletion strains, nor was it able to suppress growth of the multidrug resistance pump mutant ΔRND-4 in an agar spot assay (data not shown).

Discussion

The outer membrane channel TolC is involved in egg white survival of *Salmonella* Enteritidis and counteracts ovotransferrin-mediated iron depletion. In the current study we demonstrated that the *tolC* promoter is activated after contact with egg white and that a *tolC* deletion strain is impaired for its survival in egg white. Additionally, chromatographic separation of egg white revealed that ovotransferrin was the responsible antimicrobial agent activating the *tolC* promoter and inhibiting a *tolC* deletion strain, based on its iron sequestering capacities.

Ovotransferrin is the second major egg white protein as it accounts for about 13% of the total protein content (Guerin-Dubiard *et al.*, 2005). The protein consists of 2 similarly sized homologous lobes (N and C) each consisting of two subdomains with an inter-subdomain cleft capable of binding one Fe^{3+} ion together with two CO_3^{2-} or HCO_3^- ions (Ibrahim *et al.*, 1998). Consequently, each ovotransferrin molecule is capable of binding two iron ions limiting the amount of free iron present in egg white and thus inhibiting bacterial growth. Iron chelating antimicrobial activities of ovotransferrin have been demonstrated against a variety of bacteria, including *Salmonella* Enteritidis (Chart and Rowe, 1993). Baron *et al.* (1997) showed that iron deficiency, resulting from iron binding to ovotransferrin, was the major mechanism implicated in the inhibition of the growth of *Salmonella* Enteritidis in egg white. Additionally, Chart and Rowe (1993) demonstrated a delay in growth rate of *Salmonella* Enteritidis when incubated in TSB with ovotransferrin in comparison to non-supplemented medium. The authors attributed the delay in growth rate to the adaptations necessary for growth in an iron restricted environment. It has been shown that in response to iron deficiency, *Salmonella enterica* secretes a variety of powerful and selective iron chelators, called siderophores. In *Salmonella* 2 catecholate siderophores, enterobactin and its C-glycosylated derivate salmochelin are produced in response to iron limitation. Enterobactin is secreted through the inner membrane by both EntS and IroC, whereas salmochelin can only be exported by iroC (Crouch *et al.*, 2008). While the outer membrane transporter for salmochelin still needs to be identified, Bleuel *et al.* (Bleuel *et al.*, 2005) suggested that the TolC outer membrane channel is necessary for transport of enterobactin in *E. coli*. In the latter study, no enterobactin could be found in the supernatant of a *tolC* deletion strain. Additionally, the growth of *tolC* deletion strain was inhibited in the presence of the iron chelator 2,2'-dipyridyl. It seems plausible that under iron limiting conditions caused by ovotransferrin, a *Salmonella* Enteritidis *tolC* mutant could have difficulties to obtain iron and thus maintain growth. Bleuel *et al.* (Bleuel *et al.*, 2005) also suggested a role of the resistance nodulation cell division (RND) pumps in enterobactin export from the periplasm to the surrounding environment of *E.coli*. Nevertheless, they could not provide evidence for the role of one single transporter in this process in *E. coli*. Here we show that a *Salmonella* Enteritidis strain lacking the 4 TolC-dependent RND-pumps is inhibited by ovotransferrin and that the phenotype is restored in the presence of an excess of iron, saturating the ovotransferrin. This points to a role of TolC dependent siderophores in egg white survival.

Multidrug efflux pumps have also been shown to be involved in exporting antibacterial peptides from host environments and efflux of these compounds could be an additional mechanism of

TolC dependent survival in egg white, in synergy with counteracting iron-depletion. All 9 multidrug efflux pumps of *Salmonella* have been implicated in drug resistance (Nishino *et al.*, 2006), but the efflux of antibiotics from the bacterial cell is most likely a side-effect of their physiological role (Piddock, 2006). It has been shown that efflux pumps export bile salts (Elkins and Mullis, 2006), steroid hormones (Thanassi *et al.*, 1997) and other host derived antimicrobial agents in order to allow the bacteria to survive in a certain ecological niche. For example, the Mtr efflux pump of *Neisseria gonorrhoeae* and *meningitidis* modulates susceptibility to several antimicrobial peptides, including protegin-1 and human cathelicidin peptide LL-37 (Shafer *et al.*, 1998, Tzeng *et al.*, 2005). Bengoechea and Skurnik (Bengoechea and Skurnik, 2000) demonstrated that a *Yersinia enterocolitica* efflux pump/potassium antiporter mutant is more sensitive to the cationic antimicrobial peptides polymyxin B, cecropin P1 and melittin. In addition, a *mexAB-oprM* mutant of *Pseudomonas aeruginosa* is unable to develop tolerance against colistin (polymyxin E) in biofilms (Pamp *et al.*, 2008). Also the *acrB* efflux pump mutant of *Klebsiella pneumoniae* is more susceptible to polymyxin B and human bronchoalveolar lavage fluid and its antimicrobial peptides (NHP-1, HBD-1 and HBD-2) (Padilla *et al.*, 2010). Finally, Delgado et al (Delgado *et al.*, 2005) demonstrated the importance of the *E. coli* TolC outer membrane channel in the resistance to exogenous microcin J25, an antibacterial peptide active against *E.coli, Salmonella* and *Shigella*. Considering the role of multidrug efflux pumps and the TolC outer membrane channel in antimicrobial peptide efflux it was thus tempting to speculate that these pumps might be involved in the efflux of ovotransferrin derived antimicrobial peptides. Ibrahim *et al.* (2006) demonstrated that ovotransferrin undergoes autocleavage at high pH as a consequence of thiol-based reduction, with the possible release of functional domains, such as a kringle between residues 115-211 and 454-544. Due to the high pH and maintenance of a thiol-linked steady reducing state, it could be hypothesized that autocleavage of ovotransferrin occurs within egg white, with release of antimicrobial peptides. Ibrahim *et al.* (Ibrahim *et al.*, 1998) also identified an ovotransferrin derived bacterial peptide named OTAP-92 by cleaving ovotransferrin by diluted-acid hydrolysis. OTAP-92 consists of 92 residues located within the 108-200 sequence of the N-lobe of ovotransferrin. It has been shown that OTAP-92 is capable of killing gram negative bacteria by crossing the outer membrane and damaging the biological function of the cytoplasmic membrane (Ibrahim *et al.*, 2000). Nevertheless, the agar spot assay used in our study could not demonstrate any activity of OTAP-92 against the *Salmonella* Enteritidis *tolC* or *RND-4* deletion strains. It cannot be ruled out that other peptides derived from ovotransferrin might exhibit an antimicrobial function against these mutants. While egg white had a

bactericidal activity against the *Salmonella* Enteritidis *tolC* deletion strain, the iron depletion effect caused by ovotransferrin is bacteriostatic rather than bactericidal. Therefore it could be hypothesized that a synergistic mechanism of ovotransferrin together with other egg white proteins must exist to induce the lethal effects of egg white on a *tolC* mutant. For example, synergistic effects of lactoferrin and lysozyme have already been shown against *Salmonella* Typhimurium and *E. coli* (Ellison and Giehl, 1991). The identification of egg white proteins which mediate killing of a *tolC* deletion strain after contact with egg white, in synergy with ovotransferrin, requires further studies.

Conclusion

In summary, we provide evidence that the outer membrane channel TolC is necessary for the survival of *Salmonella* Enteritidis in egg white, most likely related to the function of RND multidrug efflux pump systems. We also demonstrate that TolC protects against ovotransferrin-mediated iron depletion, presumably due to siderophore secretion.

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3.4 A genome-wide screen identifies *Salmonella* Enteritidis LPS biosynthesis and the HtrA heat shock protein as crucial factors involved in egg white persistence at the chicken body temperature

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A genome-wide screen identifies *Salmonella* Enteritidis LPS biosynthesis and the HtrA heat shock protein as crucial factors involved in egg white persistence at the chicken body temperature

Abstract

Eggs contaminated with *Salmonella* Enteritidis are an important source of human foodborne *Salmonella* infections. *Salmonella* Enteritidis is able to contaminate egg white during formation of the egg within the chicken oviduct and it has developed strategies to withstand the antimicrobial properties of egg white in order to survive in this hostile environment. The mechanisms involved in the persistence of *Salmonella* Enteritidis in egg white are likely to be complex. To address this issue, a microarray-based transposon library screen was performed to identify genes necessary for survival of *Salmonella* Enteritidis in egg white at chicken body temperature. The majority of identified genes belonged to the lipopolysaccharide (LPS) biosynthesis pathway. Additionally, we provide evidence that the serine protease/heat shock protein (HtrA) appears essential for the survival of *Salmonella* Enteritidis in egg white at chicken body temperature.

Introduction

Salmonellosis is one of the most important foodborne threats to human health worldwide, with eggs and egg products being the main food vehicle for *Salmonella* Enteritidis (EFSA, 2012, Braden, 2006). Contamination of eggs with *Salmonella* Enteritidis most likely occurs in the reproductive tract during egg formation (Gantois *et al.*, 2009a, Gast and Holt, 2000a, Keller *et al.*, 1995). Whilst the serotype Typhimurium has also been shown to contaminate eggs during their formation in the reproductive tract, it is almost exclusively *Salmonella* Enteritidis strains that are found in eggs post lay (Keller *et al.*, 1997). The prime contamination site for *Salmonella* Enteritidis in the eggs of naturally infected hens is the egg white (Humphrey *et al.*, 1991b), a matrix equipped with multiple antimicrobial substances to prevent bacterial contamination. Although the survival of *Salmonella* Enteritidis in egg white is superior to other serotypes (Gantois *et al.*, 2008b, Clavijo *et al.*, 2006, De Vylder *et al.*, 2013), the strategy used by this serotype to do so remains unclear. Nevertheless, several studies have been performed to identify *Salmonella* Enteritidis genes involved in this process. Genes that are expressed in whole eggs at room temperature have been identified using an '*in vivo'* expression technology screening. This study showed transcriptional activation of the lipopolysaccharide (LPS) biosynthesis gene *rfbH* in whole eggs and demonstrated that a *rfbH* deletion mutant was growth restricted in egg white at room temperature, while growth in yolk was not affected (Gantois *et al.*, 2009b). Lu *et al.* (Lu *et al.*, 2003) demonstrated that DNA repair enzymes (YafD and XthA) are necessary for the survival of *Salmonella* Enteritidis in egg white at 37°C. Mutants that were more susceptible to egg white at 37°C were identified using a transposon mutant library (Clavijo *et al.*, 2006). The majority of the mutants identified were in genes involved in cell wall structure and function or nucleic or amino acid metabolism. While survival at room temperature is of importance because of the storage conditions of eggs (refrigerator or room temperature), studies at 37°C are not practically relevant, because the chicken body temperature is 42°C. Data regarding survival strategies of *Salmonella* Enteritidis in egg white at 42°C are limited. Only recently, Coward *et al.* (2013) demonstrated the involvement of both long and very long LPS O-antigen chains. Identification of genes involved in egg white survival at chicken body temperature may contribute significantly to the understanding of the behavior of *Salmonella* Enteritidis in the albumen of forming eggs. For this reason, a genome-wide screen, using a microarray-based tracking strategy, identifying genes necessary for survival of *Salmonella* Enteritidis in egg white at chicken body temperature, was performed. In addition, two of the identified genes (*rfaI* and *htrA*) were deleted to confirm the phenotype in egg white survival assays.

Materials and methods

Bacterial strains and growth conditions

An isogenic streptomycin resistant variant of the *Salmonella* Enteritidis 147 strain (147^{str}), originally isolated from egg content, was used (Methner *et al.*, 1995). Bacteria were cultured aerobically at 37°C in Luria-Bertani broth (LB) (Sigma-Aldrich, St Louis, MO, USA) with agitation. When required, media were supplemented with antibiotics (Sigma-Aldrich, St Louis, MO, USA).

Construction of transposon insertion library

A *Salmonella* Enteritidis 147^{str} transposon insertion library was constructed according to the method previously described (Badarinarayana *et al.*, 2001, Lawley *et al.*, 2006, Chan *et al.*, 2005). In a first step, the pJA1 plasmid was transferred to the *Escherichia coli* (*E. coli*) SM10 λ*pir* strain by electroporation and maintained under ampicillin (100 µg/ml) selective pressure. The pJA1 suicide vector contains IS10 inverted repeats flanking a kanamycin resistance cassette with an adjacent, outward-directed T7 transcriptional promoter. In addition the plasmid harbors a mini-Tn10 transposase under control of an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible $LacI^q/P_{tac}$ promoter and a RP4 mob region for conjugation. A standard overnight conjugation reaction, in the presence of IPTG (Sigma-Aldrich, St Louis, MO, USA) but without antibiotics, was performed between the *E. coli* SM10 λ*pir* and the *Salmonella* Enteritidis 147^{str} strain. Under influence of IPTG the transposable element, containing a kanamycin resistance gene and T7 promoter, was excised from the pJA1 plasmid and integrated randomly into the genome of *Salmonella* Enteritidis 147^{str}. The next day, all colonies were scraped from the plate into 10mM $MgSO_4$ (Sigma-Aldrich, St Louis, MO, USA), and dilutions were plated onto LB agar plates containing 200 µg/ml streptomycin and 30 µg/ml kanamycin to select for successfully transposed *Salmonella* Enteritidis 147^{str} mutants. About 50,000 mutants, all harboring an insertion of the transposable cassette at different positions in the genome, were collected and stored in 15% glycerol (Sigma-Aldrich, St Louis, MO, USA) solution at -80°C.

Negative selection of transposon insertion library in egg white

Shells of freshly laid eggs were rinsed in lugol solution (Sigma-Aldrich, St Louis, MO, USA), and decontaminated by immersion in disolol (Chem-Lab, Zedelgem, Belgium). Egg albumen

was aseptically separated from egg yolk, collected in a sterile receptacle recipient and mixed for 5 min using a magnetic stirrer. The transposon library, which had been growing for 7 hours at 37°C with agitation in LB broth supplemented with streptomycin (200 µg/ml) and kanamycin (30 µg/ml), was centrifuged and resuspended in Hanks Buffered Salt Solution (HBSS) (Life Technologies, Bleiswijk, The Netherlands). Bacterial cell counts were determined by serial dilutions on LB agar plates. The library was stored at 4°C until the next day and further diluted in HBSS to a concentration of 1 x 10^8 cfu/ml. 500 µl of the bacterial suspension was added to 4.5 ml of egg albumen, to obtain a final concentration of approximately 1 x 10^7 cfu/ml egg albumen. Inoculated egg albumen was incubated at 42°C for 24 hours after which the surviving bacteria were recovered by addition of 45 ml LB broth and grown for 7 hours at 37°C with agitation. In this way, 5 independent experiments were performed.

Microarray-based comparison of initial and egg white selected transposon library

Genomic DNA (gDNA) of the input library (grown for 7 hours at 37°C in LB broth with streptomycin) and the egg white selected bacterial pool (output library) was isolated by phenol-chloroform extraction. The gDNA (10 µg) was digested with HinP1I (New England Biolabs, Ipswich, MA, USA) and 2 µg was ligated to the Y-linkers described by Tavazoie and Church (Tavazoie and Church, 1998). A PCR, with a linker and transposon specific primer, was performed as described by Badarinarayana *et al.* (2001). One µg of PCR product was used in an *in vitro* transcription reaction using a T7 polymerase megascript kit (Ambion, Austin, Texas, USA), and thus transcribing the region adjacent to the T7 promoter. RNA was purified by lithium chloride precipitation and Cy5-labeled in a reverse transcription reaction with random hexamer primers and Klenow fragment. Labelled cDNA together with Cy3-labelled genomic DNA of the wild type *Salmonella* Enteritidis strain as a reference were hybridized on the SALSA microarray containing PCR fragments of all the ORFs of Typhimurium and Enteritidis. Two microarrays were hybridized for each biological replicate of the input or output library. Microarray slides were scanned on an axon 4000A scanner (Axon. Instruments, Foster City, CA, USA). Spots showing a reference signal lower than the background plus 2 standard deviations or obvious blemishes were excluded from subsequent analysis. Local background was subtracted from spot signals and fluorescence ratios were calculated using GenePix version 1.4 software (Agilent, Santa Clara, CA, USA). To compensate for unequal dye incorporation or any effect of the amount of template, data centering was performed by bringing the median natural logarithm of the ratios for each group of spots printed by the same pin to zero. Data that

passed the quality controls were analyzed using Genespring version GX7.3 software (Agilent, Santa Clara, CA, USA). Signal values of the output library were normalized against those of the input library and used to identify mutants for which the gene value had at least a 2-fold decrease (fold difference < 0,5) after egg white selection compared to the input library grown in LB. Significance of the centered data at $p \leq 0,05$ was determined using a parametric-based statistical test adjusting the individual p-value with the Benjamini and Hochberg false discovery rate multiple test correction. Microarray protocols were performed as previously described (Hautefort *et al.*, 2008) and are shown in detail at http://www.ifr.ac.uk/safety/microarrays/protocols.html)

Construction of deletion mutants

Deletion mutants of the *rfaI* and *htrA* genes were constructed in the *Salmonella* Enteritidis 147^{str} strain using the one-step inactivation method of Datsenko and Wanner (2000). A kanamycin resistance cassette, flanked by FRT-sites, was amplified from the pKD4 helper plasmid using P-primers (Table 3.4.1) that had a 50bp extension at the 5' side of the pKD4 specific primers, homologous with the flanking region of the target gene. The resulting PCR product was used for recombination on the *Salmonella* Enteritidis 147^{str} chromosome using the pKD20 helper plasmid encoding the λ Red system, promoting recombination between the native and PCR adjusted antibiotic resistance cassette. Recombinant clones were selected on kanamycin containing plates and replacement of the target gene by the resistance cassette was confirmed by PCR. The *htrA* deletion was P22-transduced into a new 147^{str} background (Gemski and Stocker, 1967). This step was not performed for the *rfaI* mutant as this deletion mutant was no longer P22 sensitive. Later, the antibiotic resistance cassette was eliminated using the pCP20 helper plasmid, encoding the FLP-recombinase mediating recombination between the FRT-sites flanking the kanamycin resistance cassette. Finally, PCR and sequence analysis was performed to confirm complete gene deletion of the genes from start to stop codon without the occurrence of any polar events.

Table 3.4.1: Primers used to create deletion mutants

Primer	Sequence
***rfaI*-P1**	5'-TTCAGCTATTTCTATCTCAGGAAATGAATCCATTACATCACCTATGGGTTTGTGTAGGCTGGAGCTGCTTC-3
***rfaI*-P2**	5'-TTTAAAAATTTTAATAATGCAATATTCTCGAAATTACAAAAGTGATCACTCATATGAATATCCTCCTTAG-3'
***htrA*-P1**	5'-TCTGACGTACACAGCAATTTTGCGTTACCTGTTAATCGAGATTGAAACACTGTGTAGGCTGGAGCTGCTTC-3'
***htrA*-P2**	5'-TTCACAGAAAAGTGTTGCCCCCTTCCGTGGTGGAAGGGGGACAAAGGTGACATATGAATATCCTCCTTAG-3'

Survival in egg white at 42°C

To compare the egg white survival potential of the mutants with that of the wild type, all strains were grown overnight in the presence of streptomycin (100 µg/ml). Cultures were centrifuged for 10 min at 4000 rpm and bacterial pellets were resuspended in HBSS. Bacterial cell counts were determined by plating 10-fold serial dilutions onto LB agar plates. Cultures were stored at 4°C until the next day when they were diluted to 10^6 cfu/ml. Subsequently, 50 µl of these cultures were mixed with 450 µl egg white, prepared as previously described (De Vylder *et al.*, 2013). Suspensions were incubated for 24 hours at 42°C, where after 200 µl was plated on streptomycin (100 µg/ml) containing agar plates. Plating is necessary to obtain quantitative data on bacterial cell numbers. Three independent experiments were performed, with three repeats per experiment. Additionally, growth of the wild type and mutants in tryptic soy broth (TSB) (Oxoid, Basingstoke, UK) was evaluated at 37 and 42°C. On cultures were diluted to 10^5 cfu/ml and bacterial concentrations were determined by OD_{550} measurement and by serial dilutions on LB agar plates at different time points.

Results

Genes important for egg white survival identified by microarray-based negative selection strategy

A transposon insertion library, comprising approximately 50,000 mutants, was used to identify *Salmonella* Enteritidis genes that are essential for the survival in egg white at 42°C. Mutants with a disruption in a gene necessary for egg white survival at 42°C can no longer survive in this environment and thus these genes will show reduced representation in the egg white selected bacterial pool as compared to the initial transposon library that was used for egg white inoculation. When comparing the egg-white selected transposon library with the initial library, 23 genes were reduced significantly with at least a 2-fold (fold difference < 0.5) (Table 3.4.2). Sixteen of these genes are involved in LPS biosynthesis, including 11 genes of the *rfb* locus. This in addition to a penicillin binding protein (*ampH*), a biotin synthase (*bioB*), a DNA-binding transcriptional regulator involved in sugar metabolism (*fruR*), a protease Do precursor/heat shock protein (*HtrA*) and a *Salmonella* Enteritidis specific gene (SEN1974b) which is part of the region of difference (ROD) 21 cluster and only present in the genome of *Salmonella* Enteritidis and *Salmonella* Gallinarum but not *Salmonella* Typhimurium (Thomson *et al.*, 2008).

Finally, two plasmid encoded genes (*pSENV traK/pSELA5 traE* and *pSENV_080/SELA5_p0075/Vet* gene) were picked up.

Table 3.4.2: Overview of transposon inserted mutants that are defective in egg white survival after 24h at 42°C.

Gene name	Function	Fold difference (p-value)
rfbA	Glucose-1-phosphate thymidylyltransferase	0.266 (1.93E-04)
rfbB	dTDP-glucose 4,6 dehydratase	0.255 (1.61E-05)
rfbC	dTDP-4, deoxyrhamnose 3,5 epimerase	0.130 (2.29E-05)
rfbD	Putative RBS for rfbD	0.332 (2.32E-04)
rfbF	Glucose-1-phosphate cytidylyltransferase	0.109 (2.14E-03)
rfbI	CDP-6-deoxy-delta 3,4 glucoseen reductase	0.371 (1.34E-04)
rfbK	Phosphomannomutase	0.189 (5.27E-05)
rfbM	Mannose-1-phosphate guanylyltransferase	0.276 (2.14E-03)
rfbN	Rhamnosyl transferase	0.171(1.63E-05)
rfbP	Undecaprenol-phosphate	0.365 (7.28E-03)
rfbU	Mannosyl transferase	0.200 (3.51E-04)
rfaI	Lipopolysaccharide 1,3 -galactosyltransferase	0.244 (8.40E-04)
rfaJ	Lipopolysaccharide 1,2-glucosyltransferase	0.232 (2.63E-04)
rfaL	O-antigen ligase	0.487 (3.01E-03)
rfe	Undecaprenyl-phosphate N-acetylglycosaminyltransferase	0.299 (9.48E-04)
rfc	Putative O-antigen polymerase	0.460 (5.08E-05)
ampH	Penicillin binding protein	0.223 (2.79E-06)
bioB	Biotin synthase	0.419 (8.53E-04)
htrA	Putative periplasmic serine protease Do	0.341 (1.57E-03)
fruR	Fructose repressor	0.461 (2.16E-02)
pSENV_080	Putative DNA polymerase III epsilon subunit	0.440 (5.76E-04)
traK	Putative conjugative transfer	0.473 (2.96E-03)
PT4-0182	Unknown function	0.474 (2.46E-03)

Survival of rfaI and htrA mutants in egg white at chicken body temperature

To confirm the role of a selection of genes in the survival of *Salmonella* Enteritidis in egg white, deletion mutants were constructed and evaluated for their potential to survive in egg white. In this study we chose to study LPS involvement by deletion of the *rfaI* gene, as the enzyme it encodes (lipopolysaccharides 1,3 galactosyltransferase) catalyzes an early step in LPS biosynthesis. Consequently, *rfaI* mutants most likely harbor a defective outer core and O-antigen structure. Additionally, we also studied the role of the serine protease/heat shock protein gene *htrA*. Deletion mutants in these 2 genes were constructed. The mutants and the wild type strain were incubated for 24 hours at 42°C in egg white after which the remaining bacterial cell counts were determined. It was found that bacterial cell counts/ml for the wild type strain dropped from approximately log 5 to log 2.35 whilst neither of the two mutant strains could be recovered from egg white after the incubation period and this with a detection limit of 5 cfu/ml. In addition, none of the mutants exhibited a growth defect in TSB at 37 nor at 42°C (data not shown). The data thus show that both the *rfaI* and *htrA* genes are essential for the survival of

Salmonella Enteritidis in egg white at the chicken body temperature, and confirm the data from the microarray-based negative selection screening.

Discussion

For many years *Salmonella* Enteritidis has been the most predominant serotype linked to egg-borne cases of human salmonellosis (Braden, 2006, EFSA, 2012). In principle, eggs can be contaminated with *Salmonella* Enteritidis on the outer shell surface or internally. Internal contamination can result from penetration through the eggshell (Messens, 2005, De Reu *et al.*, 2006) or by colonization of the reproductive tract, with subsequent incorporation into the forming egg (Keller *et al.*, 1995, Miyamoto *et al.*, 1997, Okamura *et al.*, 2001a). In comparison to outer shell contamination and membrane penetration, high-level reproductive tract colonization seems to be a trait more or less specific for the serotype Enteritidis. *Salmonella* Typhimurium is also capable of colonizing the reproductive tract and contaminating eggs during their formation (Keller *et al.*, 1997), but this serotype is rarely found in eggs post lay. It has been suggested that *Salmonella* Enteritidis migrates into the chicken egg through the upper oviduct in association with the egg white (Gast and Beard, 1990b, Hoop and Pospischil, 1993, Cogan *et al.*, 2004, Keller *et al.*, 1995), containing many antimicrobial components. Consequently, it could be hypothesized that *Salmonella* Enteritidis developed the potential to withstand the antibacterial activities of egg white better than *Salmonella* Typhimurium at the chicken body temperature. The validity of this hypothesis has recently been supported by in vitro experiments (De Vylder *et al.*, 2013). However, until now the exact mechanisms exploited by *Salmonella* Enteritidis to cope with this hostile antimicrobial environment at chicken body temperature are still not completely understood. In the present study, 23 genes with a potential role in egg albumen resistance at 42°C were identified (Table 2) using a genome-wide microarray-based transposon insertion library screening. Sixteen of these genes are involved in biosynthesis of the outer core or O-antigen part of the outer membrane lipopolysaccharide structure. It has been demonstrated that the amount of O-antigen polysaccharide is doubled in a virulent *Salmonella* Enteritidis strain, compared to an avirulent isolate (Rahman *et al.*, 1997). LPS biosynthesis genes were also identified in previous studies searching for *Salmonella* Enteritidis genes needed for egg white survival at room temperature (Gantois *et al.*, 2009b) or at 37°C (Clavijo *et al.*, 2006). Coward *et al.* (2013) have recently shown that the lack of a very-long O-antigen chain diminishes the ovary and oviduct colonization capacities of a *Salmonella* Enteritidis strain leading to *Salmonella* Enteritidis free eggs. These authors also demonstrated

that the lack of long and very long O-antigen side chains severely diminished the potential of *Salmonella* Enteritidis to survive in egg white at the chicken body temperature. Additionally, it has been shown that *Salmonella* Enteritidis strains isolated from eggs are characterized by their ability to produce high molecular weight LPS structures (HMW LPS) (Guard-Petter *et al.*, 1997). In contrast to *Salmonella* Enteritidis, *Salmonella* Typhimurium is less able to produce these HMW LPS structures (Guard-Bouldin *et al.*, 2004). Consequently, it could be hypothesized that the production of HMW LPS increases the resistance of *Salmonella* Enteritidis to egg white antimicrobial factors. This could explain why, although both serotypes are found in the forming egg of infected hens during passage of the egg through the oviduct, mostly the serotype Enteritidis is detected in eggs post lay (Keller *et al.*, 1997). LPS acts as a first line bacterial defense mechanism and is able to protect bacteria against harmful substances such as lysozyme, which mediates peptidoglycan hydrolysis. It is clear that an aberrant LPS production enhances the accessibility of the cytoplasmic membrane for antimicrobial substances.

In addition to LPS biosynthesis genes, our study shows that the protease Do precursor protein/ heat shock protein gene *htrA*, is essential for the survival of *Salmonella* Enteritidis in egg white at 42°C. HtrA is a stress-induced protein involved in the clearance of damaged and misfolded proteins in the periplasm. Protein misfolding results from exposure to a myriad of stresses such as high temperatures, extreme pH values, nutrient starvation and oxidative stress (Basnak'ian *et al.*, 2001). HtrA possesses chaperone activity at low temperatures, while the protease activity takes over at high temperatures (Spiess *et al.*, 1999). It is essential for the survival of *E. coli* at temperatures above 42°C (Lipinska *et al.*, 1989) and in conditions of oxidative stress (Skorko-Glonek *et al.*, 1999). For the *Salmonella* Typhimurium *htrA* mutant growth was only inhibited once the temperature reached 46°C (Lewis *et al.*, 2009), but the mutant was sensitive to oxidizing agents and was less able to survive within macrophages (Baumler *et al.*, 1994, Johnson *et al.*, 1991, Humphreys *et al.*, 1999), resulting in a decreased virulence potential in mice (Humphreys *et al.*, 1999, Chatfield *et al.*, 1992). Exposure to the stressful environment of the hen's egg white at the chicken body temperature of 42°C combined with a high pH and a nutrient shortage might cause accumulation of misfolded proteins in the periplasm of the *htrA* deletion mutant, which eventually may lead to bacterial cell death.

This study also revealed the involvement of the ROD21 PT4-0182 gene, in the survival of *Salmonella* Enteritidis in egg white. As the ROD21 gene cluster is absent in the genome of *Salmonella* Typhimurium, it could be hypothesized that this island can facilitate the survival of

Salmonella Enteritidis in egg white. Several studies demonstrated that a *Salmonella* Enteritidis ROD21 mutant strain was less efficient in liver and spleen colonization of mice, compared to the wild type (Quiroz *et al.*, 2011, Silva *et al.*, 2012). A decreased spleen colonization of a *Salmonella* Enteritidis ROD21 mutant was also observed in adult laying hens, while no difference in ovary or oviduct colonization could be observed (Coward *et al.*, 2012). The role of the PT4-0182 gene in egg white survival at 42°C of *Salmonella* Enteritids needs further investigation.

Other genes identified in our transposon screening as being potentially important for egg white survival include *bioB* which encodes a biotin synthase. Biotin is an essential cofactor for various enzymes and is necessary both for the developing chicken embryo and for bacterial growth. In the egg white biotin is bound to a carrier proteins such as avidin to reduce its availability for bacterial growth (White *et al.*, 1992). In response, bacteria have developed their own biotin synthesis pathway and it seems logical that a deficiency in biotin synthesis would reduce bacterial fitness in an environment with low free biotin levels. Another gene identified in the transposon screen was, *fruR*, a regulator of the central carbon metabolism. It was previously shown that deletion of *fruR* in *Salmonella* Typhimurium reduced its virulence potential in mice.

The ampicillin binding protein, *ampH*, was also picked up in this screening. In *E. coli*, *ampH* is a low molecular-mass penicillin binding protein (PBP), probably involved in peptidoglycan remodeling and recycling (Gonzalez-Leiza *et al.*, 2011). Although individual and combined low molecular weight PBP mutants of *E.coli* didn't seem to experience any growth defect in rich laboratory medium (Denome *et al.*, 1999), a firm peptidoglycan structure might be necessary for the survival of *Salmonella* Enteritidis in egg white.

Finally, two virulence plasmid encoded proteins (*pSENV traK/pSELA5 traE* and *pSENV_080/SELA5_p0075/Vet* gene) were identified to be important for egg white survival at 42°C. The function of these genes in egg white survival is difficult to predict.

This study could not confirm the involvement of DNA damage repair systems (*yafD* and *xthA*) which were previously identified by Lu *et al.* as being essential for the survival of *Salmonella* Enteritidis in egg white at 37°C.

Conclusion

In conclusion, we identified 23 genes with a potential function in the survival of *Salmonella* Enteritidis in egg white of which most are genes involved in LPS biosynthesis. Additionally, using *in vitro* egg white survival assays, we provide evidence for the role of the LPS biosynthesis gene *rfaI* and the protease/heat shock protein gene *htrA* in survival of *Salmonella* Enteritidis in egg white at chicken body temperature of 42°C. It could thus be hypothesized that LPS structures and stress induced proteins might be necessary for *Salmonella* Enteritidis to cope with the antimicrobial environment of the egg white at the chicken body temperature of 42°C.

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4 General discussion

General discussion

An increase in the number of human *Salmonella* Enteritidis cases due to contamination of chicken eggs and egg products has been observed since the mid 1980s as this serotype had acquired the ability to colonize the chicken reproductive tract, from where it could be easily incorporated into the forming egg (Rodrigue *et al.*, 1990). It is generally accepted that *Salmonella* Enteritidis colonizes the chicken reproductive tract more efficiently than most other serotypes (Okamura *et al.*, 2001a, Okamura *et al.*, 2001b, Gantois *et al.*, 2008b) and that the serotype Entertidis has developed an enhanced potential to survive in the highly antimicrobial egg white of the forming egg (Keller *et al.*, 1997, De Vylder *et al.*, 2013). Until now, the molecular mechanisms underlying the *Salmonella* Enteritidis conquest of the chicken reproductive tract and egg contamination are still unknown, and could be related to gene content changes. However, no significant consistent differences in gene content were observed between 60-year-old and more recent *Salmonella* Enteritidis strains (Porwollik *et al.*, 2005). Alternatively, it must be mentioned that the *Salmonella* Enteritidis pandemic was preceded by the endemic presence of the avian adapted *Salmonella enterica* serotype Gallinarum in poultry, until its eradication by the 1970s. Consequently, some authors have speculated that presence of *Salmonella* Gallinarum might have inhibited *Salmonella* Enteritidis colonization in poultry by competitive exclusion and cross-immunity, while *Salmonella* Gallinarum eradication from these animals led to the availability of this ecological niche for *Salmonella* Enteritidis (Rabsch *et al.*, 2000). Even then, strains from this serotype must have some special characteristics making them superior in oviduct colonization and egg contamination.

Salmonella Enteritidis bacteria have been detected immunohistochemically inside the tubular gland cells of the magnum and isthmus of laying hens after natural and experimental infection (Hoop and Pospischil, 1993, Keller *et al.*, 1995, De Buck *et al.*, 2003). It might be suggested that the bacteria reside intracellularly in the reproductive tract to escape the local defense mechanisms. As *Salmonella* Enteritidis is present intracellulary inside the tubular gland cells of the oviduct, it could be hypothesized that bacteria are transported out of these cells in association with their secretory granules. Association of *Salmonella* Enteritidis with these granular secretions would indicate a long term exposure of the bacteria to the antimicrobial content of these granules, and thus the antimicrobial egg white components. Although *Salmonella* Enteritidis is superior in tolerating this matrix at the chicken body temperature during a limited period of time, it appears to experience difficulties when the exposure time at

this high temperature is prolonged. This was demonstrated by Guan *et al.* (2006) as *Salmonella* Enteritidis organisms could no longer be detected in egg white after a 96 hours incubation. Additionally, confocal laser scanning microscopy could not demonstrate the presence of *Salmonella* Enteritidis inside these granules (Appia–Ayme personal communication). Finally, the temporal clustering with which *Salmonella* positive eggs are found suggests that *Salmonella* Enteritidis might sense some specific environmental cues which in turn induce their secretion and incorporation into the forming egg. It was suggested that stress might be of relevance in this process (Humphrey *et al.*, 1989, Humphrey, 2006) and this in accordance with several observations: pre-treatment of chickens with the stress hormone norepinephrine before *Salmonella* Enteritidis challenge caused a dramatic increase in caecal colonization and systemic spread to the liver (Methner *et al.*, 2008). Introduction of new young chickens and water and feed withdrawal resulted in a short term increased shedding in chickens which were previously infected with *Salmonella* Enteritidis (Nakamura *et al.*, 1994). It was also demonstrated that feed removal to induce molting decreases the resistance of hens to *Salmonella* infection, resulting in an increased severity of infection, an increased intestinal shedding and a higher risk for *Salmonella* Enteritidis positive eggs during the first week after molting (Moore and Holt, 2006, Rostagno, 2009).

Whatever the environmental conditions that stimulate *Salmonella* Enteritidis colonization of the oviduct and contamination of eggs, and whatever the route taken by the bacteria to end up inside the egg, it is clear that the chicken reproductive tract and the environment of the forming egg are both highly stressful for bacteria. This is due to the high chicken body temperature but also because of the presence of the local innate and adaptive immunity. In this work we aimed to elucidate the molecular mechanisms exploited by *Salmonella* Enteritidis to colonize the reproductive tract and to contaminate the forming egg.

4.1 Colonization of the reproductive tract by genetic variance

As *Salmonella* Enteritidis might reside intracellularly, it is not surprising that the transposon insertion library, used to identify genes necessary for persistence inside the reproductive tract pointed to the major determinants of *Salmonella* virulence, SPI1 and 2. Despite the fact that effectors of these islands are known to modulate the chicken immune response (Li *et al.*, 2009, Ebers *et al.*, 2009), these islands are probably not of specific importance to explain the superior

ability of *Salmonella* Enteritidis to colonize the reproductive tract as they are present in all virulent *Salmonella* serotypes.

A comparison between the complete genome sequences of *Salmonella* Enteritidis and Typhimurium has led to the identification of genes which are present in the genome of *Salmonella* Enteritidis but that are absent or degenerated in the genome of *Salmonella* Typhimurium and vice versa. The majority of these specific coding sequences (CDS) is clustered and therefore referred to as regions of difference (ROD) (Thomson *et al.*, 2008). Genes belonging to ROD9, 21 and 40, which are present in *Salmonella* Enteritidis but absent in Typhimurium, were identified during our transposon library screening (chapter 3.2) as genes that are likely to be necessary for or involved in the persistence inside the reproductive tract. Genes belonging to these islands are also found in *Salmonella* Gallinarum (Thomson *et al.*, 2008). *Salmonella* Gallinarum is a host adapted serotype causing fowl typhoid which is generally presented as a systemic disease with a high mortality rate. Infection with *Salmonella* Gallinarum may occur through the fecal-oral route or by vertical transmission as a consequence of egg contamination, indicating that this serotype is also able to colonize the reproductive tract and to contaminate eggs (Uzzau *et al.*, 2000). Additionally, it must be mentioned that the serotype Gallinarum is most likely a direct evolutionary descendant of *Salmonella* Enteritidis (Thomson *et al.*, 2008). One way to explain how these serotypes developed the potential to colonize the reproductive tract could be by the presence of unique sets of genes. The identified RODs might interfere with the potential of the serovar Enteritidis to colonize the reproductive tract and eventually contaminate eggs. As the majority of the genes identified belonged to the ROD21 gene cluster, a complete deletion mutant of this island was constructed in *Salmonella* Enteritidis and used in an intravenous and intra-oviduct infection trial to compare the reproductive tract colonization potential of this mutant with that of the wild type strain. A colonization defect for the ROD21 mutant could not be observed (own unpublished data). Additionally, individual deletion mutants of five genomic islands present in *Salmonella* Enteritidis but absent in Typhimurium (ROD9, ROD13, ROD17, ϕSE20 and ROD21) were constructed by Coward *et al.* (2012) and used in a chicken *'in vivo'* trial by oral inoculation. None of these islands seemed to be involved in colonization of the reproductive tract, while all mutants were less recovered from the spleen after 14 days of infection. Although no difference in reproductive tract colonization could be observed during these trials, it could be that redundancy exists between these gene clusters and that strains with multiple deletions are necessary to study the functions of these RODs in oviduct colonization.

Additionally, it is possible that small changes in gene sequences (SNPs) are responsible for the reproductive tract tropism and/or the ability to survive in egg albumen (Guard-Bouldin, 2006). The importance of SNPs was recently demonstrated by Van Immerseel *et al.* (2013), as a few single-base-pair substitutions made the difference between the *Salmonella* Gallinarum SG9R vaccine and a systemic disease causing field strain. Additionally; pathogenicity might be altered as a consequence of gene deletion or a loss of gene function. For example, the genome sequence of *Salmonella* Enteritidis and Gallinarum are highly similar, though mutations in 5 of 50 motility genes of *Salmonella* Gallinarum made the difference between a motile *Salmonella* Enteritidis strain and a non-motile *Salmonella* Gallinarum strain (Thomson *et al.*, 2008). It is thus possible that mutations in genes exist that make the serotype Enteritidis more capable of colonizing the chicken reproductive tract and the egg. Ultimately, differences in gene expression profile between various *Salmonella* serotypes could lead to an enhanced interaction of *Salmonella* Enteritidis with the laying hen compared to other serotypes. Nevertheless, to our knowledge no information regarding the global gene expression in the reproductive tract or the forming egg are available for different *Salmonella* serotypes.

4.2 Survival strategies in the reproductive tract and inside the forming egg

During colonization of the reproductive tract and after contamination of the forming egg, *Salmonella* Enteritidis encounters an arsenal of environmental stresses, including oxidative and heat stress, DNA damaging mechanisms, nutrient starvation and numerous antimicrobial peptides. The genome wide IVET (chapter 3.1 and 3.3) and transposon library (chapter 3.2 and 3.4) screening methods used in this thesis showed that *Salmonella* Enteritidis utilizes several survival strategies to protect itself from the harmful environment of the chicken reproductive tract and the forming egg white. Identified genes necessary for these survival mechanisms are discussed below.

4.2.1 Protection against heat, oxidative stress and DNA damage

The existence of peroxidase activity (Yoshimura *et al.*, 2006) together with the presence of endo- and exo-nucleases (Lu *et al.*, 2003) at the chicken body temperature of 42°C might pose a serious threat on *Salmonella* Enteritidis bacteria colonizing the oviduct tissue and

contaminating the forming egg. In order to survive in these environments *Salmonella* Enteritidis might activate several stress management responses.

A previously performed IVET-screening identified the universal stress proteins (*usp*) A and B and the heat shock protein 33 (*hsp33* or *yrfI*) as being highly expressed inside the reproductive tract and in forming eggs (Gantois *et al.*, 2008a). The role for *uspA* and *uspB* in oviduct colonization and egg contamination was confirmed in this thesis (chapter 3.1), as promoters of both genes were activated after contact with egg white and a *uspBA* double mutant was less able to colonize the reproductive tissue and to contaminate eggs. This is probably because these proteins might modify the membrane composition and protect against DNA damage. The *yrfI* and the *hsp60* promoters were also detected in our IVET-screening in egg white at 42°C (chapter 3.3). Nevertheless, the role of these heat shock proteins in oviduct colonization and egg white survival remains to be confirmed. Subsequently, we also identified several stress related genes of which 2 (*ogt* and *yciG*; chapter 3.2) are shown to be activated by the alternative sigma factor σ^S (RpoS), involved in the survival of bacteria under starvation or stress conditions (Ibanez-Ruiz *et al.*, 2000). Although the role for *yciG* still remains a mystery it has been shown that *ogt* might be involved in oxidative stress repair as this O6-methylguanine-DNA transferase is responsible for the elimination of the O6-methyl group on guanine and thus prevents the mutagenesis of GC to AT (Yamada *et al.*, 1995). A single mutation in the *ogt* gene however had no effect on bacterial virulence in an oral or intraperitoneal mouse model. Decreased virulence was only seen after oral inoculation with a mutant devoid of 5 genes (*ogt*, *ada*, *tag*, *uvrA* and *mfd*) that were all involved in the repair of alkylation damage (Alvarez *et al.*, 2010). Additionally we identified the two genes known to be involved in the recovery of heat injured *Salmonella* Enteritidis, *htrA* and *hslV* (chapter 3.2) (Kobayashi *et al.*, 2005). As the *htrA* gene was also identified as important for survival in egg white in our transposon library screen (chapter 3.4), a deletion mutant of this gene was constructed and showed that this gene is indeed involved in survival of *Salmonella* Enteritidis during the formation of the egg.

4.2.2 Protection against nutrient starvation

In order to support the complete development of the embryo, the chicken egg must be provided with all essential nutrients. To prevent bacterial uptake and growth, many of these nutrients are bound to carrier proteins, such as avidin and the biotin binding protein (BBP) for biotin, and ovotransferrin for iron.

Biotin, also called vitamin H, vitamin B7 or coenzyme R, is a cofactor for different biotin-dependent carboxylases involved in fatty acid synthesis, leucin degradation and the amino acid metabolism in both pro- and eukaryotic cells. It is an essential cofactor for the developing embryo and is present in both egg white and yolk where it is bound respectively to avidin and BBP (White *et al.*, 1992). Nonetheless, bacteria have developed their own biotin synthesis pathway in which they produce the cofactor from pimeloyl-coA through four enzymatic steps. In the last step, dethiobiotin is converted to biotin by the biotin synthase encoded by *bioB* (Streit and Entcheva, 2003). The *bioB* gene had previously been demonstrated to be important for the replication of *Salmonella* Typhimurium inside macrophages (Shi *et al.*, 2009) and was subsequently identified as important in survival in egg white in our transposon library screening (chapter 3.4). Although a defined *bioB* deletion strain did not seem to experience any diminished survival after a 24 hour incubation period in the antimicrobial egg white at 42°C, it must be mentioned that the mutant had some difficulties to grow through the egg white drops in our agar spot assay (own unpublished data). It could thus be hypothesized that the *bioB* gene of *Salmonella* Enteritidis may be involved in bacterial replication after the egg is laid and when the vitelline membrane starts to degrade with the release of nutrients in a biotin limited environment rather than during the formation of the egg.

Additionally, we demonstrate that the outer membrane channel TolC and a combination of RND-pump inner membrane transporters are essential to overcome the iron deprivation caused by ovotransferrin (chapter 3.3). This is most likely a result of siderophore secretion through these channels, as *Salmonella* Typhimurium has been shown to react to iron restriction with the secretion of enterobactin and salmochelin (Crouch *et al.*, 2008). Enterobactin is encoded by the *ent* operon and transported through the inner membrane using the EntS and IroC channel (Crouch *et al.*, 2008), while passage through the outer membrane is mediated by the TolC channel (Bleuel *et al.*, 2005). A proportion of enterobactin is C- glycosylated by IroB encoded within the *iroA* gene cluster to form salmochellin, a siderophore which is transported by the IroC inner membrane channel and a yet unidentified outer membrane transporter (Crouch *et al.*, 2008). Our agar spot assay with ovotransferrin showed a growth inhibition defect for the *entB*, *entC* and *iroC Salmonella* Typhimurium deletion mutant, while this could not be observed for the *entS*, *iroB* or deletion of the entire *iroA* gene cluster (own unpublished data). These results are in line with a study performed by Kang *et al.* (2006), who demonstrated that a deletion in the *entF* gene impaired the egg white survival capacities of *Salmonella* Enteritidis. It could thus

be hypothesized that siderophores are essential to cope with the iron limiting conditions in egg white.

4.2.3 Protection against antimicrobial peptides

A decreased siderophore secretion caused by the deletion of the *tolC* gene cannot explain why this mutant experiences an enhanced lethality in egg white at 42°C, as the iron sequestering capacities of ovotransferrin are rather bacteriostatic than bactericidal. This means that CAMPs present in egg white might function in addition to or even collaborate with ovotransferrin to kill the *tolC* deletion strain. TolC functions as an outer membrane channel for the majority of the *Salmonella* multidrug efflux pumps (Horiyama *et al.*, 2010), which have been studied intensively for their role in antibiotic resistance, through efflux from the bacterial cell. It has been speculated that these pumps are mainly developed to allow the bacteria to survive in certain ecological niches and that antibiotic resistance is a by-product of their biological role (Piddock, 2006). It is thus tempting to speculate that the TolC outer membrane channel of *Salmonella* Enteritdis is necessary for the efflux of egg white antimicrobial components from the bacterial cell. Until now, no such egg white antimicrobial peptides could be demonstrated, but the hypothesis is further strengthened by the observation that the *baeR* promoter is activated after contact with egg white at the chicken body temperature (chapter 3.3). BaeR is part of the BaeSR two-component system which increases multidrug resistance by regulating efflux systems (Nishino *et al.*, 2007).

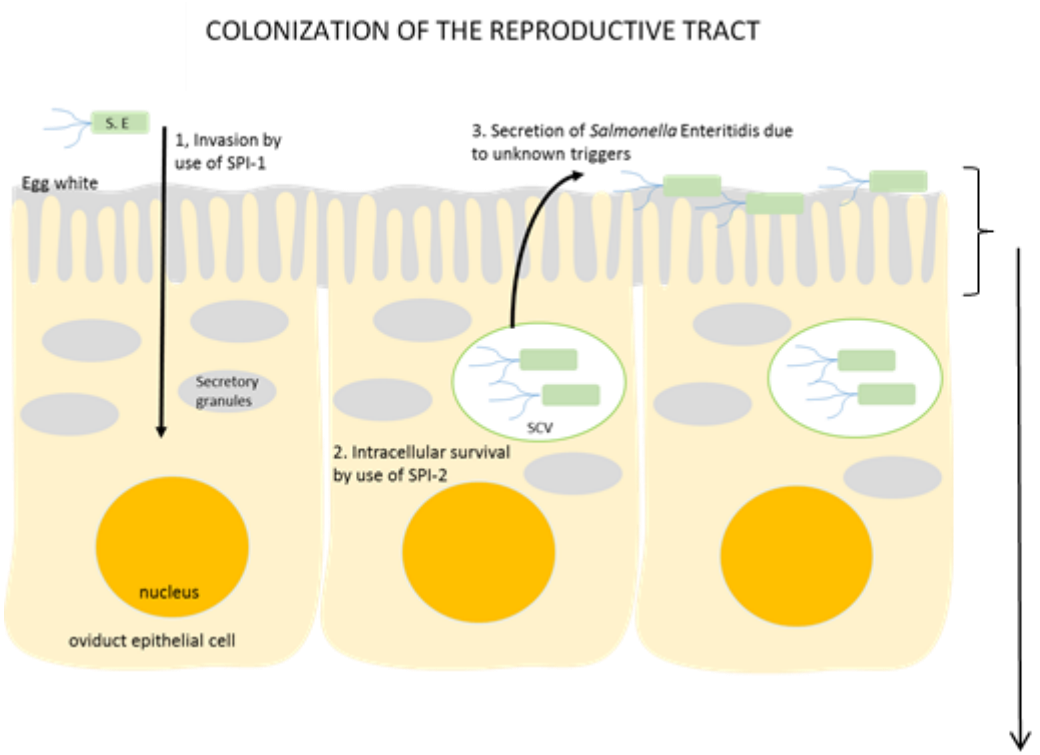
Additionally, it has been suggested that *Salmonella* might recognize CAMPs through the PhoPQ/PmrAB two-component regulatory systems (Bader *et al.*, 2003, Bader *et al.*, 2005, Richards *et al.*, 2012), which may in turn initiate modification of the LPS lipid A and core component but also O-antigen length by the *wzz* and *febE* genes (Chen and Groisman, 2013) leading to the formation of long (16-35 subunits) and very long (> 100 subunits) O-antigen chains. The role of LPS modification for oviduct colonization was detected in our transposon library screening, as the UDP-glucose 6-dehydrogenase (*ugd, udg, pagA* or *pmrE*) gene was identified (chapter 3.2). Gunn *et al.* (1998) showed that this gene is involved in lipid A modification with 4-aminoarabinose masking the negative charges and thus reducing the affinity for antimicrobial peptides. Additionally we provided evidence for a role of the LPS structure in egg white survival as a *rfaI* deletion strain was no longer capable of surviving in egg white at the chicken body temperature (chapter 3.4). Furthermore, the need for very long O-antigen synthesis was demonstrated by Coward *et al.* (Coward *et al.*, 2013), as a *febE* deletion

strain exhibited reduced colonization of the reproductive tissue. This phenotype could not be observed for the *wzz* deletion strain as removal of this gene even resulted in an increased frequency of egg contamination, a phenomenon which was also described by Parker *et al.* (2002). It was hypothesized that deletion of the *wzz* gene probably resulted in a higher production of very-long O-antigens, and that since these structures might have a higher contribution to the survival in egg white, their increased presence probably leads to a higher rate of egg contamination in the wzz deletion strain (Coward *et al.*, 2013). Furthermore, it must be mentioned that *Salmonella* Enteritidis strains, in comparison to *Salmonella* Typhimurium, have an increased potential to produce high molecular weight (HMW) LPS and that egg white is an excellent source for *Salmonella* Enteritidis strains producing these structures (Parker *et al.*, 2001).

4.3 Vertical transmission of *Salmonella* Enteritidis: A unifying hypothesis

Taking all the above into consideration, it is clear that *Salmonella* Enteritidis is a unique and intriguing serotype as it causes gastro-intestinal disease in a wide variety of animals and even humans, while it has adapted itself to persistently colonize the chicken reproductive tract and contaminates eggs. As the avian adapted *Salmonella* Gallinarum serotype is probably a descendent from *Salmonella* Enteritidis, it can be hypothesized that reproductive tract colonization by these serotypes arose before their divergence, while the development of their specific disease characteristics evolved afterwards. Consequently, the existence of RODs in *Salmonella* Enteritidis and Gallinarum but not Typhimurium might suggest a role for these genes in the adaptation of *Salmonella* Enteritidis and Gallinarum to the avian species. Although until now conflicting results regarding the function of these islands in reproductive tract colonization exist, further studies are needed to elucidate their exact role. Secondly, it can be assumed that *Salmonella* Enteritidis utilizes SPI1 and 2 to invade and survive inside the chicken oviduct epithelial cells and this probably to protect themselves from the hen's local immune system. Consequently, during periods of immune suppression, such as stress, *Salmonella* Enteritidis might be excreted from these cells and incorporated into the forming egg. During their persistence inside the epithelial cells and their incorporation into the forming egg *Salmonella* Enteritidis might activate some stress responses to further withstand the hostile environment of this ecological niche. For example, the high chicken body temperature, the presence of reactive oxygen species and other DNA damaging components might induce stress

response systems leading to activation of heat shock proteins and DNA repair mechanisms. Additionally, a dual role for multidrug efflux pumps was demonstrated during this thesis as these pumps are probably necessary for the secretion of siderophores to cope with the iron depletion and, although speculative at this point, as they might enhance the export of host antimicrobial components out of the bacterial cells. It is clear that resistance against antimicrobial peptides is also achieved by the LPS structures and most likely HMM OAg chains which may cause a steric structure on the bacterial membrane masking it for antimicrobial factors. Subsequently, once the eggs is laid *Salmonella* Enteritidis is capable of surviving for a prolonged period of time inside the egg white by use of its LPS-structure and some genes belonging to the ROD gene clusters. After about 3 weeks the vitelline membrane starts to lose some of its integrity, leading to leakage of yolk nutrients in the albumen and mobilization of *Salmonella* Enteritidis to the yolk. Once the perivitelline membrane is reached, *Salmonella* Enteritidis probably uses its curli fimbriae to attach to the vitelline membrane and to invade the egg yolk were it can easily multiply. Additionally, numerous metabolic genes were identified during this screening. Although identification of these genes might be caused by a disruption of an important metabolic pathway, some metabolic genes might also be participating in pathogenesis. This was recently demonstrated by Flahou *et al.* (2011) who showed that the γ-glutamyl transpeptidase of *Helicobacter suis* and *pylori* was involved in apoptosis and necrosis of gastric epithelial cells. A combination of differences in presence or function of all these virulence factors between *Salmonella* Enteritidis and other serotypes might enable the former to colonize the reproductive tract more efficiently than other serotypes. Additionally, further studies are necessary to determine whether these genes are restricted for colonization of the reproductive tract or rather if they are necessary for the overall pathogenesis of *Salmonella* Enteritidis in the chicken. An overview of the different confirmed virulence factors used by *Salmonella* Enteritidis is during the whole egg contaminating process is given in figure 4.3.1.



1. Invasion of *Salmonella* Enteritidis (S.E) in oviduct epithelial cells by SPI-1
2. Intracellular survival in *Salmonella* containing vacuole (SCV) by SPI-2
3. Release of *Salmonella* Enteritidis into the egg white of the forming egg as a consequence of unknow triggers
4. Attachment of *Salmonella* Enteritidis to oviduct secretions by type 1 fimbriae
5. *Salmonella* Enteritidis under attack of antimicrobials components such as lysozyme, ovotransferrin and its derived peptides, defensins and peroxidase. Membrane damage with influx of antimicrobial components leading to DNA damage and interuption of protein function
6. *Salmonella* Enteritidis response mechanisms leading to membrane reorganisation (*usp*), formation of long chain LPS (*wzy, febE, rfbH* and *rfaI*), DNA repair (*usp, yafD* and *xthA*) and efflux of siderophores and antimicrobial peptides (*tolC* and *MDR*-pumps)

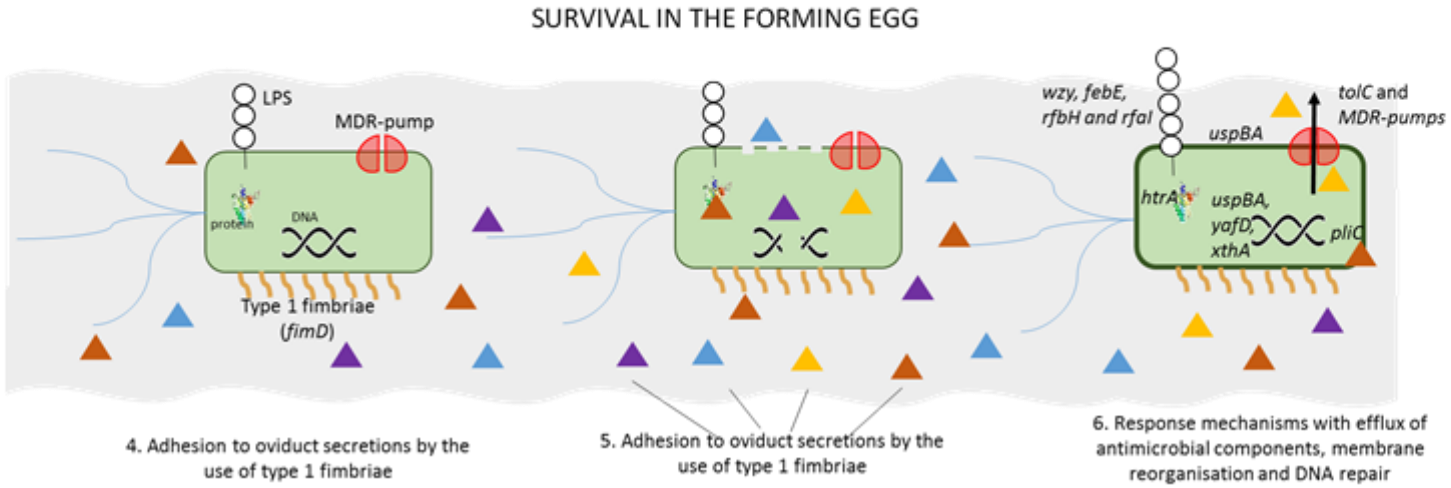


Figure 4.3.1: Summary of *Salmonella* Enteritidis pathogenesis to contaminate eggs

4.4 *Salmonella* control strategies

4.4.1 Vaccination

In order to constrain the *Salmonella* Enteritidis pandemic caused by contaminated eggs, several national control programs implemented vaccination of laying hens. The ideal vaccine should possess the following characteristics: a) reduce or prevent the intestinal colonization and thus fecal shedding and egg shell contamination and b) prevent systemic infection and decrease colonization of the reproductive tissues and thus reduce internal egg contamination. In Belgium, both killed inactivated and live attenuated vaccines are on the market. After intramuscular vaccination with the killed inactivated Nobilis® *Salmonella* ET vaccine, previously known as Nobilis® Salenvac or Nobilis® Salenvac T, a significant reduction in the positive tissue and faecal samples in addition to a decreased egg contamination after intravenous challenge with *Salmonella* Enteritidis was observed (Woodward *et al.*, 2002). Nevertheless, there is accumulating evidence that attenuated live vaccines are more immunogenic as they have the potential to induce a cell-mediated immune response. Live attenuated vaccines available in Belgium are the Avipro *Salmonella* vac®E, Avipro *Salmonella* vac®T and the Avipro *Salmonella* Duo, which are metabolic drift mutants. Oral vaccination with these vaccines at day 1, week 6 and 16 triggered the induction of specific antibody response and did not lead to egg contamination with these strains. Additionally, this vaccination protocol decreased internal organ colonization, including reproductive tract colonization and egg contamination by a challenge strain which was given intravenously at week 24 (Gantois *et al.*, 2006).

4.4.2 Towards a more resistant chicken breed

In addition to vaccination, additional measures to avoid the introduction of *Salmonella* and to reduce the infection pressure must be taken into account. The breeding and rearing of chickens which are more resistant to *Salmonella* infection might be an attractive option. Between-line differences have been demonstrated, as different lines of mature chickens infected with *Salmonella* Enteritidis exhibited different incidences of fecal shedding, organ colonization and egg contamination (Protais *et al.*, 1996). To identify host genes involved in this process, several chicken lines have been studied and a number of candidate genes, mainly involved in the innate and adaptive immunity, have been identified so far (Calenge *et al.*, 2010). Since *Salmonella* Enteritidis has developed a higher potential to survive in the forming egg at the chicken body temperature, difference in egg white proteins may be of particular interest. Avian embryonic

intestinal cell lines that exhibited a higher expression of the AvBD1 and 2 harbored less intracellular *Salmonella* Enteritidis bacteria after infection (Derache *et al.*, 2009), while genetic variation in AvBD11, 12 and 13 seems to be associated with the bacterial burden in caecal content (Hasenstein and Lamont, 2007).Nevertheless, although studies regarding other antimicrobial egg white components are currently missing, it can be assumed that also variations in their content might make a difference in the reaction of a chicken to a *Salmonella* Enteritidis infection. Consequently, genetic selection might generate a chicken breed which is more resistant to intestinal and systemic colonization of *Salmonella* Enteritidis and in which a higher concentration of antimicrobial peptides in the forming egg may kill *Salmonella* Enteritidis more efficiently.

4.5 In conclusion

To colonize the chicken reproductive tract and to survive inside the forming egg, *Salmonella* Enteritidis probably relies on a unique set of genes. The bacteria adequately orchestrate a joint collaboration between genes involved in the protection against several stresses, nutrient starvation, and the defense against antimicrobial peptides. Although vaccination of laying hens has significantly reduced the number of *Salmonella* Enteritidis positive laying hen farms and subsequently the number of human cases, additional control measures must be taken to improve food safety. These should be based on knowledge of the pathogenesis of egg infections, and can include breeding towards resistant lines.

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summary

Summary

Salmonella is one of the most important foodborne threats to human health worldwide. An important change in the epidemiology of *Salmonella* occurred since the mid-80s, when *Salmonella enterica* subspecies *enterica* serovar Enteritidis (*Salmonella* Enteritidis) became a major contaminant of eggs and egg products. Although many other serovars are present in the environment of the laying hen, *Salmonella* Enteritidis is the main serotype associated with eggs. Consequently, during the past few years several hypotheses have been put forward to explain the tropism of *Salmonella* Enteritidis for eggs. Although eggs can be contaminated on the outer shell, extensive washing and disinfection of the eggshell in the USA have not eliminated *Salmonella* Enteritidis from eggs, indicating that the bacteria might be present internally. In order to contaminate the egg internally, *Salmonella* Enteritidis might penetrate the egg shell or be incorporated directly into egg white of the forming egg through colonization of the upper reproductive tract. While the former is not a unique trait for *Salmonella* Enteritidis it has been demonstrated that *Salmonella* Enteritidis is superior in the colonization of the reproductive tissue in comparison with other serotypes. It is now increasingly accepted that the upper oviduct is the main colonization site for *Salmonella* Enteritidis and that the bacteria are most likely incorporated into the egg white of the forming egg. These observations suggest that strains from the *Salmonella* serotype Enteritidis harbor some intrinsic characteristics, leading to a unique interaction with the chicken reproductive tract. Nevertheless, until now only limited information regarding the *Salmonella* Enteritidis virulence factors mediating oviduct colonization and egg contamination are available. Fortunately, genome-wide screening methods have been developed to provide insights in our understanding of the bacterial pathogenesis. One of these techniques, the *in vivo* expression technology or IVET screening was previously used to identify genes that are activated inside the reproductive tract and in eggs but not under standard laboratory conditions. Seven promoters were induced in both the reproductive tract and in laid eggs, suggesting a potential role for these genes in the colonization of this organ and the contamination of eggs.

As both the universal stress proteins (*usp*) A and B were identified during this screening, the first study in this thesis was performed to confirm the role of these genes in reproductive tract colonization **(chapter 3.1)**. To verify that the expression of both genes was induced by egg white, their promoters were cloned in front of the *luxCDABE* genes which enabled us to monitor gene expression by light measurement. These results clearly showed that both promoters were

induced after contact with egg white. Additionally, defined single (*uspA* and *uspB*) and double (*uspBA*) deletion mutants were constructed and used in *in vivo* experiments. Intra-oviduct inoculations showed that the *uspB* and *uspBA* mutants had a decreased ability to colonize the magnum and isthmus of the oviduct 7 days post-inoculation. This phenotype was confirmed in an intravenous challenge model in which the *uspBA* deletion strain was less able to colonize the chicken reproductive tract and eggs of chickens infected with the *uspBA* knock out were significantly less contaminated than those of chickens infected with the wild type. Consequently, analogous to the function of universal stress proteins in other species, we hypothesize that these genes are involved in long term persistence of *Salmonella* Enteritidis in the harmful environment of the reproductive tract and the egg and this probably by altering the composition of the bacterial membrane and by repair or protection against DNA damage.

To avoid the local immunity of the reproductive tract, *Salmonella* Enteritidis most likely resides intra-cellular inside the oviduct epithelial cells. No data were yet available on *Salmonella* Enteritidis genes that are of importance to colonize the oviduct. Consequently, a genome wide microarray based transposon library of 50.000 mutants was used in **chapter 3.2** to extend our knowledge on how this bacterium persists inside these cells. The idea behind this technique is that mutants harboring deletions in essential genes are no longer capable to persist or multiply inside the oviduct cells and decrease in numbers compared to mutants in which genes that are not essential for oviduct colonization are mutated. Using this technique a total of 81 potential essential genes was identified. The results suggest that *Salmonella* Enteritidis invades and survives inside the chicken oviduct epithelial cells using its pathogenicity islands. To further cope with the antimicrobial factors present in these cells, the bacteria might alter the membrane composition, activate DNA repair and stress management strategies to cope with the high chicken body temperature and antimicrobial stressors. Finally, a potential role for the Region Of Difference Genomic islands 9, 21 and 40 was demonstrated.

It is increasingly accepted that S*almonella* Enteritidis colonization of the upper reproductive tract might lead to incorporation of the bacteria into the egg white or the egg shell membranes of the forming egg. Although egg white at the chicken body temperature of 42°C is a highly antimicrobial matrix, it has been demonstrated that *Salmonella* Enteritidis has developed an enhanced potential to survive in this hostile environment compared to other serotypes. Consequently, to obtain information about the underlying mechanisms exploited by *Salmonella* Enteritidis, the above mentioned IVET and transposon library were used to identify genes involved in egg white survival at 42°C.

in **chapter 3.3** the IVET-screening was used to identify genes which are induced after contact with egg white at the chicken body temperature of 42°C. During this screening the *tolC* gene was identified as highly expressed in egg white. This gene encodes an outer membrane channel involved in siderophore export and is part of the multidrug resistance pumps, conferring resistance against antibiotics and host-derived antimicrobial agents. It was shown that a *tolC* deletion strain exhibits a decreased survival in the chicken egg white at 42°C compared to the wild type. Using chromatographic separation techniques and subsequent testing of antimicrobial activities of purified egg white fractions by an agar spot assay, ovotransferrin was identified as the egg white antimicrobial factor inhibiting the *tolC* deletion strain, due to its iron depriving characteristics. To determine whether the multidrug efflux pumps themselves are involved in this process a strain harboring deletions in the inner membrane channels was constructed. It was shown that a mutant with deletions in 4 genes encoding different inner membrane channels was inhibited in the presence of ovotransferrin. Although the iron restriction caused by ovotransferrin on its own probably can not explain the lethality of a *tolC* deletion strain in egg white, it could be that a disturbed efflux pump system in this mutant leads to accumulation of other antimicrobial peptides which in turn may cause bacterial cell death.

Finally, **chapter 3.4** describes the use of the transposon library to identify mutants that are no longer capable of surviving in egg white at 42°C. This screening identified a lot of genes involved in LPS biosynthesis and the heat shock protein HtrA as important for survival of *Salmonella* Enteritidis in egg white at the chicken body temperature of 42°C. Subsequently, deletion mutants in the *rfaI* and *htrA* gene were constructed and used in an egg white survival assay at 42°C for 24 hours. These mutants were no longer capable of surviving in egg white at 42°C.

In conclusion, this thesis offers new insights in the pathogenesis of *Salmonella* Enteritidis reproductive tract colonization and egg contamination. It was shown that *Salmonella* Enteritidis utilizes a set of unique genes for colonization and that colonization is further promoted by the use of stress response systems leading to the activation of heat shock proteins and DNA repair mechanisms. Furthermore, it was shown that *Salmonella* Enteritidis survival in egg white is mediated by siderophore secretion through multidrug efflux pumps and that LPS biosynthesis might increase the resistance against antimicrobial peptides present in egg white.

samenvatting

Samenvatting

Salmonella vormt wereldwijd een belangrijke bron van voedselintoxicatie bij de mens. Een stijging in het aantal humane salmonellose gevallen werd opgemerkt sinds de jaren tachtig door de opkomst van het *Salmonella enterica* subspecies *enterica* serotype Enteritidis (*Salmonella* Enteritidis) in eieren en eiproducten. Hoewel meerdere *Salmonella* serotypes geïsoleerd kunnen worden uit de omgeving van de leghen, is *Salmonella* Enteritidis bijna het enige serotype dat geassocieerd kan worden met humane *Salmonella* besmetting via consumptie van eieren. Om dit tropisme van *Salmonella* Enteritidis voor eieren te verklaren werden dan ook enkele hypothesen naar voor gebracht. Eicontaminatie door *Salmonella* Enteritidis kan in principe het gevolg zijn van uitwendige schaalbesmetting, maar aangezien het wassen en desinfecteren van de eischaal geen invloed had op de prevalentie van het aantal salmonellose gevallen, kon worden aangenomen dat *Salmonella* Enteritidis de ei-inhoud contamineert. Inwendige ei-contaminatie kan in principe veroorzaakt worden door penetratie van de bacterie doorheen de eischaal na uitwendige schaalbesmetting of door infectie van het ovarium of oviduct met incorporatie van de bacterie in het vormende ei tot gevolg. Terwijl eischaalpenetratie geen eigenschap blijkt te zijn die specifiek is voor het *Salmonella* serotype Enteritidis, werd aangetoond dat dit serotype de oviduct van de leghen beter koloniseert in vergelijking met andere serotypes. Het wordt dan ook meer en meer aanvaard dat *Salmonella* Enteritidis eieren besmet via kolonisatie van het oviduct waardoor de bacterie makkelijk in het eiwit of de eischaalmembranen van het vormende ei kan terecht komen. Deze data suggereren dan ook dat *Salmonella* Enteritidis over bepaalde eigenschappen beschikt die de bacterie in staat stellen om de oviduct van de leghen op een unieke wijze te koloniseren. Gezien tot voor kort nog maar weinig geweten was over de interactie van *Salmonella* Enteritidis met de oviduct, werd tijdens een voorgaand onderzoek gebruik gemaakt van genoom omvattende *in vivo* expressie technologie of IVET screening, om genen die geactiveerd worden in de oviduct omgeving te identificeren. Dit onderzoek leidde bijgevolg naar 7 promoters die zowel in de oviduct als in eieren geïnduceerd werden, wat vervolgens deed vermoeden dat ze een belangrijke rolspelen in de kolonisatie van de oviduct en eibesmetting.

Daar zowel de promotor van het universal stress protein (*usp*) A als B werd opgepikt tijdens deze screening werd het eerste hoofdstuk van deze thesis gewijd aan het bevestigen van de rol van deze genen in oviductkolonisatie **(hoofdstuk 3.1)**. Eerst en vooral werd nagegaan of de promotor van deze genen effectief geactiveerd werd door contact van *Salmonella* Enteritidis

met eiwit. Hiervoor werden de beide promotors voor de *luxCDABE* genen gekloneerd zodat promotor expressie kon gemeten worden aan de hand van licht productie. De resultaten van dit experiment toonden inderdaad aan dat de promotors geactiveerd werden na contact met eiwit. Daarnaast werd ook van beide genen een deletiemutant en een dubbel mutant aangemaakt die dan verder gebruikt werden in *in vivo* proeven bij de leghen. Hierbij toonde een intra-oviduct inoculatie experiment aan dat de *uspB* and *uspBA* deletiemutanten een verminderd vermogen hadden om de oviduct te koloniseren. Ook een intraveneus infectiemodel toonde het belang van deze genen in oviduct kolonisatie aan, daar de *uspBA* deletie mutant minder aanwezig was in de oviduct en in eieren in vergelijking met de wild type stam. Op basis van deze resultaten en door vergelijking van studies over deze genen in andere species kan vervolgens gesuggereerd worden dat de universal stress proteins nodig zijn voor de persistentie van *Salmonella* Enteritidis in de oviduct en in eieren en dit waarschijnlijk door hun rol in resistentie tegen membraan en DNA beschadigende componenten aanwezig in deze omgeving.

Er wordt verondersteld dat *Salmonella* Enteritidis binnenin de epitheelcellen van de oviduct persisteert om locale immuunreacties te ontwijken. Om genen essentieel in oviductkolonisatie op te sporen werd gebruik gemaakt van een transposonbank met 50.000 mutanten die in de oviduct en op oviductcellen werd gebracht **(hoofdstuk 3.2)**. Op basis van deze techniek werden 81 mogelijk belangrijke genen geïdentificeerd. Er werd aangetoond dat *Salmonella* Enteritidis de epitheelcellen van de oviduct binnendringt en erin overleeft door gebruik te maken van zijn pathogeniciteits eilanden. Daarnaast kan ook aangenomen worden dat de bacterie de samenstelling van zijn membraan verandert en dat systemen verantwoordelijk voor herstel van DNA schade en voor responsen op stress belangrijk zijn. Een mogelijks belangrijk resultaat in deze studie was dat genen die behoren tot de zogenaamde regions of Difference 9, 21 en 40, die aanwezig zijn in *Salmonella* Enteritidis maar niet in Typhimurium, mogelijks belangrijk zijn voor oviductkolonisatie.

Het is duidelijk dat infectie van de oviduct kan leiden tot incorporatie van de bacterie in het eiwit of de eischaalmembranen van het vormende ei. Hoewel eiwit op de lichaamstemperatuur van de kip (42°C) een heel antimicrobiële matrix is, werd reeds aangetoond dat *Salmonella* Enteritidis beter in staat is te overleven in deze antimicrobiële omgeving dan andere serotypes. Bijgevolg werd tijdens dit onderzoek gebruik gemaakt van de bovenstaande genoom omvattende methoden om informatie te verkrijgen over welke genen belangrijk zijn in dit proces.

Zo werd in **hoofdstuk 3.3** gebruik gemaakt van de IVET-screening om genen te identificeren die tot expressie komen in eiwit op 42°C, de lichaamstemperatuur van de kip. Tijdens deze screening werd het *tolC* gen opgepikt als potentieel belangrijk. TolC is een proteïne in de buitenste membraan van de gram-negative bacteriële celwand, waar het een kanaal vormt voor de efflux van sideroforen maar ook deel uitmaakt van de zogenaamde multidrug efflux pompsystemen die verantwoordelijk zijn voor antibiotica resistentie en het naar buiten pompen van gastheer geproduceerde antimicrobiële componenten. Tijdens deze studie werd aangetoond dat een *tolC* deletiemutant niet langer in staat was te overleven in eiwit op 42°C en dat ovotransferrine de groei van deze mutant in agar spot assay inhibeerde. Om te bepalen of de multidrug efflux pompen een rol speelden in de groei van *Salmonella* Enteritidis in aanwezigheid van ovotransferrine werden mutaties aangebracht in de binnenste membraan kanalen van deze pompen. Hieruit bleek dat na deletie van 4 efflux pompen een mutant gecreëerd werd die niet langer in staat was te groeien in aanwezigheid van ovotransferrine. Hoewel de ijzer restrictie veroorzaakt door ovotransferrine wellicht niet verantwoordelijk is voor de afdoding van een *tolC* mutant in eiwit kan het zijn dat door de verstoorde efflux functie in deze mutant andere antimicrobiële componenten worden opgestapeld dewelke dan uiteindelijk kunnen leiden tot bacteriële celdood.

Naast een IVET-screening werd de transposonmutantenbank gebruikt ter identificatie van *Salmonella* Enteritidis specifieke factoren betrokken in eicontaminatie **(hoofdstuk 3.4)**. Deze screening leidde tot de identificatie van een aantal LPS biosynthese genen en ook het heat shock protein HtrA als potentieel noodzakelijk voor de overleving van *Salmonella* Enteritidis in eiwit op 42°C. Deletiemutanten werden aangemaakt in de *rfaI* en *htrA* genen waarna de mutanten getest werden op hun overleving in eiwit. De mutanten bleken niet langer in staat te overleven in eiwit.

De resultaten uit dit proefschrift geven nieuwe inzichten in de pathogenese van eibesmetting door *Salmonella* Enteritidis. Er werd aangetoond dat *Salmonella* Enteritidis voor de kolonisatie van de oviduct mogelijks gebruik maakt van een unieke set genen. Deze kolonisatie wordt dan waarschijnlijk verder versterkt door de activatie van stress respons systemen bij dewelke heat shock proteïnen en DNA herstel mechanismen een centrale rol spelen. Eens geïncorpeerd in het eiwit van het vormende ei kan deze bacterie beter weerstaan aan de antimicrobiële eigenschappen met behulp van de multidrug effluxpompen door de excretie van sideroforen en eventueel andere antimicrobiële peptiden. Verder kan ook gesteld worden dat de LPS structuur een belangrijke rol speelt in de resistentie tegen deze antimicrobiële componenten.

Curriculum Vitae

Curriculum Vitae

Ruth Raspoet werd op 29 november 1985 geboren te Aalst. Na het beëindigen van haar studies algemeen secundair onderwijs, richting Wetenschappen-Wiskunde, aan het Instituut voor Katholiek Secundair onderwijs te Denderleeuw, begon ze in 2003 aan de kandidaturen en licenties Biomedische Wetenschappen aan de Universiteit Gent. In 2007 behaalde ze met onderscheiding het diploma Licentiaat/Master in de Biomedische Wetenschappen. Vervolgens begon ze in 2007 aan de Engelstalige master na master Molecular Biotechnology waarvan ze in 2008 het diploma Master of Molecular Biotechnology behaalde met grote onderscheiding.

In oktober 2008 startte ze haar doctoraatsonderzoek aan de vakgroep Pathologie, Bacteriologie en Pluimveeziekten met Prof. Dr. Ir. Van Immerseel en Prof. Dr. Ducatelle als promotoren. In december 2008 behaalde zij een specialisatiebeurs toegekend door het Instituut voor de Aanmoediging van Innovatie door Wetenschap en Technologie in Vlaanderen (IWT). Dit liet haar toe om gedurende vier jaar onderzoek te verrichten naar de virulentiefactoren van *Salmonella* Enteritidis betrokken in ei-besmetting. Gedurende dit onderzoek nam ze deel aan twee buitenlandse stages: In 2009 verbleef ze 5 weken aan het Institute of Food Research in Norwich (United Kingdom) en in 2012 verbleef ze gedurende 6 weken in Japan, waarvan 3 weken aan Kagoshima University, Department of Biochemistry and Biotechnology (Kagoshima) en 3 weken aan het Institute of Scientific and Industrial Research, Departement of Microbiology and Infectious Diseases (Osaka).

Vanaf 2013 werd de doctoraatsstudie verder gefinancieerd door Lohmann Animal Health.

In 2014 voltooide zij de doctoraatsopleiding in de Diergeneeskundige Wetenschappen.

Ruth Raspoet is auteur of medeauteur van meerdere wetenschappelijke publicaties in internationale tijdschriften. Zij nam deel aan congressen en presenteerde de resultaten van haar onderzoek in de vorm van posters en voordrachten.

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Dankwoord

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