Analysis and characterization of the prophage content in *Salmonella* Enteritidis

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Zusammenfassung

Die Zahl der *Salmonella*-Infektionen mit gesundheitlicher und wirtschaftlicher Bedeutung ist seit Mitte der 1980er Jahre angestiegen. In einigen europäischen Staaten wurde ein bis zu zwanzigfacher Anstieg der Fälle beobachtet.

Trotz der genetischen Verwandtschaft der mehr als 2500 Serovaren von *Salmonella (S.) enterica* zeigen sie eine erhebliche Vielfalt in der Art und Schwere der Erkrankung die sie hervorrufen, und in ihrem Wirtsspektrum. Die Aufnahme neuer Gene durch horizontalen Gentransfer wird weithin als der Hauptmechanismus angesehen, der die Evolution der *Salmonella*-Pathogenität vorantreibt. Bakteriophagen spielen in diesem Prozess eine wichtige Rolle. Ein vielversprechender Ansatz zum besseren Verständnis der am *Salmonella*-Wirtsspektrum und der *Salmonella*-Virulenz beteiligten Faktoren ist der Vergleich des Prophagengehalts verschiedener *Salmonella*-Serovaren.

Im ersten Teil dieser Studie wurden durch eine *in-silico* Genomanalyse fünf Prophagen im Genom des *S.* Enteritidis Stammes SE125109 identifiziert, welche ΦSE10, ΦSE12, ΦSE12A, ΦSE14 und ΦSE20 genannt wurden. Diese Prophagen-Regionen wurden vollständig annotiert und für die Annotierung des *S.* Enteritidis Stammes SE125109 verwendet.

Eine repräsentative *S.* Enteritidis Stammsammlung mit Isolaten verschiedener Phagentypen und Herkünfte und nicht-Enteritidis-Isolate wurden mittels PCR auf das Vorhandensein der zuvor identifizierten Prophagen-Abschnitte hin untersucht. Die PCR-Reaktionen wurden so entworfen, dass sie jeweils das 5'-Ende, 3'-Ende oder die Mitte der jeweiligen Prophagen replizieren. Dieser Teil der Untersuchung wurde durch Microarray-Experimente ausgewählter *S.* Enteritids Isolate verschiedener Phagentypen aus der Stammsammlung komplementiert. Die PCR-Ergebnisse zeigten eine Konservierung des Prophagengehalts für die *S.* Enteritidis-Isolate, wobei die Isolate der Phagentypen 9b, 11 und 20 die größte Variation zeigten. Die Microarray-Experimente zeigten hingegen eine deutliche Variabilität zwischen den Isolaten der verschiedenen Phagentypen. Innerhalb der zum gleichen Phagentyp gehörenden Isolate konnte eine starke Homogenität beobachtet werden. Die Prophagenbereiche scheinen aus einer Zusammenstellung von Phagengenen, die auch in anderen Serovaren vorhanden sind, zu bestehen. Diese werden jeweils entsprechend rekombiniert.

Im letzten Abschnitt dieser Arbeit wurden Untersuchungen zur spontanen Induktion von Bakteriophagen in Form eines klassischen Fisk-Tests durchgeführt. In diesen Untersuchungen zeigten die zu den Phagentypen 8, 9b, 11, 13a und 20 gehörenden Isolate das individuellste Verhalten in Bezug auf Phagenfreisetzung und –empfänglichkeit. Dies stimmte mit den PCR- und Microarray-Ergebnissen überein, bei denen diese Phagentypen sich am meisten von den Phagentyp 4-Isolaten zu unterscheiden schienen, was darauf hindeutet, dass sie wahrscheinlich einen anderen Satz Prophagen beinhalten.

Schlüsselwörter: *Salmonella* Enteritidis, Prophage, Virulenzgene

Abstract

The number of *Salmonella* infections of economic and health significance has increased since the mid 1980s and some European countries witnessed a 20-fold increase in incidents. Besides the genetic relatedness of the more than 2500 *Salmonella enterica* serovars, they show a considerable variety in severity and characteristics of the diseases they cause and in their host range. The acquisition of new genes by horizontal gene transfer is widely regarded as the main mechanism driving the evolution of *Salmonella* pathogenicity. Bacteriophages play a major role in this process. A promising approach to reveal more knowledge about the factors involved in *Salmonella* host range and virulence is to compare the prophage content of different *Salmonella* serovars.

In the first part of this study five prophage regions were identified in the genome of *Salmonella* Enteritidis 125109 by *in silico* genome analysis, which were named ΦSE10, ΦSE12, ΦSE12A, ΦSE14 and ΦSE20. These prophage regions were fully annotated and included into the annotation of the *Salmonella* Enteritidis 125109 genome.

A representative strain collection containing *S.* Enteritidis isolates covering different phage types and origins as well as non-Enteritidis isolates was screened by PCR for the presence of the previously identified prophage regions. The PCR reactions were designed to target the 5'-, 3'- and central region of the respective prophages. This part of the study was complemented by microarray analysis of selected *S.* Enteritidis isolates from the strain collection covering different phage types. According to the PCR results, the prophage content seemed to be quite conserved between the *S.* Enteritidis isolates, with those isolates belonging to the phage types 9b, 11 and 20 showing the biggest variation, but the microarray results showed the prophage content to differ enormously between the isolates belonging to different phage types. Homogeneity in prophage content could be seen in isolates belonging to the same phage type. The prophage locations seemed to consist of an assortment of phage genes also present in other serovars that are recombined frequently.

In the last part of this study spontaneous phage release experiments were performed as a classical Fisk test. In these the isolates belonging to phage types 8, 9b, 11, 13a and 20 showed the most unique behaviour in terms of phage induceability and susceptibility, which is in accordance with the PCR and microarray results where these phage types seemed to be the most diverse from phage type 4 isolates, indicating them to putatively harbour a different set of prophages.

Keywords: *Salmonella* Enteritidis, prophage, virulence genes

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1 Introduction

1.1 *Salmonella* as a source of human food-poisoning

The genus *Salmonella* consists of the two species *enterica* and *bongori*. While the latter is represented by 17 serotypes and associated with disease in cold-blooded animals, the species *enterica* contains over 2,500 serovars (Smith-Palmer *et al.*, 2003) and is responsible for a variety of diseases in warm-blooded animals including gastroenteritis and typhoid fever, depending on the nature of the infected host and on the serovar of the infecting bacteria. The subspecies IIIa (*arizonae*) in the species *enterica* is usually associated with disease in cold-blooded organisms, but occasionally responsible for systemic disease in humans (Blanc-Potard *et al.*, 1999; Chan *et al.*, 2003; Wain *et al.*, 2001).

Based on infection biology and pathogenesis, *Salmonella enterica* can be divided into two groups. One group consists of a smaller number of "host specialized" serovars causing systemic typhoid-like disease in a restricted range of host species like *Salmonella* Typhi in humans and chimpanzees or *Salmonella* Pullorum and Gallinarum in poultry. *Salmonella* Gallinarum is non-motile and causes a septicaemic disease primarily in chickens and turkeys, which is referred to as fowl typhoid. It does not colonise the gut well (Berchieri *et al.*, 2001; Poppe, 2000). The larger number of serovars belongs to the second group that consists of "host generalists" like *Salmonella* Typhimurium and *Salmonella* Enteritidis, which infect a wide range of animals (including wild rodents, poultry, pigs and cattle). They are motile and capable of efficiently colonising the gut. In a range of hosts including humans they cause gastroenteritis, in mice they cause systemic infection, while an asymptomatic chronic infection is seen in chickens. Chickens infected with *Salmonella* Enteritidis often do not show any signs of illness at all. (Alokam *et al.*, 2002; Bäumler *et al.*, 1998; Encheva *et al.,* 2007; Uzzau *et al.*, 2000; Wigley *et al.*, 2001). While the host generalists tend to colonize young animals, which suggests that they struggle to adapt to a fully mature immune system, do the host-adapted serovars, on the other hand, tend to cause disease with equal frequency in all age groups and are more virulent, which is illustrated by the higher mortality rates they exhibit (Bäumler *et al.*, 1998). The *Salmonella* serovars Gallinarum and Enteritidis are believed to arise from a common ancestor together with *Salmonella* Pullorum, which is considered to be a biotype of *Salmonella* Gallinarum (Li *et al.*, 1993).

Introduction

The number of *Salmonella* infections that are of worldwide economic and health significance has increased since the mid 1980s, and some European countries witnessed a 20-fold increase in incidents during the last 15-20 years (Hartung, 1992; WHO, 1997). The *Salmonella* serovars Gallinarum, Pullorum and Enteritidis are of major economic importance concerning animal disease, while *Salmonella* Enteritidis phage type 4 (PT4) together with *Salmonella* Typhimurium definitive type 104 (DT104) are the main cause of human zoonotic infections (Smith-Palmer *et al.*, 2003). In the EU *Salmonella* infects an estimated 160,000 individuals every year. The costs of foodborne *Salmonella* infections are estimated at up to 2.8 billion Euro annually (European Union 2002). The Economic Research Service (ERS) of the United States Department of Agriculture (USDA) has published similar data for the United States: Annual economic costs due to foodborne *Salmonella* infections add up to $2.9 billion, and the number of infections is even higher than in the EU and is estimated to reach 1.4 million annual cases (ERS 2004).

The consumption of poultry meat and eggs, which represent a major source of cheap high energy protein for much of the world, is believed to be the main cause for *Salmonella* infections in humans. For this reason control programmes to limit *Salmonella* infections in poultry are being developed in many countries (Zhang-Barber *et al.*, 1999; Immerseel, van *et al.*, 2002). In the case of *S.* Enteritidis, an epidemiological association with eggs is observed, which is probably caused by still undefined intrinsic characteristics (reviewed in Gantois *et al.*, 2009). The majority of the foodborne cases of *Salmonellosis* reported worldwide since the mid-1980s were caused by *S.* Enteritidis, and 80 % of the 371 outbreaks with a known source that were registered in the US between 1985 and 1999 were egg-associated (Patrick *et al.*, 2004). 62.5 % of the 165,023 confirmed human *Salmonellosis* cases reported through the European Surveillance System (TESSy) in 2006 were caused by *S.* Enteritidis followed by 12.9 % caused by *S.* Typhimurium. All other serotypes were responsible for less than 2 % of the cases in humans (EFSA, 2007a). A link between eggs and human *S.* Enteritidis infections is clearly illustrated by the observation that eggs and egg products were most often identified as the food vehicles in the *S.* outbreaks (Braden, 2006; Gantois *et al.*, 2009). An EU-wide analysis of faecal and dust samples from layer houses showed 30.8 % of the 5.310 commercial large-scale laying hen holdings to be *Salmonella* positive, with *S.* Enteritidis being the most prevalent serotype found in 52.3 % of the holdings. The observation that almost 50 % of the isolates from layer farms were non-Enteritidis isolates didn't match with the findings seen in the table eggs, of which 0.8 % were *Salmonella* positive in the EU in 2006 (EFSA, 2007b). *S.* Enteritidis could be confirmed in more than 90 % of the positive

eggs, which indicates *S.* Enteritidis to possess intrinsic properties that allow a specific interaction with the egg components or the hen's reproductive organs, although the data has to be interpreted cautious because the sampling points were not specified (EFSA, 2007b: Gantois *et al.*, 2009).

1.2 Approaches to infection control

The economic necessities that are connected with the poultry slaughter process make it much more practical to control the *Salmonella* infection on the poultry farm than trying to do that in the slaughterhouse. In many countries including the EU a treatment of table eggs is not allowed, which also requires *Salmonella* control on the layer farm (HMSO, 1995; European Union, 1991). Totally *Salmonella*-free poultry can be raised, but this requires a number of cost intensive measures as described for example in the OIE terrestrial animal health code including enclosed poultry housing facilities and strict control of feed quality, hygiene and management (OIE, 2009a; OIE, 2009b). Additionally, the consumption of meat and eggs from poultry raised in a free range system and the rearing of poultry in small "back-yard" flocks in developing countries make the application of improved hygiene to poultry raised under these conditions quite difficult and lead to increased environmental contamination with *Salmonella*. Under high temperatures open sided poultry houses add to the risk of environmental infection. The costs and impracticability of the necessary improvements in hygiene and management to achieve *Salmonella* free poultry flocks make biological measures important actions in the control programmes set up to control *Salmonella* infections in poultry. These biological approaches include the use of antibiotics, competitive exclusion (CE) products and vaccines or combinations of these measures (Zhang-Barber *et al.*, 1999).

Antibiotics have been increasingly used as growth promoting agents for many years. The application of such antibiotics has been discussed very critically during the last years, because the widespread use of antibiotics in livestock production has been connected with the rise of multiple drug resistant bacteria (Threlfall *et al.*, 1998; Wray and Davies, 2000). Another problem is the appearance of unwanted antibiotic residues in animal products, which essentially boosted public concerns regarding the use of antibiotics in feed. As a consequence, most of the antibiotics have been banned within the European Union as growth promoters since the end of June 1999 (Immerseel, van 2004).

Competitive exclusion, also named the 'Nurmi concept' or exclusion flora (EF) consists of the administration of cultures of intestinal flora to newly hatched chickens to enhance the resistance of the chicks to intestinal infections. The cultures are derived from gut contents of adult, healthy birds (Nurmi and Rantala, 1973). As the bacterial composition of the administrated suspensions is unknown and not defined, such treatments are not acceptable to regulatory agencies in some countries. Therefore, efforts have been made to identify those components of the intestinal microflora, which are responsible for the protective effect in chicks. The aim of this research is to identify bacteria that could be used alone or in mixtures of defined cultures to protect chicks from *Salmonella* colonisation as an alternative to the undefined solutions currently used. The successfully used mixtures contain about 50 isolates of different bacteria. Since the mechanism of the protection is not yet fully understood, it is difficult to select the required strains, and prospects in developing efficient defined preparations are not yet very promising (Stavric, 1992).

1.3 Vaccination

The vaccination of poultry has become one of the most important measures to control *Salmonella* infections of the birds because of the costs, impracticability and disadvantages of the other approaches mentioned above. Live vaccines produce better protection than killed vaccines. Killed vaccines have been tested with varying results and only stimulate antibody production (Barrow, 1996; Chatfield *et al.*, 1993). They may also lead to poor immune protection due to the destruction of relevant antigens during vaccine preparation and the fast destruction and elimination of the vaccine from the inoculated animals (Barrow, 1991). They can present only those antigens that were induced under the conditions of the fermentation process (Barrow and Wallis, 2000). Their protective efficacy is additionally restricted by their low immunogenicity in unprimed hosts and the fact that they do not induce cytotoxic T cells (Nagaraja and Rajashekara, 1999). Furthermore killed vaccines do not elicit secretory IgA responses, which play an important role in protecting mucosal surfaces (Barrow and Wallis, 2000).

Live vaccines reduce the colonisation of the intestine more efficiently. They stimulate a prevailing Th1 rather than a Th2 response. The Th1 response is assumed to be important for the elimination of the bacteria from the gut or the tissues. Studies from Desmidt *et al.* showed that the administration of live *Salmonella* to 1-day-old chicks activated an antibody response

to the LPS antigen from day 18 post infection (Desmidt *et al.*, 1997). There is only little knowledge about the starting point of a specific cellular immune response in the chicken after the administration of the live *Salmonella*. Newly hatched chickens have an immunity gap during their first days of life due to their immunological immaturity. But also non-specific resistance effects to invasion and intestinal colonisation with a number of *Salmonella* challenge strains are conferred by live vaccines. These effects are based on colonisation exclusion and neutrophil infiltration, which leads to a reduced invasion and enteritis (Foster *et al.*, 2003; Immerseel, van *et al.*, 2002). Little is known about the mechanism underlying this early colonisation inhibition, but it is believed that microbiological and host-related factors contribute to the effect. *In vitro* studies by Zhang-Barber *et al.* support the hypothesis that the colonisation-inhibition is a microbiological effect aroused by growth suppression because of the absence of an utilizable carbon source or electron acceptor (Zhang-Barber *et al.*, 1997).

Live vaccines should meet certain conditions in terms of efficacy and safety. They should provide an effective protection against intestinal and systemic infection. At the same time they have to be avirulent for man. The public acceptance of live vaccines will probably increase with the risen awareness of food poisoning caused by *Salmonella* infections. Considering practicability and costs, the ideal administration route of live vaccines for poultry is orally via drinking water, feed, or by spray (Zhang-Barber *et al.*, 1999). The vaccine strain should be avirulent in chicken but stimulate maximum immunity through high invasiveness. As residual virulence may lead to vertical transmission, the vaccine should not produce disease in the offspring and not decrease performance. Protection nevertheless should last as long as possible. In broilers the protection should last a matter of weeks (Zhang-Barber *et al.*, 1999). As chickens are very susceptible to *Salmonella* infection during the first days of their life, as mentioned above, a protection against *Salmonella* infection at this point is only successful through competitive exclusion because of the immunological immaturity of the chicks and the time a protective immunity needs to develop. For this reason live vaccines that show competitive exclusion effects should be used.

In general, live vaccines should be cleared from the animal within a certain time period. Therefore, attenuated *Salmonella* strains, which do not revert to virulence, should be used (Immerseel, van 2004). However, the degree of attenuation must be adapted to the chicken's lower susceptibility to *Salmonella* infections in terms of achieving an optimised vaccine action. For this reason virulence should not be reduced as strongly as in other vaccine strains (Linde *et al.*, 1997). The *Salmonella* vaccine strains that are currently developed possess defined nonreverting mutations of metabolic functions and virulence factors. The metabolic

functions affected by these mutations include the biosynthesis of aromatic amino acids (*aro*A, *aro*C, *and aroD*), purines (*pur*A, *pur*E), adenylate cyclase (*cya*) and the cyclic AMP receptor protein (*crp*) (Cooper *et al.*, 1990, 1992, 1994a, 1994b; Hassan and Curtis, 1990; McFarland and Stocker, 1987). In vaccine strains with an attenuated virulence, the mutations usually have an impact on the *phoP/phoQ* two-component regulatory system or the genes located in SPI 2 (Medina *et al.*, 1999; Raupach and Kaufmann 2001).

At the present time there are a number of *Salmonella* live vaccines licensed in the EU. In other countries, a different range of live vaccines is available, and there are also countries that do not allow vaccination with live vaccines at all. The currently licensed live vaccines are genetically undefined mutants of *Salmonella* Enteritidis, *Salmonella* Typhimurium and *Salmonella* Gallinarum. In future, the use of defined deletion mutants as vaccine strains should be favoured for many reasons. These include a higher stability, a better understanding of the strain itself, and the mechanisms underlying its virulence and colonisation, and the feasibility of a good differentiation between wild-type and vaccine strains for example by PCR. All these properties of a defined deletion mutation will lead to a bigger acceptance of the use of a strain as a vaccine strain for a live vaccine by the public and regulatory agencies responsible for the licensing of new vaccines.

Currently, Lohmann Animal Health (LAH) offers two very effective *Salmonella* live vaccines, a *Salmonella* Enteritidis (AviPro® SALMONELLA VAC E) and a *Salmonella* Typhimurium (AviPro® SALMONELLA VAC T) vaccine. The vaccine strains are drift mutants carrying minus mutations in essential enzymes and metabolic compartments, which lead to longer generation times resulting in a decreased virulence. As the metabolic compartments are points of action for antibiotics, the mutative structural changes simultaneously produce an antibiotic resistance in the mutant strains through a loss of antibiotic binding sites. This resistance has no effect on therapeutic mechanisms, but is used to identify the vaccine strain (Linde *et al.*, 1997). In the *Salmonella* live vaccines produced by LAH, the following metabolic compartments are genetically altered by the use of chromosomal antibiotic-resistant mutations: RNA polymerase (giving resistance to Rifampicin (Rif)) and ribosomal protein S12 (giving resistance to Streptomycin (Sm)) in AviPro® SALMONELLA VAC E, and RNA polymerase (giving resistance to Rifampicin (Rif)) and gyrase (giving resistance to nalidixic acid (Nal)) in AviPro® SALMONELLA VAC T (Linde *et al.*, 1997). A cell membrane mutation increasing the cell's permeability to erythromycin and other antibiotics acts as an additional marker optimising the vaccine strain (Hancock, 1984; Vaara, 1993). By definition by the WHO, it also serves as an attenuation

marker (WHO, 1972). It provides the vaccine strain with three additional safety functions: (1) a limitation of the shedding of the vaccine strain to a maximum of 21 days (Linde *et al.*, 1993; Hahn *et al.*, 1993); (2) a reduced survival of the strain in the environment resulting in a faster elimination in the faeces (Linde and Randhagen, 1986), and (3) a fourfold increased sensitivity to antibiotics such as doxycycline, chloramphenicol and others used in human medicine (and also to quinolines for the Sm/Rif metabolic drift combination) (Linde, 1993). Orally administered live vaccines can be inactivated by bile if they are sensitive to bile and surfactant. AviPro® SALMONELLA VAC T possesses a reversion of bile and surfactant sensitivity to bile and surfactant tolerance (Rtt), which inhibits the inactivation by bile. This reversion is an anti-epidemic marker that reduces shedding and leads to a shortened survival time in the environment (Linde, 1982; Linde *et al.*, 1987). AviPro® SALMONELLA VAC E is supersensitive to quinolones (Ssq), especially ciprofloxaxin. This feature is simultaneously a safety and therapeutical marker, as ciprofloxaxin is the most effective antibiotic in the therapy of *Salmonella* infections (Simon and Stille, 1993; Linde *et al.*, 1993).

1.4 *Salmonella enterica* and modern approaches to taxonomy

As enterobacteria *Salmonella* share a common ancestor together with *Escherichia coli*. The genera diverged between 100 and 150 million years ago (Euzéby, 1999; Ochman and Wilson 1987). Their genomes share extensive regions of homology, and the order of orthologous genes is strongly conserved (Krawiec and Riley, 1990; Sanderson, 1976). Probably this core of conserved genes is responsible for efficient transmission between hosts and survival in the intestine (Thomson *et al.*, 2004). However, differences between the genomes exist as a result of DNA inversions, deletions and insertions and the presence or absence of a plasmid (Liu and Sanderson 1996; Wain *et al.*, 2001). The integration of novel DNA sequences into the genome is preferred at specific sites: between the *rrn* (rRNA) operons and in the TER (terminus of replication) region. At these sites the rearrangements can be tolerated without affecting the cells' fitness, while it is strongly selected against rearrangements at other sites during evolution (Achtman and Pluschke, 1986; Alokam *et al.*, 2002; Liu and Sanderson 1996). The insertion sites of bacteriophages frequently lie within transfer RNA (tRNA) genes. The association of horizontally acquired DNA with tRNA genes indicates a bacteriophage origin for the DNA integrated at these integration sites (Campbell, 2003a; Wain *et al.*, 2001).

Genome rearrangements and homologies in the genome of different *Salmonella* serovars have been analysed by broad spectrum of methods including pulsed-field gel electrophoresis (PFGE) (Liu and Sanderson, 1995), multilocus enzyme electrophoresis (MLEE) (e.g. Boyd *et al.*, 2003), PCR, DNA-DNA hybridisation (e.g. Porwollik *et al.*, 2004), DNA sequencing (e.g. McClelland *et al.*, 2001; Parkhill *et al.*, 2001) and microarray analysis (e.g. Porwollik *et al.*, 2004; Thomson *et al.*, 2004). For the different *Salmonella* serovars investigated, sequence identities of house-keeping genes and 16S rRNA of 96-99 % have been found (Porwollik and Mc Clelland, 2003). Despite this genetic relatedness among the *Salmonella enterica* serovars, they show a big variability in their pathogenic properties, as they differ deeply in their disease spectrum and host range as mentioned above. This adaptation to a broad range of ecological niches while maintaining a high degree of genetic relatedness may be based on the occurrence of lateral gene transfers, which are responsible for qualitative leaps in evolution of many bacterial species and mean the transfer of foreign genetic material into recipient cells (Porwollik and McClelland, 2003). Several mobile genetic elements (insertion sequences, plasmids, pathogenicity islands and bacteriophages) have been associated with the horizontal transfer of virulence genes (Davis and Waldor, 2002).

1.5 The *Salmonella* life cycle: infection, disease and bacterial virulence determinants

Salmonella enterica has a complex life cycle in infected animals, and a large number of virulence genes have been identified that contribute to the two key virulence traits involved in the interaction of bacteria and host cells: *Salmonella enterica* invades into nonphagocytic cells like the epithelial cell of the gastrointestinal mucosa. It is also a facultative intracellular pathogen that can withstand phagocytosis by macrophages and replicate inside eucaryotic host cells (Blanc-Potard *et al.*, 1999; Hansen-Wester *et al.*, 2004). A huge number of virulence factors contribute to the multiple steps involved in the infection process. Approximately 4 % of the *Salmonella* Typhimurium genome is believed to be required for fatal infection of mice, covering over 200 virulence genes, each of which might make only a small contribution to overall pathogenesis (Bowe *et al.*, 1998; Ho *et al.*, 2002).

Additionally, the survival of the bacteria in the host seems to be based on an accurate balance of many gene products acting at the right time in the correct location (Bowe *et al.*, 1998). These genes are located on plasmids or within the chromosome as islets of single or few

virulence genes or as large cassettes composed of a series of genes and operons. At least 17 of such large *Salmonella* pathogenicity islands (SPIs), which contribute to a coordinated expression of virulence genes, have been previously identified. Some of them have already been studied extensively and are characterized briefly below. The acquisition of SPIs represent major events in the evolution of bacterial pathogens, because their incorporation by horizontal transfer from a different bacterial genus can transform a normally benign organism into a pathogen in a single step. SPIs usually have a GC-content lower than that of the rest of the chromosome and are often inserted into tRNA genes, which indicates a bacteriophage origin (Chiu *et al.*, 2005; Hensel, 2004; Marcus *et al.*, 2000; Vernikos and Parkhill, 2006; Wain *et al.*, 2001). SPI 1 controls the ability to invade epithelial cells and is required for *Salmonella*-induced apoptosis of macrophages (Chen *et al.*, 1996; Collazo and Galan, 1997; Marcus *et al.*, 2000; Mills *et al.*, 1995). SPI 2 contains genes necessary for intramacrophage survival and systemic infection (Ochman *et al.*, 1996; Shea *et al.*, 1996). The acquisition of SPI 2 marked the divergence of *Salmonella* into the two species *Salmonella enterica* and *Salmonella bongori,* which was together with the acquisition of SPI 1 a "quantum leap" in *Salmonella* evolution (Groisman and Ochman, 1996). Two of the genes encoded by SPI 3 are related to the transport of magnesium at low Mg^{2+} conditions, while they seem not to be required for virulence in mice but might be involved in other aspects of pathogenesis like chronic infection and host specificity (Blanc-Potard and Groisman, 1997, Blanc-Potard *et al.*, 1999). Sequence analysis suggests that SPI 4 encodes a type I secretion system and it has been speculated that SPI 4 is involved in the secretion of a cytotoxin. A locus within SPI 4 is required for intramacrophage survival. The main function of SPI 4 remains to be determined (Bäumler *et al.*, 1994; Marcus *et al.*, 2000; Mecsas and Strauss, 1996; Wong *et al.*, 1998). SPI 5 finally contains genes that mediate the enteropathogenesis of *Salmonella* (Wallis and Galyov, 2000; Wood *et al.*, 1998). Like many other pathogenic bacteria, *Salmonella* employ type III secretion systems to translocate bacterial effector proteins directly into the cytosol of host cells and to modulate responses of host cells through interactions with eukaryotic proteins (Galan and Collmer, 1999; Hueck, 1998; Mirold *et al.*, 2001; Wallis and Galyov, 2000). Effector proteins, which are translocated by the SPI 1-encoded type III secretion system, mediate early stages of the infection (Galan and Collmer, 1999; Wallis and Galyov, 2000). The SPI 2 encodes a type III secretion system (T3SS), which plays a central role in systemic infections by *Salmonella enterica* and for the intracellular phenotype as it is used to translocate a set of effector proteins into the host cell. The effector proteins modify host cell functions enabling intracellular survival and replication of the bacteria (Hansen-Wester *et al.*,

2004; Hensel, 2000). The virulence of mutant strains deficient in SPI 2 is severely attenuated, and these strains are deficient in intracellular survival and proliferation. In contrast to SPI 1, the SPI 2 locus is only present in *Salmonella enterica* and not in *Salmonella bongori* and its acquisition is thought to be a major step towards successful systemic colonisation of host organisms (Bäumler, 1997). After cloning of the SPI 2 virulence locus, it could be functionally transferred into SPI 2-negative *Salmonella bongori* (Hansen-Wester *et al.*, 2004). The transfer of the SPI 2 locus and a single effector locus did not confer the ability to cause systemic infections to *Salmonella bongori*, probably due to the absence of further gene loci outside the SPI 2 locus, which encode additional members of the group of STE (*Salmonella* translocated effector) proteins that have to be present to produce the full phenotype of intracellular virulence (Hansen-Wester *et al.*, 2004).

1.6 Bacteriophages

The name bacteriophage was introduced by the Canadian bacteriologist Felix Hubert d'Herelle working at the Institute Pasteur in Paris for a bacteriolytic substance that he isolated from faeces in 1917 (d'Herelle, 1917; Adams, 1959). It means "eater of bacteria" and usually the short form "phages" is used today (Adams, 1959). d'Herelle shares credit for the discovery of phages with the British bacteriologist Frederick William Twort, who independently described an acute infectious disease of staphylococci that produced marked changes in colony morphology in 1915 (Adams, 1959; Twort, 1915). Twort considered a filterable virus analogous to the viruses of animals and plants to be responsible for his observations (Adams, 1959; Twort, 1915). Today bacteriophages are universally recognized to form a group of bacteria-specific viruses (Adams, 1959). Most bacteria are susceptible to infection by bacteriophages, and bacteriophages can be found in all habitats of bacteria including plants, animals, soil, lake and marine waters (Griffiths *et al.*, 1999). They are believed to be evolving since 3 billion years or more, and their population size is estimated to be in the order of 10^{31}, making phages to be the majority of organisms on Earth (Hendrix, 2005).

Based on their infection cycle, bacteriophages can be divided into two groups, virulent phages and temperate phages. Virulent phages are always lytic. They infect and lyse the host cell, resulting in progeny phages. After attachment of the phage to a bacterium, it injects its genetic material into the bacterial cytoplasm. Strong viral promoters control the genes of virulent

phages, and the machinery of the bacterial cell is taken over by turning off the synthesis of bacterial components. The phage genetic material is replicated in high copy numbers, and the bacterial synthetic system is redirected to make phage components. The new bacteriophage virions are assembled and the host cell finally bursts releasing 100-200 phage particles that can start a new lytic cycle. The second group, temperate phages, can undergo a lytic cycle under certain conditions, but more often they integrate into the bacterial chromosome at specific insertion sites, very often tRNA genes. The inserted phage, which is referred to as a prophage in this condition, is replicated along with the bacterial chromosome, and the lysogenic state can be transmitted genetically through many bacterial generations. A lysogenic bacterium, carrying a prophage is resistant to subsequent infection, because immunity is conferred by the presence of the prophage. Specific phage repressors act to prevent the autonomous replication of the phage DNA and the expression of phage functional proteins required for the lytic cycle. The prophage can be induced by exogenous physical or chemical stress factors that cause DNA damage. The prophage can than excise from the bacterial chromosome and enter a lytic cycle, leading to lysis of its host cell and the production of a large number of progeny phages (Ackermann, 1998; Campbell, 2003b; Canchaya *et al.*, 2004; Griffiths *et al.*, 1999; Yang *et al.*, 2006). Traditionally, lysogenic cultures were induced by UV light or mitomycin C. (Bainbridge, 2000; Gemski *et al.*, 1978; Yee *et al.*, 1993). Nevertheless, a variety of other inducing agents has been described, including hycanthone, chlorophenols, and hydrogen peroxide (DeMarini *et al.*, 1990; Figueroa-Bossi and Bossi, 1999; Shungu and Cook, 1974). The SOS regulon allows bacteria to withstand DNA damaging agents (Walker, 1984). In *E. coli* it comprises at least 20 genes whose expression is regulated by LexA and RecA and is involved in physiological responses like DNA repair and mutagenesis (Lewis *et al.*, 1994; Peterson *et al.*, 1988; Walker, 1984). DNA damage activates RecA, which enables the autoproteolytic inactivation of the SOS repressor LexA and subsequent derepression of the SOS-regulated genes (Little *et al.*, 1980; Little, 1983; Little 1984; Little 1991). Components of the SOS regulon including the *recA* locus have also been identified in *S.* Typhimurium (Pierré and Paoletti, 1983). The RecA protein promotes the autoproteolytic cleavage of prophage repressors like *cI* enabling the prophage to enter the lytic cycle (Bainbridge, 2000; Craig and Robets, 1980; Herskowitz and Hagen, 1980; Yang *et al.*, 2006). In many lambda-strains the repressor protein is thermo labile at 45 ° C, so that lysogenic cultures can be induced by a short treatment at this temperature (Bainbridge, 2000). Phages can also enter the lytic phase in the absence of inducing agents, which is referred to as spontaneous induction. This is believed to be a property of the host

bacterium and not one of the prophage itself, and it occurs likely due to spontaneous DNA damage. Recent studies have shown that for some phages, like for example P22, it occurs with the onset of host stationary phase (Abedon, 2008; Lunde *et al.*, 2003; Lwoff, 1953; Ramirez *et al.*, 1999). The extent of DNA damage necessary for induction varies between phage strains (Little, 2005). The spontaneous induction rate of Stx-encoding phages has been shown to be much higher than that of lambdoid phages, and a study of the spontaneous production of phages by *P. pyocyanea* revealed that one bacterium in 600 produces phages spontaneously (Bail, 1921; Delbruck, 1950; Livny and Friedmann, 2004; Lwoff, 1953). When a lysogen carries two lambdoid prophages with different repressors, usually both prophages in the same cell are induced (Livny and Friedmann, 2004). It has been described that the spontaneous production of inducible phages is much higher than the spontaneous production of non-inducible phages (Lwoff, 1953).

1.7 Prophages as determinants of bacterial virulence

In addition to those virulence genes present in SPIs, other virulence-related *Salmonella* genes that have probably been acquired horizontally can be found in bacteriophages. Bacterial hosts can be transformed from a non-pathogenic strain to a virulent strain or a strain with increased virulence by bacteriophages encoding virulence genes in a process called phage lysogenic conversion. These virulence factors are located on a number of morphologically diverse bacteriophages that belong to the virus families *Podoviridae* (short tail stub), *Siphoviridae* (long flexible non-contractile tail), *Myoviridae* (contractile tail) and *Inoviridae* (filamentous). Within one family, bacteriophages with little sequence homology among each other or with characteristics similar to members of another family can be found because the current classification for the first three bacteriophage families is based on virion morphology (Boyd and Bruessow, 2002; Lawrence at al., 2002).

The proteins encoded by the bacteriophages, which are involved in lysogenic conversion, provide mechanisms to invade host tissues, damage host cells and avoid host immune defences. The ecological success of a lysogenic bacterium contributes to the dissemination of bacteriophage genes and is also in the interest of the bacteriophage for this reason (Boyd and Bruessow, 2002).

The lysogenic transformation by bacteriophages is efficient and does not require intimate contact between bacteria (Miao and Miller, 1999). It allows the acquisition and exchange of

virulence factors on a much more rapid time scale (Ho *et al.*, 2002). Bacteriophages can carry large blocks of DNA. They can withstand hostile conditions, which eliminate bacterial populations, and the DNA important to a bacterial population can be preserved, until a host for lysogenic conversion is reintroduced into the environmental niche. The DNA can be spread directly into an entire population of bacteria, which eliminates the necessity for clonal expansion of a specific population (Miao and Miller, 1999).

Toxins as bacteriophage-encoded virulence factors have been found in a range of both Gram-negative and Gram-positive bacteria (reviewed in Bishai and Murphy 1988; Wagner and Waldor, 2002) since in 1951 it was discovered that the diphtheria toxin is encoded on the β-phage genome from *Corynebacterium diphtheriae* (Freeman, 1951; Uchida *et al.*, 1971). The structural genes encoding botulinum toxins C_1 and D (Eklund *et al.*, 1971; Eklund *et al.*, 1972; Fujii *et al.*, 1988; Inoue and Iida, 1970; Inoue and Iida, 1971) streptococcal erythrogenic toxin (Goshorn and Schlievert, 1989; Johnson and Schlievert, 1984; Weeks and Ferretti, 1984), staphylococcal enterotoxin A (Betley and Mekalanos, 1985; Casman, 1965; Coleman *et al.*, 1989; Jarvis and Lawrence, 1971), Shiga toxins 1 and 2 (Stx1 and Stx2) (McDonough and Butterton, 1999), the Shiga-like toxins (SLT) of *E. coli* (Huang *et al.*, 1986; Newland *et al.*, 1985; Strockbine, *et al.*, 1986; Willshaw *et al.*, 1985), *Pseudomonas* cytotoxin (Hayashi *et al.*, 1990; Nakayama *et al.*, 1999), and cholera toxin (CT) (Waldor and Mekalanos, 1996) are further examples for bacteriophage-encoded virulence factors. The genes are located in the genomes of temperate bacteriophages that confer toxinogenicity upon their hosts (Bishai and Murphy, 1988). Besides toxins, there are other potential virulence factors encoded by bacteriophages. Among these are enzymes that alter the antigenic properties of lipopolysaccharide (LPS) (Waldor, 1998). Since it was first shown in 1971 that O-antigen genes were encoded by phage ε from *Salmonella* (Wright 1971), bacteriophage-encoded O-antigen modification genes have been detected in a range of Gram-negative bacteria. The O-antigen modification proteins produce antigenic variation. This alters the host recognition of the infecting bacteria and enables the bacteria to avoid the host immune system (Boyd and Bruessow, 2002).

Many of the genes for virulence and host adaptation in *Salmonella* are encoded either adjacent to bacteriophage-like elements or by temperate bacteriophages (Boyd and Bruessow, 2002). These will be discussed in more detail below and include effector proteins, which are translocated by a type III secretion system and are required for uptake by intestinal epithelial cells. Other examples are a copper, zinc superoxide dismutase (SodC), which probably mediates bacterial defence against the oxidative burst (De Groote *et al.*, 1997; Farrant *et al.*,

1997) and neuraminidase (Figueroa-Bossi *et al.*, 2001). Proteins involved in bacterial attachment to host cells can be added to the list of bacteriophage-encoded virulence factors (Karaolis *et al.*, 1998; Karaolis *et al.*, 1999; Kovach *et al.*, 1996).

A range of bacteriophage-encoded virulence genes has been described for *Salmonella* Typhimurium. Among the prophages described for *S.* Typhimurium are two prophage-like elements named Gifsy-1 and Gifsy-2, which carry a sequence reportedly transcriptionally activated during *Salmonella* adaptation to oxidative stress (Figueroa-Bossi and Bossi, 1999; Wong and Mc Clelland, 1994). These lambdoid bacteriophages have the same relative gene order as the prototype phage lambda (Ho *et al.*, 2002). Genetic analysis indicates that the Gifsy-2 bacteriophage significantly contributes to *Salmonella* pathogenesis. *Salmonella* Typhimurium strains that are cured of Gifsy-2 are attenuated over 100-fold in their virulence in mice, indicating that Gifsy-2 contributes virulence factors to its host (Figueroa-Bossi and Bossi, 1999; Ho *et al.*, 2002). One of these factors is SodCl. The *sodCl* gene encodes a periplasmatic Cu/Zn superoxide dismutase, which probably increases the virulence of serovar Typhimurium by reducing the antimicrobial effects of the oxidative burst produced by host macrophages (De Groote *et al.*, 1997; Farrant *et al.*, 1997). *Salmonella* Typhimurium strains mutant in *sodCl* are attenuated in macrophages as well as in mice (De Groote *et al.*, 1997; Farrant *et al.*, 1997). Another virulence factor encoded by Gifsy-2 identified by deletion analysis is *gtgE*. The *gtgE*-gene encodes a putative protein of 228 amino acids for which no significant homologues in other bacteria have been detected so far (Ho *et al.*, 2002). The contribution of the Gifsy-1 prophage to virulence, which is undetectable in the presence of the Gifys-2, as Gifsy-2 can fully substitute for Gifsy-1, becomes significant in cells lacking Gifsy-2 but containing the *sodC* gene in the chromosome. This indicates that Gifsy-1 carries one or more virulence genes besides *sodC* that have functional equivalents in Gifsy-2 and also verifies the role of Gifsy-2-encoded SodC protein for *Salmonella* virulence (Figueroa-Bossi and Bossi, 1999). One virulence gene that has been identified in the Gifsy-1 genome is *gipA*, which is involved in the bacterial colonisation of the small intestine and necessary for Salmonella survival in the Peyer's patches (Stanley *et al.*, 2000). Similar to these findings, *gtgE* and *sodC1*, which are carried by the Gifsy-2AO-bacteriophage in *Salmonella* Abortusovis have been identified as the main virulence determinants contributing to virulence in lambs for this serovar (Bacciu *et al.*, 2004). Gifsy-2 also contains the gene *grvA*, which encodes GrvA that probably decreases the virulence of *Salmonella* Typhimurium in a wild-type situation in mice most likely by affecting the bacteria's resistance to toxic oxygen. *grvA* is therefore termed an antivirulence gene (Ho and Slauch, 2001).

The survival of *Salmonella* Typhimurium within macrophages is an essential virulence property and requires a coordinated transcriptional activation of virulence genes. The two-component transcriptional system PhoP/PhoQ comprising the transcriptional activator PhoP and the sensor-kinase PhoQ responds to signals within the acidified macrophage phagosome environment to induce *phoP*-activated gene (*pag*) transcription, but PhoP/PhoQ-dependent genes are not necessarily macrophage specific (Alpuche Aranda *et al.*, 1992; Belden and Miller, 1994; Miller *et al.*, 1989; Valdivia and Falkow; 1997). The lambdoid prophage Gifsy-3 is present in the *Salmonella* Typhimurium strain ATCC14028. Gifsy-3 contains the *phoP/phoQ*-activated *pagJ* gene, but bacteria cured of this prophage showed no detectable attenuation in their ability to cause systemic infection and death in mice after oral inoculation (Figueroa-Bossi *et al.*, 2001). Gifsy-3 also contains a gene for the secreted leucine repeat protein (SspH1), which is a substrate of a type III secretion system. Further members of this family of bacteriophage-encoded virulence proteins that share translocation signals and are translocated by a type III secretion system include the SseI protein of Gifsy-2 and the putative GogB protein of Gifsy-1 (Figueroa-Bossi *et al.*, 2001; Miao and Miller, 2000). The P2-like phage SopEΦ contains the *sopE* gene, which codes for another effector protein translocated *via* a type III secretion system in *Salmonella* Typhimurium (Hardt *et al.*, 1998a). SopE stimulates cytosceletal reorganisation and Jun N-terminal kinase (JNK) activation in a CDC42- and Rac-1-dependent manner. Purified SopE has also been shown to stimulate GDP/GTP nucleotide exchange in several Rho GTPases *in vitro* (Hardt *et al.*, 1998b). SopE increases the pathogenicity of *Salmonella* Typhimurium in calves (Zhang *et al.*, 2002). A bacteriophage released from *Salmonella* Typhimurium strain LT2 has been identified as a lambda-like phage Fels-1. It carries the *nanH* gene that codes for a neuramidinase for which the importance in pathogenesis is not clearly defined, but which is present mainly in pathogenic isolates suggesting a role in virulence (Figueroa-Bossi *et al.*, 2001; Boyd and Bruessow, 2002). Fels-1 was also found to encode a novel *sodC* gene, *sodCIII*.

The repertoire of translocated effector proteins varies even between closely related *Salmonella* strains and is believed to define host specificity and epidemic virulence. Lysogenic conversion with a *sopE*-encoding bacteriophage is one of the mechanisms allowing *Salmonella* to modify the effector protein repertoire in order to optimise the interaction with host animals. This can lead to the emergence of new epidemic clones and adaptation to new animal hosts (Mirold *et al.*, 2001). In *Salmonella* Typhimurium and *Salmonella* Typhi, SopE is encoded by a P2-like phage. Contrary to that, it is encoded in a lambda-like bacteriophage in the *Salmonella* serovars Hadar, Gallinarum, Enteritidis and Dublin (Mirold *et al.*, 2001).

This indicates that the same bacterial virulence factor can move horizontally between different *Salmonella* serovars via lysogenisation with a certain bacteriophage, and it can be transferred between different types of bacteriophages, which increases the flexibility of the reassortment of effector protein repertoires by avoiding restrictions caused by immunity functions or the occupancy of attachment sites by resident prophages (Mirold *et al.*, 2001). The organisation of the phage-encoded virulence modules (morons) as independent transcriptional units allows autonomous expression even from repressed prophages, which ensures that the virulence factors can be transferred freely between phages, without affecting orderly and timely gene expression (Hendrix *et al.*, 2000; Mirold *et al.*, 2001). These autonomous genetic modules were termed morons in accordance with the fact that their addition to the genome means that there is "more DNA" than there is without the element (Juhala *et al.*, 2000).

In addition to the virulence factors encoded in the chromosome, like SPIs and bacteriophages, many *Salmonella* serovars harbour virulence plasmids that play an important role in the systemic infection of experimental animals after oral inoculation and enhance the strains' virulence. The virulence plasmids are involved in the ability of *Salmonella* to invade from the intestines into deeper tissues, such as the mesenteric lymph nodes and spleens, after oral inoculation of mice. The plasmids contain highly homologous *Salmonella* plasmid virulence genes (*spv*), which are involved in systemic infection by increasing the replication rate of the bacteria in host tissues. The *spv* genes can enhance the severity of the enteric infection and produce lethal disease in those serovars which carry virulence plasmids (Gulig, 1990; Gulig *et al.*, 1997; Libby *et al.*, 1997; Marcus *et al.*, 2000).

1.8 *Salmonella* genes, genomes and virulence

Many of the *Salmonella* genes that have been identified as genes required for virulence and particularly for intracellular survival and multiplication are also regarded as housekeeping genes, entitling genes expressed in all cells and coding for molecules necessary for basic maintenance and essential cellular functions (Turner *et al.*, 2003). Among those are genes affecting DNA supercoiling like *hupA* and *hns* (Harrison *et al.*, 1994; Turner *et al.*, 1998). A lot of genes are associated with environmental sensing and transcriptional regulation: *cya/crp, ompR/envZ, phoP/phoQ, rpoS,* and *rpoE* (Chatfield *et al.*, 1991; Curtiss *et al.*, 1987; Fang *et al.*, 1992; Humphreys *et al.*, 1999; Miller *et al.*, 1989). To resist bacterial stress induced by

the intracellular environment, another set of genes is required: *htrA, clpB,* and *dksA* (Chatfield *et al.*, 1992; Turner *et al.*, 1998; Webb *et al.*, 1999). To develop full virulence, genes related with the biosynthesis of nutrients like purines, pyrimidines, and aromatic amino acids, are required, too (Fields *et al.*, 1986; Hoiseth and Stocker, 1981). The same has been described for genes involved in the uptake of mineral nutrients and trace elements like Mg^{2+}, Fe^{2+}, and Cu^{2+} for *Salmonella* and other bacteria (Heithoff *et al.*, 1997; Wang *et al.*, 1996). The simultaneous prevention of the synthesis and high-affinity transport of the primary nitrogen donor glutamine attenuates the virulence of *Salmonella* Typhimurium (Klose and Mekalanos, 1997). *Salmonella* invading the gastrointestinal tract encounter anaerobic stress, which has been suggested to enhance their virulence: anaerobically grown *Salmonella* serovars Typhi and Typhimurium showed higher cell surface hydrophobicity, induced expression of five outer membrane proteins (OMPs) and significantly higher levels of antioxidant enzymes like superoxide dismutase (SOD) and catalase (Kapoor *et al.*, 2002; Singh *et al.*, 2000).

As mentioned earlier, *Salmonella* show considerable variability in severity and characteristics of the diseases they cause and have extremely different host ranges besides their genetic relatedness (Porwollik and Mc Clelland, 2003). The virulence functions determining an epidemic strain and the genetic determinants of *Salmonella* host range have remained largely unknown (Figueroa-Bossi *et al.*, 2001; Mirold *et al.*, 2001). The acquisition of new genes by horizontal gene transfer is widely regarded as the main mechanism driving the evolution of *Salmonella* pathogenicity (Bäumler, 1997; Boyd and Bruessow, 2002; Ochman *et al.*, 2000; Porwollick and McClelland, 2003). Bacteriophages play a major role in the movement of virulence factors among bacteria (Bacciu *et al.*, 2004; Cheetham and Katz, 1995; Figueroa-Bossi *et al.*, 2001; Miao and Miller, 1999; Miao and Miller, 2000; Waldor, 1998). The genetic mechanisms, which facilitate the integration of bacterial virulence factors into bacteriophage genomes and transfer of morons between different bacteriophages are not yet completely understood and have moved into the focus of scientific interest (Hendrix *et al.*, 2000; Mirold *et al.*, 2001). With the availability of multiple complete bacterial genome sequences, the important role of prophages in the diversification of strains within a bacterial species has been shown for example for *Streptococcus* (Beres *et al.*, 2002) or *E. coli* (Ohnishi *et al.*, 2001) by comparative bacterial genomics. A promising approach to reveal more knowledge about the factors involved in *Salmonella* host range and virulence is to compare and analyse the prophage content of different *Salmonella* serovars based on the available *Salmonella* sequence data, which will be one main objective of this study.

During the course of this study the genomes of the *Salmonella* strains *Salmonella* Enteritidis PT4, *Salmonella* Typhimurium DT104 and SL1344, *Salmonella* Gallinarum 287/91 and *Salmonella* Bongori 12419 were sequenced by the Sanger Institute in Cambridge. The sequencing of the 4,809,037 bp genome of *Salmonella* Typhi CT18 had already been completed and was published by the group that performed the sequencing, including the Sanger Institute (Parkhill *et al.*, 2001). The CT18 strain harbours two plasmids, a 218,150 bp multiple drug resistance incH1 plasmid (pHCM1), and a 106,516 bp cryptic plasmid (pHCM2). The chromosome contains 4,599 coding sequences (CDS), 204 of these are predicted to be pseudogenes, which is a remarkably high number for an organism capable of growing in- and outside of the host. 124 of the pseudogenes have been inactivated by the introduction of a stop codon or single frameshift. 27 are the remains of integrases, insertion sequence (IS) transposases and genes of bacteriophage origin. 75 of the pseudogenes are believed to be involved in housekeeping functions (Parkhill *et al.*, 2001). A lot of mutations have been found in genes probably involved in virulence or host interaction. These include components of seven of the twelve chaperone-usher fimbrial operons (Townsend *et al.*, 2001), genes, which are within or associated with previously described SPIs (Blanc-Potard *et al.*, 1999; Hensel *et al.*, 1999; Tsolis *et al.*, 1999) and genes coding for type-III-secreted effector proteins (Bakshi *et al.*, 2000; Miao and Miller, 2000). With 59 %, a greater proportion of pseudogenes than expected lies within islands unique to *Salmonella* Typhi relative to *E. coli*, as for all genes, this proportion is only 33 % (Parkhill *et al.*, 2001). The inactivation of many of the mechanisms of host interaction resulting from this distribution may be an approach to explain the host restriction of *Salmonella* Typhi compared to other *Salmonella* serovars (Parkhill *et al.*, 2001). In general, the genomes of *Salmonella* Typhi and *E. coli* (Blattner *et al.*, 1997) are essentially collinear along their entire length. Most of the differences are the result of insertions, deletions or replacements, but there are also some cases of small gene blocks being translocated. Among the larger of the 290 blocks containing genes unique to *Salmonella* Typhi compared to *E. coli* are the previously described SPIs 1-5. There are also at least five more islands with the characteristics of SPIs (SPI 6-10) and 7 prophage elements (Parkhill *et al.*, 2001). Additionally, there are many insertions of smaller gene blocks and individual genes, which may be involved in pathogenicity. While the gene clusters unique to *Salmonella* Typhi in comparison to *E. coli* probably contribute to the adaptation to environmental niches and to pathogenicity, the conserved genes may be needed for the basic lifestyle of enteric bacteria that requires intestine colonisation, environmental survival and transmission (Parkhill *et al.*, 2001).

The comparison of *Salmonella* Typhi CT18 with *Salmonella* Typhimurium LT2 (Mc Clelland *et al.*, 2001) shows that *Salmonella* Typhi is much closer related to *Salmonella* Typhimurium than to *E. coli*. Only 13 % of the genes are unique to *Salmonella* Typhi compared to *Salmonella* Typhimurium, and 11 % of the genes are unique to *Salmonella* Typhimurium compared to *Salmonella* Typhi (Parkhill *et al.*, 2001). Analogous with *E. coli*, the differences are not limited to a few large blocks. 42 unique genes are single gene insertions, and 103 genes are located in insertions of 5 genes or less. These unique insertions include the phages ST10, ST15, ST18 and ST48 and SPIs 7, 8 and 10 (Parkhill *et al.*, 2001).

The pHCM1 plasmid with 249 CDS / 8 pseudogenes encodes resistances to multiple drugs, including all of the first-line drugs used for the treatment of typhoid fever. Genes, apparently virulence-associated have not been found on pHCM1 (Parkhill *et al.*, 2001). The second plasmid, pHCM2, contains 131 CDS / no pseudogenes and is phenotypically cryptic. It shares over 56 % of its sequence with the plasmid pMT1 from *Yersinia pestis* (Hu *et al.*, 1998), which encodes the main virulence-associated determinants of *Yersinia pestis*. The CDS unique to pHCM2 show similarities to several bacteriophage genes and genes with direct or indirect involvement in DNA biosynthesis and replication including a gene cluster encoding genes similar to thymidylate synthetase, dihydrofolate reductase, ribonuclease H and ribonucleotide diphosphate reductase and also a putative primosomal gene cluster (Parkhill *et al.*, 2001). These genes form an integral part of the primase replication complex in the bacteriophage T4 (Jing *et al.*, 1999). Plasmids related to pHCM2 have been found in *Salmonella* Typhi strains from Southeast Asia only, while most *Salmonella* Typhi strains do not harbour this plasmid (Parkhill *et al.*, 2001).

The complete genome sequence of *Salmonella* Typhimurium LT2 has been published by a group in the US (McClelland *et al.*, 2001). The genome consists of 4,857,432 bp and harbours a 93,939 bp virulence plasmid (pSLT). The publishing group compared the data with genome data from eight related enterobacteria (*Salmonella* Typhi CT18[1], *Salmonella* Paratyphi A, *Salmonella* Paratyphi B, *Salmonella arizonae*, *Salmonella bongori*, *E. coli* K12[2], *E. coli* O157:H7[3] and *K. pneumoniae*) to determine the distribution of close homologues of the *Salmonella* Typhimurium LT2 genes. The chromosome contains 4,489 CDS, and 55 % of these have close homologues in all eight of the other bacterial genomes that it was compared to (McClelland *et al.*, 2001). In contrast to *Salmonella* Typhi CT18 with 204 pseudogenes,

[1] Parkhill *et al.*, 2001

[2] Blattner *et al.*, 1997

[3] Perna *et al.*, 2001

only 39 pseudogenes have been detected in the genome of *Salmonella* Typhimurium LT2, which might allow *Salmonella* Typhimurium to infect a broader range of hosts compared to *Salmonella* Typhi (McClelland *et al.*, 2001). 145 of the 204 pseudogenes in *Salmonella* Typhi are present as intact genes in *Salmonella* Typhimurium. Only 23 are present as pseudogenes in *Salmonella* Typhimurium as well (Parkhill *et al.*, 2001). Usually, the consequences of the loss of function for the pseudogenes in *Salmonella* Typhimurium are unclear, because the function of the intact homologues in other organisms is unknown. Some pseudogenes may be unrecognised, because there is no intact homologue available for 11 % of the *Salmonella* Typhimurium genome (McClelland *et al.*, 2001). Some genes are only found in *Salmonella*, and 1,106 CDS in this group have a close homologue in at least one of the other five *Salmonella* from the comparative analysis. Many of the pathogenesis-associated genes like invasion genes, genes coding for type-III-secreted proteins and some secretory system genes are in this group (McClelland *et al.*, 2001). In the comparative analysis, close homologues have been found in one or more of the other three subspecies I genomes (*Salmonella* Typhi, *Salmonella* Paratyphi A and *Salmonella* Paratyphi B), but not in the five other genomes including *Salmonella arizonae* and *Salmonella bongori* for 352 CDS, indicating that these may include genes for specialisation of subspecies I to warm-blooded animals. 121 CDS have no close homologues in any of the eight genomes from the comparative analysis. A + T-rich CDS are almost threefold over-represented among those genes with no close homologues outside of subspecies I, indicating that these genes might have been acquired from a A + T-rich source (McClelland *et al.*, 2001). The *Salmonella* Typhimurium LT2 genome contains four functional prophages: Gifsy-1 and -2 and Fels-1 and -2. The comparative analysis showed that these phages are not present in the eight other genomes, but homologues have been found for some of the genes, which are probably parts of related prophages in these genomes. By homology to other bacteriophages, a previously unknown bacteriophage or phage remnant that includes the genes STM4201 and STM4219 has been detected in the *Salmonella* Typhimurium LT2 genome (McClelland *et al.*, 2001).

The pSLT plasmid (Matsui *et al.*, 2001) contains 108 CD / 6 pseudogenes. Close homologues in *Salmonella* Typhi, *Salmonella* Parathyphi A or *Salmonella* Parathyphi B have been found for only three CDS, because these strains do not harbour this plasmid. Close homologues in plasmids from other *Salmonella* serovars have been found for 50 pSLT genes (McClelland *et al.*, 2001).

A group including members of the Pathogen Sequencing Unit at the Sanger Institute, Cambridge, UK has performed an *in silico* analysis of the prophage-like elements harboured

by *Salmonella* Typhi CT18 and compared these against 40 other *Salmonella* isolates by DNA microarray technology utilizing the available sequence data from the sequencing of *Salmonella* Typhi (Deng *et al.*, 2003; McClelland *et al.*, 2001) and *Salmonella* Typhimurium (Parkhill *et al.*, 2001; Thomson *et al.*, 2004). This study indicated *Salmonella* Typhimurium to harbour the lysogenic bacteriophages Gifsy-1, -2 and –3, Fels-2 and the P2-like phage SopE (Figueroa-Bossi *et al.*, 1997; Figueroa-Bossi and Bossi, 1999; Hardt *et al.*, 1998a; Miao and Miller, 1999; Mirold *et al.*, 1999; Thomson *et al.*, 2004). In a detailed bioinformatic analysis displaying regions unique to *Salmonella* Typhi with respect to *Salmonella* Typhimurium seven prophages or prophage-like elements were identified in the *Salmonella* Typhi genome, representing 3.76 % of the genome: ST10, ST15, ST18, ST27, ST35, $SopE_{ST}$ (ST44) and ST46. ST10 and ST18 have extensive regions of sequence similar to lambdoid phages, whereas ST15 is a chimera with similarities to P2-family phages, bacteriophages Mu and lambda. ST46 has a high similarity to the satellite phages of the P4-family (Thomson *et al.*, 2004). It lies within the SPI 10 region of *Salmonella* Typhi CT18 and appears to retain many of the essential genes for phage proliferation (Briani *et al.*, 2001; Pierson and Kahn, 1987; Thomson *et al.*, 2004). The prophage-like elements ST27, ST35 and $SopE_{ST}$ share significant sequence homology with each other and members of the *Myoviridae*. They are very similar to members of the P2-family (Bertani and Bertani, 1971; Thomson *et al.*, 2004). A global comparison of the two sequenced *Salmonella* Typhi genomes (CT18 and Ty2) showed that most of the prophage-like regions are similar for both isolates (Deng *et al.*, 2003; Parkhill *et al.*, 2001; Thomson *et al.*, 2004). Differences include ST18, which is totally absent from *Salmonella* Typhi Ty2 and a novel P4-family prophage (ST2-27), which is only present in *Salmonella* Typhi Ty2. In *Salmonella* Typhi, the location and orientation of ST10 and ST15 has been altered by a chromosomal inversion (Deng *et al.*, 2003; Thomson *et al.*, 2004). Compared to *Salmonella* Typhimurium LT2, ST27, ST35 and $SopE_{ST}$ are similar to the P2-family Fels-2 phage (Parkhill *et al.*, 2001; Pelludat *et al.*, 2003; Pickard *et al.*, 2003; Thomson *et al.*, 2004). $SopE_{ST}$ and ST35 showed high levels of similarity to the sequenced SopE phage from *Salmonella* Typhimurium DT204. The genetic organisation and gene complement of these putative phages most closely matches the *E. coli* bacteriophage 186 (Mirold *et al.*, 1999; Pelludat *et al.*, 2003; Thomson *et al.*, 2004). DNA microarray data showed that all of the *Salmonella* Typhi isolates tested hybridised to the majority of the $SopE_{ST}$-like phage genes, including the *sopE* virulence gene itself (Thomson *et al.*, 2004). A significant level of hybridisation could also be seen for all *Salmonella* Paratyphi A isolates tested (36 out of 46 $SopE_{ST}$ phage genes). Interestingly enough these data suggest that both *Salmonella enterica*

serovars, which are associated with invasive disease in humans, harbour complete $SopE_{ST}$ phage (Thomson *et al.*, 2004). Hybridisation of DNA from other *Salmonella* serovars to the $SopE_{ST}$ genes was limited. *Salmonella* Typhimurium LT2 showed no hybridisation to *sopE*, which is absent from this strain, and weak hybridisation to many of the $SopE_{ST}$ structural and replicative genes, which may be in part caused by cross-hybridisation to the Fels-2 phage, which shows strong similarity on the amino acid level, but only limited similarity on the DNA level (Thomson *et al.*, 2004). Apart from the *Salmonella* Typhi isolates, none of the *Salmonella* serovars showed hybridisation to the ST27 genes. Nevertheless, all tested *Salmonella* serovars, including *Salmonella* bongori, showed strong hybridisation to the *iro*A iron uptake locus, which is located directly downstream of the ST27 invertase gene (Bäumler *et al.*, 1996; Thomson *et al.*, 2004). Some of the *Salmonella enterica* serovars hybridised to regions within ST35. Two *Salmonella* Montevideo isolates and the *Salmonella* Dublin strain S16 hybridised strongly to the ST35 central region (Thomson *et al.*, 2004). Combining these data with the signals obtained for $SopE_{ST}$ genes suggests that these serovars harbour one or more prophages that are hybrids of $SopE_{ST}$ and ST35 (Thomson *et al.*, 2004), an event that has been previously observed in many bacteriophages (Hendrix, 2002; Juhala *et al.*, 2000). ST10 and ST18 have sequence similarities with the lambda-like phages Fels-1, Gifsy-1 and Gifsy-2 (Figueroa-Bossi *et al.*, 1997; McClelland *et al.*, 2001; Thomson *et al.*, 2004). For ST15 only a weak similarity to a prophage-like locus in *Salmonella* Typhimurium LT2 could be detected (McClelland *et al.*, 2001; Thomson *et al.*, 2004). In conclusion, the comparative analysis by Thomson *et al.* revealed that *Salmonella* Typhimurium LT2, *Salmonella* Typhi CT18 and Ty2 harbour a diverse range of prophage-like elements, which add considerable diversity to their genomes. In contrast to other *Salmonella*-specific DNA elements, which are generally conserved between *Salmonella enterica* serovars like SPIs, the unique combination of prophage-like elements distinguishes the genome of *Salmonella* Typhi (Chan *et al.*, 2003; McClelland *et al.*, 2000; McClelland *et al.*, 2001; Parkhill *et al.*, 2001; Thomson *et al.*, 2004). The results of the comparative analysis by Thomson *et al.* and the identified prophage-like elements in *Salmonella* Typhi and *Salmonella* Typhimurium was the basis for the comparative analysis to identify prophage-like elements in the genome of *Salmonella* Enteritidis in this study.

1.9 Objectives of this study

(I) Comparative *in silico* analysis of the genome of *Salmonella* Enteritidis 125109 with the existing *Salmonella* Typhi and *Salmonella* Typhimurium genomes for the presence and organisation of prophages in the genome.

(II) PCR based screening of the prophage content identified in the *in silico* analysis of SE125109 within the same phage type and other phage types in *S.* Enteritidis, and in other *Salmonella* serovars.

(III) Microarray based analysis of the prophage content in different *S.* Enteritidis phage types selected based on the results of the PCR screening.

(IV) Microbiological investigations of spontaneous release and induceability of prophages in *Salmonella* Enteritidis.

The genome comparison will identify sequence fragments in the *S.* Enteritidis genome with high similarity to prophages which might be associated with virulence genes. BLAST and FAST-A searches will be used to identify a possible gene function for these fragments. The results of this analysis will be used for annotation of the prophage regions in the *S.* Enteritidis genome and will contribute to the complete annotation of the *S.* Enteritidis genome performed by the Pathogen Sequencing Unit at the Sanger Institute, Cambridge, UK.
This study should produce a wider knowledge of the prophage content of *S.* Enteritidis and their association with *Salmonella* virulence. In subsequent studies the obtained information can be used for comparison with the genomes of other *Salmonella* serovars like *S.* Gallinarum to produce a wider knowledge and understanding of the mechanisms underlying the different host spectra, disease characteristics and colonisation properties observed in different *Salmonella* serovars. This knowledge can be included in the criteria applied on the selection of candidate vaccine strains and in the development of future, genetically modified *Salmonella* live vaccines with defined virulence properties.
This study is integrated into a scientific network. At the Institute of Animal Health, Compton Laboratory, Compton, Newbury, UK, other members of the group of Paul A. Barrow have used the same *Salmonella* sequence data to look at other aspects of *Salmonella* virulence

including fimbrial genes, SPIs, and genes associated with *Salmonella* energy generation and storage. The entire work is integrated into the SUPASALVAC project (FP7 505523) funded by the European Union, which deals with the production of *Salmonella*-free broilers by live-vaccine induced innate resistance to colonisation and invasion and novel methods to eliminate vaccine and field strains.

2 Material and Methods

2.1 Material

2.1.1 Laboratory Apparatus

Apparatus	Type	Manufacturer
Stereomicroscope	Stemi 2000	Zeiss
Colony Counter		IUL Instruments
Digital Camera	DS-5M	Nikon
Digital Camera Control Unit	DS-L1	Nikon
Gel Electrophoresis Apparatus	Agagel Standard without cooling	Whatman Biometra
Polaroid Camera	DS 34	Polaroid
Power Supply	M 200 / 2.0	Biorad
Spectrophotometer	Lambda 2	PerkinElmer
Steam Pot		Varioklav
Thermocycler	Mastercycler gradient	eppendorf
Thermocycler	T1	Biometra
UV-Transilluminator	TFX-20M	Vilber Lourmat
Centrifuge	Biofuge Fresco	Heräus
Centrifuge	Micro Centaur	Sanyo
Sonicator	Virsonic 300	Virtis
Hybridization Chamber	10 Slide Chamber	Genetix
Microarray Scanner	Axon 4000B	Axon Instruments, Inc.

Table 2-1: Laboratory Apparatus

2.1.2 Software

OLIGO 4.1 Primer Analysis Software, National Biosciences, Inc. Plymouth, MN, USA
Artemis Release 6 (Java2), Genome Research Limited, The Sanger Centre, Hinxton, Cambridge, UK
ACT Artemis Comparison Tool, Release 3, Genome Research Limited, The Sanger Centre, Hinxton, Cambridge, UK
GACK 3.631, Department of Microbiology and Immunology, Stanford University Medical Center, Stanford, CA, USA
Genepix, MDS, Inc., Toronto, Canada
GeneSpring 7.2, Silicon Genetics, Agilent Technologies, Inc, Santa Clara, CA, USA

Table 2-2: Software

2.1.3 Chemicals and Products

Substance	Company
Agarose	Merck KgaA
Cryobank	Mast Diagnostica GmbH
dCTP-Cy3, dCTP-Cy5	GE Healthcare
DNA standard size marker lambda DNA *Hind* φX174 DNA *Hae* III	Finnzymes Oy
Enteroclon anti-*Salmonella* sera	Sifin GmbH
Ethidium bromide 1 % solution	Carl Roth GmbH + Co. KG
TAE buffer 50X, DNA typing grade	GIBCO BRL
DyNAzyme II DNA polymerase	Finnzymes Oy
dNTP Mix	Finnzymes Oy
LifterSlips	Nunc, Thermo Fisher Scientific
Mg^{2+}-free DyNAzyme buffer	Finnzymes Oy
Mg^{2+}-solution	Finnzymes Oy
$MgSO_4$ * 7 H_2O	Merck KgaA
Mitomycin C from *Streptomyces caespitosus*	Sigma-Aldrich
Tryptose Phosphate	invitrogen
Standard I Nutrient Agar	Merck KgaA
Standard I Nutrient Solution	Merck KgaA
Columbia Blood Agar	heipha Dr. Müller GmbH
PCR Primer	Whatman Biometra
NucleoSpin® Tissue Kit	Macherey-Nagel
NaAc	Sigma-Aldrich
Swarm Agar	Sifin GmbH
Trizma Base	Sigma-Aldrich
Trizma HCl	Sigma-Aldrich
BioPrime® DNA Labeling System	invitrogen
AutoSeq G-50 Dye Terminator Removal Kit	GE Healthcare
Membrane filters 0.45 µm	Sartorius
Yeast t-RNA	Sigma-Aldrich

Table 2-3: Chemicals and Products

2.1.4 Salmonella strain collection

To check whether the putative prophage regions identified by *in silico* analysis in the sequenced *S.* Enteritidis strain SE125109 are representative for the whole serovar, a strain collection was set up. This collection contains *S.* Enteritidis strains representing different phage types. For a few isolates however, the phage type was unknown. To make this collection more representative, isolates coming from different sources (species; geographical regions) were included into the collection. It was especially focussed on having phage type 4 isolates from various sources in the collection to be able to analyze if the sequenced strain SE125109 is typical for prophage content of *S.* Enteritidis phage type 4 isolates. To complete the picture, non-Enteritidis isolates both from group D1, which also harbours *S.* Enteritidis and from other groups were added to the strain collection.

The strains in the strain collection were characterized by the methods described below and used for the PCR-based screening for the presence of the putative prophage regions identified in SE125109. Based on the results of the PCRs, a panel of 11 strains was selected for further analysis by DNA microarray as described below. The available information for the strains in the strain collection is displayed in Table 2-4.

Group	Serovar	Strain	PT	Source	Provided by
D1	***S.* Enteritidis**	**125109**	**4**	**human food poisoning**	**Dr. Barrow, IAH, Compton, UK**
	***S.* Enteritidis**	**Leipzig**	**4**	**vaccine parent strain**	**Prof. Linde, University of Leipzig, Germany**
	***S.* Enteritidis**	**VAC E[1]**	**4**	**vaccine strain**	**Lohmann Animal Health, Cuxhaven, Germany**
	S. Enteritidis	FUR Working Seed	4	modified candidate vaccine strain	Dr. Rabsch, RKI, Wernigerode, Germany
	S. Enteritidis	05-00229	4	calf, faeces	Dr. Schroeter, BfR, Berlin, Germany
	S. Enteritidis	04-01518	4	cattle, diagnostic sample	Dr. Schroeter, BfR, Berlin, Germany
	S. Enteritidis	05-00213	4	pig, faeces	Dr. Schroeter, BfR, Berlin, Germany
	S. Enteritidis	05-00264	4	horse, diagnostic sample	Dr. Schroeter, BfR, Berlin, Germany
	S. Enteritidis	04-00319	4	dog, faeces	Dr. Schroeter, BfR, Berlin, Germany
	S. Enteritidis	03-01771-1	4	colubrid, faeces	Dr. Schroeter, BfR, Berlin, Germany
	S. Enteritidis	1004	4	reisolate, chickbox	Dr. Löhren, PHW-Group, Rechterfeld, Germany
	S. Enteritidis	1007	4	reisolate, chickbox	Dr. Löhren, PHW-Group, Rechterfeld, Germany
	S. Enteritidis	03-03058	4	duck	Dr. Rabsch, RKI, Wernigerode, Germany
	S. Enteritidis	02-02864	4	goose	Dr. Rabsch, RKI, Wernigerode, Germany
	S. Enteritidis	125589	4	human food poisoning, invH	Dr. Methner, FLI, Jena, Germany
	S. Enteritidis	1135	4	broiler, liver	Dr. Bolte, Vechta, Germany
	S. Enteritidis	Salmovac SE	4	vaccine strain	IDT, Dessau, Germany
	S. Enteritidis	K482/91	4	layer, ileoceacal tonsil	Prof. Urbaneck, Dessau, Germany
	***S.* Enteritidis**	**04-03158**	**1**	**dog, diagnostic sample**	**Dr. Schroeter, BfR, Berlin, Germany**
	S. Enteritidis	02-07368	1	chicken, meconium	Dr. Löhren, PHW-Group, Rechterfeld, Germany
	S. Enteritidis	02-07381	1	surface swab	Dr. Löhren, PHW-Group, Rechterfeld, Germany
	S. Enteritidis	02-07396	1	chicken, sock swab	Dr. Löhren, PHW-Group, Rechterfeld, Germany
	S. Enteritidis	02-00900	4b	sheep, organ sample	Dr. Schroeter, BfR, Berlin, Germany
	S. Enteritidis	451/02	6a	reisolate, caecum	Dr. Löhren, PHW-Group, Rechterfeld, Germany
	S. Enteritidis	809/02	6a	reisolate, meconium	Dr. Löhren, PHW-Group, Rechterfeld, Germany
	***S.* Enteritidis**	**05-01906**	**8**	**calf, diagnostic sample**	**Dr. Schroeter, BfR, Berlin, Germany**
	S. Enteritidis	03-01087	8	sheep, diagnostic sample	Dr. Schroeter, BfR, Berlin, Germany
	S. Enteritidis	K1298/05	8	chicken, sock swab	Lohmann Tierzucht, Cuxhaven, Germany
	***S.* Enteritidis**	**03-03059**	**9b**	**duck**	**Dr. Rabsch, RKI, Wernigerode, Germany**
	***S.* Enteritidis**	**04-03092**	**11**	**cat, diagnostic sample**	**Dr. Schroeter, BfR, Berlin, Germany**
	***S.* Enteritidis**	**03-03561**	**13a**	**pig**	**Dr. Schroeter, BfR, Berlin, Germany**
	***S.* Enteritidis**	**02-00191**	**20**	**duck**	**Dr. Rabsch, RKI, Wernigerode, Germany**
	***S.* Enteritidis**	**02-06391**	**21**	**duck**	**Dr. Rabsch, RKI, Wernigerode, Germany**
	S. Enteritidis	05-01372	21	horse, faeces	Dr. Schroeter, BfR, Berlin, Germany
	S. Enteritidis	518/02	21	reisolate, sock swab	Dr. Löhren, PHW-Group, Rechterfeld, Germany
	***S.* Enteritidis**	**04-03909**	**21c**	**cattle**	**Dr. Schroeter, BfR, Berlin, Germany**
	S. Enteritidis	86/360	34	broiler-breeder	Dr. Terzolo, INTA, Mar del Plata, Argentina
	S. Enteritidis	1005	na*	reisolate, chickbox	Dr. Löhren, PHW-Group, Rechterfeld, Germany
	S. Enteritidis	1006	na*	reisolate, chickbox	Dr. Löhren, PHW-Group, Rechterfeld, Germany
	S. Enteritidis	7497	na	turkey	Lohmann Tierzucht, Cuxhaven, Germany
	S. Enteritidis	7499	na	turkey	Lohmann Tierzucht, Cuxhaven, Germany
	S. Enteritidis	7661	na	turkey	Lohmann Tierzucht, Cuxhaven, Germany
	S. Enteritidis	1607	na		Lohmann Tierzucht, Cuxhaven, Germany
	S. Enteritidis	K229/63	na		Lohmann Tierzucht, Cuxhaven, Germany
	S. Enteritidis	K482/91	4	layer, ileoceacal tonsil	Prof. Urbaneck, Dessau, Germany
	S. Enteritidis	F971/82 (669)	na	chicken	Lohmann Tierzucht, Cuxhaven, Germany
	S. Gallinarum	K517/94-5	na	nr	Dr. Barrow, IAH, Compton, UK
	S. Eastbourne	S2 (R22)	na	nr	Lohmann Animal Health, Cuxhaven, Germany

Group	Serovar	Strain	PT	Source	Provided by
B	*S.* Typhimurium	576	na	nr	Dr. Barrow, IAH, Compton, UK
	S. Indiana	R1	na	nr	Dr. Zucker, FU Berlin, Berlin, Germany
	S. Saint Paul	898/1	na	nr	Dr. Böhland, Deersheim, Germany
	S. Agona	533-4	na	nr	Dr. Böhland, Deersheim, Germany
	S. Paratyphi B	B 1086/00	na	nr	Dr. Miko, BfR, Berlin, Germany
	S. Stanley	R20	na	nr	Lohmann Animal Health, Cuxhaven, Germany
C1	*S.* Virchow	V1	na	nr	Dr. Müller-Molenar, Köthen, Germany
	S. Infantis	6633	na	nr	Dr. Methner, FLI, Jena, Germany
C2-3	*S.* Hadar	18UM	na	nr	Dr. Methner, FLI, Jena, Germany
	S. Albany	2713	na	nr	Dr. Löhren, PHW-Group, Rechterfeld, Germany
E1	*S.* Anatum	4279	na	nr	Prof. Linde, University of Leipzig, Germany
E4	*S.* Senftenberg	1331/7	na	nr	Dr. Müller-Molenar, Köthen, Germany
I	*S.* Yoruba	322 SII	na	nr	I. Wiebelitz, Möckern, Germany

Table 2-4: *Salmonella* strain collection featuring the *Salmonella* strains used in this study. The isolates printed in bold were selected for the microarray analysis.

[1]AviPro® SALMONELLA VAC E

*** serologically rough; na: information not available; nr: not relevant**

2.1.5 Media

All media were prepared using demineralised water.

Tryptose Phosphate Broth (TPB) (per 1l)

250 mg $MgSO_4$
30 g tryptose phosphate

pH 7.4
autoclave 20 min at 121 ° C

Standard I Nutrient Agar

Typical composition per 1l:

15 g peptones
3 g yeast extract
6 g sodium chloride
1 g D(+) glucose
12 g agar-agar

pH 7.5

Preparation:

37 g Standard I Nutrient Agar per 1l
autoclave 20 min at 121 ° C

The solid agar was stored at 4 ° C for up to 3 months after autoclaving. Before use it was distributed to petri dishes after melting in a steam pot.

A semi-solid Standard I Nutrient Agar containing only 50 % of the agar concentration of the regular Standard I Nutrient Agar was used in the phage induction experiments. To produce this agar, only half of the regular amount of Standard I Nutrient Agar was used, substituted with the corresponding amount of Standard I Nutrient Bouillon.

Typical composition of the semi-solid agar per 1l:

15 g peptones
3 g yeast extract
6 g sodium chloride
1 g D(+) glucose
6 g agar-agar

pH 7.5

Preparation:

18.5 g Standard I Nutrient Agar and
12.5 g Standard I Nutrient Bouillon per 1l
autoclave 20 min at 121 ° C

Columbia Blood Agar

Typical composition per 1l:

23 g special peptone
1 g starch
5 g sodium chloride
50 ml sheep blood
14 g agar

pH 7.3

semisolid

2.2 Methods

2.2.1 Identity testing of the *Salmonella* used for the analyses

All bacterial strains received for the strain collection used for the analyses of this study were tested for their identity. The strains received were inoculated onto blood agar and standard I agar and incubated overnight at 37 ° C. After a visual control of culture purity and morphology, the bacteria were inoculated from the blood agar or the standard I agar plate onto swarm agar and again incubated overnight at 37 ° C. Slide agglutination was performed with the bacteria grown on the swarm agar according the manufacturer's manual. In case of *S.* Enteritidis strains Enteroclon anti-*Salmonella* sera O 9 and H g,m were used. For non-Enteritidis strains Enteroclon anti-*Salmonella* polyspecific group sera were used. Identity testing of the strains by slide agglutination was used as one criterion for inclusion of the strains into this study together with the *Salmonella spp.* PCR and the *Salmonella* Enteritidis PCR described below.

2.2.2 Long term storage of bacteria from the strain collection

The bacteria were stored using a cryobank system according to the manufacturer's instruction at -80 ° C.

2.2.3 Genome comparisons for the analysis of the prophage content in *Salmonella* Enteritidis strain SE125109

To identify prophage regions in the *S.* Enteritidis PT4 genome recently sequenced by the Sanger Centre, Hinxton, Cambridge, UK (http://www.sanger.ac.uk/Projects/Salmonella/), the sequence data of the sequenced strain SE125109 was compared with the published annotated complete genomes of *S.* Typhi CT18 (GenBank accession number: AL513382; Parkhill *et al.*, 2001) and *S.* Typhimurium LT2 (GenBank accession number: AE006468; McClelland *et al.,* 2001). The strain SE125109 chosen for the sequencing was isolated from an outbreak of human foodpoisoning in the United Kingdom that was traced back to a poultry farm. The strain is highly virulent in newly hatched chickens and is also invasive in laying hens,

resulting in egg contamination (Barrow 1991; Barrow and Lovell 1991). The sequencing of the strain is further described in Thomson *et al.*, 2008.

The genome comparisons were based on the identified prophage regions in the genomes of *S.* Typhi CT18 and *S.* Typhimurium LT2 and the results of a comparative *in silico* analysis (Thomson *et al.*, 2004), in which the prophage-like elements harboured by *S.* Typhi CT18 were compared with 40 other S*almonella* isolates by DNA microarray technology.

The annotated *S.* Typhi CT18 and *S.* Typhimurium LT2 genomes were uploaded into the Artemis software (Rutherford *et al.*, 2000) to display the known prophages and their genomic organisation in these genomes. The Artemis software is a tool that allows the visualization and annotation of a DNA sequence, and it is especially useful in analysing the compact genomes of bacteria, *archaea* and lower *eukaryotes.* The results of any analysis or sets of analyses can be viewed in the context of the sequence and its six-frame translation. The software is available under the GNU General Public License from the Sanger Centre website (http://www.sanger.ac.uk/Software/Artemis). It is implemented in Java, and is available for UNIX, GNU/Linux, BSD, Macintosh and MS Windows systems. Sequences and annotation can be read and written directly in EMBL, GenBank and GFF format. It can also read sequences in FASTA or raw format.

All known prophage genes from the *S.* Typhi CT18 and *S.* Typhimurium LT2 genomes were extracted from these genomes and saved in FASTA format using the Artemis software. Each of these genes was then individually blasted against the *S.* Enteritidis PT4 genome using the "blast 2 sequences" tool (Tatusova and Madden, 1999) available from the NCBI homepage (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi) with standard settings to locate similar genes or gene fragments in the raw sequence of the *S.* Enteritidis PT4 genome. In addition to that each of these genes was also blasted against all GenBank, EMBL, DDBJ and PDB sequences using BLASTN 2.2.10 (Altschul *et al.*, 1997) to find similarities to known sequences of other *Salmonella* strains besides *S.* Typhi CT18 and *S.* Typhimurium LT2 including other *S.* Typhimurium and *S.* Typhi strains, already annotated *S.* Enteritidis fragments and other *Salmonella* serovars already present in the database and to other closely related *Enterobacteriaceae*. The Artemis Comparison Tool (ACT) (Carver *et al.*, 2005) was used for the comparison of the *S.* Enteritidis genome with the *S.* Typhi CT18 and *S.* Typhimurium LT2 genomes to identify prophage regions in the *S.* Enteritidis genome, and to analyse the genomic structure of these putative prophages. ACT is a DNA sequence comparison viewer based on Artemis, which allows an interactive visualisation of comparisons between complete genome sequences and associated annotations. The Artemis

components used to display the sequence are powerful tools for searching and analysis. The software is available from the Sanger Centre website (http://www.sanger.ac.uk/Software/ACT/) and distributed under the terms of the GNU General Public License. Like Artemis, ACT is written in Java and runs on UNIX, GNU/Linux, Macintosh and MS Windows systems. It can read complete EMBL and GENBANK entries or sequence in FASTA or raw format. Extra sequence features can be in EMBL, GENBANK or GFF format. The sequence comparison displayed by ACT can be generated with several different programmes; BLASTN, TBLASTX or Mummer comparisons between genomic DNA sequences, or orthologue tables generated by reciprocal FASTA comparison between protein sets. Regions of similarity, insertions and rearrangements at any level from the whole genome to base-pair differences can be identified.

Due to the high similarity of the ΦSE20 prophage region found in SE125109, the genome of SE125109 was also directly compared to the genome of *Salmonella* phage ST64B (GenBank accession number: AY055382) using ACT. To additionally identify other bacteriophages in the *S.* Enteritidis genome that are not present in the genomes of *S.* Typhimurium and *S.* Typhi, a database was set up containing the known bacteriophage sequences from the NCBI-website (http://www.ncbi.nlm.nih.gov/genomes/static/phg.html). This database was used to perform a BLAST analysis against the *S.* Enteritidis genome to detect any of these bacteriophages in this genome. The putative bacteriophage genes and prophage elements detected in the ACT-based genome comparison and the BLAST analyses were annotated in the *S.* Enteritidis PT4 genome using the ACT software. All relevant hits that were obtained in the "blast 2 sequences" analyses and all hits to Enterobacteriaceae that were obtained in the BLASTN analyses were annotated in the *S.* Enteritidis genome in a first step to enable the visualisation of clusters of putative bacteriophage genes. These clusters were then subject to manual curation using gene synteny. ORFs automatically displayed in Artemis in the non-annotated *S.* Enteritidis PT4 genome present in the previously identified clusters of bacteriophage genes were checked for their affiliation with the identified putative prophages.

After identification of the putative prophage regions in *S.* Enteritidis, the G + C content of these regions was analysed using the Artemis software.

Additionally, an analysis to identify pseudogenes and a Pfam-analysis will be performed at the Sanger Institute. Pfam is a large collection of multiple sequence alignments and hidden Markov models covering many common protein families. It is very useful to automatically assign a new protein to an existing protein family, even if the homology is weak (Bateman *et al.*, 2004).

2.2.4 DNA extraction

The NucleoSpin® Tissue Kit from Macherey-Nagel was used for the isolation of DNA from bacteria for use in the PCR applications. The basic principle underlying the DNA extraction with this kit is the reversible binding of DNA to a silica membrane depending on the ion concentration. A protocol modified for the extraction of DNA from bacteria was used.
For the DNA isolation, all bacteria were grown as overnight cultures at 37 ° C under permanent shaking. 9 ml of TPB were inoculated with one bead from the Cryobank system used for the long-term storage of bacteria.
1 ml of the overnight culture was centrifuged for 5 min at 8,000 x g in a Heräus Biofuge Fresco. The supernatant was removed and the pellet was carefully resuspended in 180 µl buffer T1 by pipetting up and down. 25 µl proteinase K solution were added and the mixture was carefully vortexed. The samples were incubated over night at 56 ° C. To remove RNA, 20 µl of a 20 mg/ml RNase solution were added to the samples and after vortexing the samples were incubated at room temperature for 5 min. 200 µl buffer B3 were added to the samples. After vortexing they were incubated at 70 ° C for 10 min. The samples were vortexed briefly, then 210 µl (96-100 %) ethanol were added to the samples and they were vortexed vigorously. The samples were loaded onto NucleoSpin® Tissue columns that were placed into 2 ml collection tubes. The columns were centrifuged for 1 min at 11,000 x g in a Heräus Biofuge Fresco. The flow-through was discarded and 500 µl buffer BW were added in a first washing step. After centrifugation for 1 min at 11,000 x g in a Heräus Biofuge Fresco, the flow-through was again discarded and 600 µl buffer B5 were added in a second washing step. After another centrifugation step with the same parameters, the flow-through was again discarded. To dry the silica membrane another similar centrifugation step followed. To elute the isolated DNA, the NucleoSpin® Tissue columns were placed into a 1.5 ml eppendorf reaction tube and 100 µl elution buffer BE prewarmed to 70 ° C were added onto the column. After incubation at room temperature for 1 min, the DNA was eluted from the columns in a final centrifugation step of 11,000 x g for 1 min in the Heräus Biofuge Fresco.
The DNA concentration was determined as described in 2.2.5. With final DNA concentrations between 45 ng/µl and 60 ng/µl, 1 µl of DNA solution was used in the consecutive PCR applications. The DNA was stored at –20 ° C.

The components of the NucleoSpin® Tissue Kit were prepared and stored according to the manufacturer's manual.

An RNase solution free of DNase was produced according to Sambrook *et al.*, 1989. 100 mg RNase were dissolved in 5000 µl of 0.01 M NaAc solution (pH 5.2). The solution was incubated at 100 ° C for 15 min to deactivate DNases. To adjust the pH-level, 500 µl of a 1 M Tris Cl solution (pH 8.0) were added. Aliquots of the RNase solution were stored at –20 ° C.

2.2.5 Determination of DNA concentration by UV-spectroscopy

The DNA concentration in aqueous solutions was determined by measurement of the absorption at a wavelength of 260 and 280 nm (A 260 and A 280) in QS 1000 quartz cuvettes using a Lambda 2 UV/VIS spectrophotometer (Perkin Elmer). The DNA solutions were diluted 1:50 in H_2O dest. (10 µl DNA solution + 490 µl H_2O dest.). Measurements were made against water as a blank value. Purity of DNA was determined by measuring the A 260 / A 280 ratio. The A 260 / A 280 ratio was expected to be 1.7-2.0 to indicate pure DNA. The DNA concentration was calculated according to Sambrook *et al.*, 1989, using the following formula:

$$[\text{dsDNA}]\ (\mu g/\mu l) = \frac{50 \times \text{Dilution} \times \text{A } 260}{1000}$$

2.2.6 Polymerase Chain Reaction (PCR)[4]

2.2.6.1 General principle

The polymerase chain reaction is an *in vitro* method, which allows the amplification of defined DNA fragments using two specific oligonucleotide primers. The primers hybridize to opposite strands and flank the region of interest in the target DNA. The principle of the PCR technique was first described in 1986 by Mullis *et al.*, and it was first applied to the amplification of human β-globin DNA for the prenatal diagnosis of sickle cell anaemia by a group in the Human Genetics Department at Cetus (Embury *et al.,* 1987; Saiki *et al.,* 1985; Saiki *et al.,* 1986). It is based on a repetitive series of cycles, which involves a heat-related

[4] The PCR process is protected under U.S. Patents 4,683,202, 4,683,195, and 4,965,188, or their foreign counterparts, owned by Roche Molecular Systems, Inc. and F. Hoffmann-La Roche Ltd ("Roche").

template denaturation, the annealing of the primers and the extension of the annealed primers by a thermostable DNA polymerase. Under ideal conditions, the amplification of the target fragment whose termini are defined by the 5' ends of the primers is exponentially.
PCR technique was used in this study for a screening analysis of DNA from different *Salmonella* isolates for the presence of the four prophage regions identified in the genome comparisons: ΦSE10, ΦSE12/ΦSE12A, ΦSE14 and ΦSE20. Additionally, two PCRs, of which one was specific for the genus *Salmonella* and the other one was initially believed to be specific for the serovar *Salmonella* Enteritidis were applied to characterize the strains used in the further analyses.

2.2.6.2 PCRs for the characterisation of the used strains

Two PCRs that are currently being evaluated in collaboration with Alejandra Velilla, Instituto Nacional de Tecnología Agropecuaria (INTA), Balcarce, Buenos Aires, Argentina, were used for the characterisation of the strains in the strain collection.

2.2.6.2.1 *Salmonella spp.* PCR

The *Salmonella spp.* PCR was modified after the PCR described by Way *et al.*, 1993 for the specific detection of *Salmonella spp.* The sequences of the primer pair used for this reaction are shown in Table 2-5. The target for the PCR is the *phoP/phoQ* locus, which is part of the phosphorylation regulon regulating the expression of genes involved in virulence and macrophage survival in *Salmonella* (Miller *et al.*, 1989).

Primer Pair Name	Sequence
PhoPBis Forward	5'-TATGCGCGGTAGCGGCGTGTTGT-3'
PhoBBis Reverse	5`-GGCAATGATCTGCCCGGCGTATTGT-3'

Table 2-5: Primer pair sequence for *Salmonella spp*. PCR

The reactions were performed in a volume of 25 µl in 0.2 ml eppendorf PCR tubes using a Biometra thermocycler T1. The composition of the reaction mixture is shown in Table 2-6.

	Final concentration	Volume per reaction
dd H_2O		**20.25 µl**
10 x buffer $MgCl_2$ free	**1 x**	**2.5 µl**
50 mM $MgCl_2$	**1,5 mM**	**0.75 µl**
PhoPBis Forward	**0,1 µM**	**0.025 µl**
PhoBBis Reverse	**0,1 µM**	**0.025 µl**
25 mM dNTP-Mix	**200 µM**	**0.2 µl**
DyNAzyme	**0,5 U**	**0.25 µl**
template DNA		**1 µl**

Table 2-6: Composition of the reaction mixture for the *Salmonella spp*. PRC

The PCR programme used for the reaction is shown in Table 2-7.

Step	Temperature	Time	Cycles
Initial Denaturation	95 ° C	10 min	1
Denaturation	95 ° C	30 sec	35
Annealing	63 ° C	30 sec	
Elongation	72 ° C	1 min	
Final Elongation	72 ° C	7 min	1

Table 2-7: PCR programme for *Salmonella spp*. PCR

To ensure the reagents used did not contain any contaminations, one reaction without addition of any template DNA was used as a negative control. DNA from the sequenced strain SE125109 was used as a template in another reaction as a positive control to ensure the overall function of the PCR.

2.2.6.2.2 Salmonella Enteritidis PCR

The *Salmonella* Enteritidis PCR was modified after the PCR described by Agron *et al.*, 2001, which was initially believed to be specific for *Salmonella* Enteritidis. The sequences of the

primer pair used for this reaction are shown in Table 2-8. The target for the PCR is the *lygD* gene, which is part of the Sdf I region according to Agron *et al.,* 2001.

Primer Pair Name	Sequence
SdfI SE1063 Forward	5'-TGTGTTTTATCTGATGCAAGAG-3'
SdfI SE1063 Reverse	5'-CGTTCTTCTGGTACTTACGATG-3'

Table 2-8: Primer pair sequence for *Salmonella* Enteritidis PCR

The reactions were performed in a volume of 25 µl in 0.2 ml eppendorf PCR tubes using a Biometra thermocycler T1. The composition of the reaction mixture is shown in Table 2-9.

	Final concentration	Volume per reaction
dd H_2O		**20.25 µl**
10 x buffer $MgCl_2$ free	**1 x**	**2.5 µl**
50 mM $MgCl_2$	**1,5 mM**	**0.75 µl**
SdfI SE1063 Forward	**0,1 µM**	**0.025 µl**
SdfI SE1063 Reverse	**0,1 µM**	**0.025 µl**
25 mM dNTP-Mix	**200 µM**	**0.2 µl**
DYNAzyme	**0,5 U**	**0.25 µl**
template DNA		**1 µl**

Table 2-9: Composition of the reaction mixture for the *Salmonella* Enteritidis PCR

The PCR programme used for the reaction is shown in Table 2-10.

Step	Temperature	Time	Cycles
Initial Denaturation	95 ° C	10 min	1
Denaturation	95 ° C	30 sec	35
Annealing	60 ° C	30 sec	
Elongation	72 ° C	1 min	
Final Elongation	72 ° C	7 min	1

Table 2-10: PCR programme for *Salmonella spp.* PCR

To ensure the reagents used did not contain any contaminations, one reaction without addition of any template DNA was used as a negative control. DNA from the sequenced strain SE125109 was used as a template in another reaction as a positive control to ensure the overall function of the PCR.

2.2.6.3 Development and optimisation of specific PCRs for prophage screening

2.2.6.3.1 Selection of target sequences and design of primers

A PCR-based screening of DNA from different *Salmonella* isolates for the presence of the four previously identified prophage loci ΦSE10, ΦSE12/ΦSE12A, ΦSE14 and ΦSE20 was performed. Three target sequences were chosen for each of the four bacteriophage loci:

1. The 5'-end of the prophage region with primers that amplify a region overlapping the border between the *Salmonella* genome and the 5'-end of the prophage region.
2. The 3'-end of the prophage region with primers that amplify a region overlapping the border between the 3'-end of the prophage region and the *Salmonella* genome.
   Due to an adjustment of the definite location of the putative prophage regions ΦSE12A and ΦSE20, after the PCR primers had been designed and the PCR based screening had been performed, the PCRs targeting the 3'-end in these putative prophage locations are located wholly within the putative prophage location.
3. A region from within the bacteriophage locus with primers that amplify a region of interest conserved in relation to the prophage regions used for the genome comparisons:
   a) the putative conserved effector protein gene *sseI* in ΦSE10 (SEN0916),
   b) the putative conserved effector protein gene *sopE* in ΦSE12 (SEN1155),
   c) a region highly conserved in relation to the *S.* Typhimurium prophage ST18 in ΦSE14 with the genes SEN1385 and SEN1386,

d) the *immC* region, a superinfection immunity system highly conserved in relation to ST64B from *S.* Typhimurium in ΦSE20 (1955).

The primers used for the analyses where designed using the DNA/RNA Primer Analysis Software OLIGO 4.1. Primers were designed to have melting temperatures (Td[5]) around 60 ° C. Primer positions were chosen to produce products sized between 788 and 1,014 bp. The primers were synthesized by Whatman Biometra. A complete list of the primers used with their sequences can be found in Table 2-13.

2.2.6.3.2 Optimisation of PCR conditions

Standard PCRs were performed in a volume of 25 µl in 0.2 ml eppendorf PCR 8-strip tubes. To find the optimal reaction conditions for every set of primers, each set was tested at different $MgCl_2$ concentrations (2 mM, 4 mM and 6 mM) and different annealing temperatures for each of the different $MgCl_2$ concentrations. To test out different annealing temperatures, the eppendorf Mastercycler gradient thermocycler, which allows creating a temperature gradient between the individual reaction tubes, was used. A default temperature of 58 ° C was used with a gradient of 10 ° C. The default temperature was adjusted, if no satisfying results were obtained. This procedure allowed the determination of optimised PCR conditions for each set of primers by choosing those conditions, which gave a good product yield and a specific amplification of the target sequence. See Table 2-11 for the PCR programme used for the optimisation of the annealing temperature.

[5] Oligonucleotide Tm calculated according to the nearest neighbour method by the Oligo 4.1 software.

Step	Temperature	Time	Cycles
Initial Denaturation	94 ° C	4 min	1
Denaturation	94 ° C	30 sec	30
Annealing	58 ° C R= 3.0 ° C / sec G= 10 ° C	30 sec	
Elongation	72 ° C	1 min	
Final Elongation	72 ° C	7 min	1

R= Ramp Increment; G= Gradient

Table 2-11: PCR programme for temperature optimisation

The volumes and final concentrations of the PCR components used for the optimisation of the $MgCl_2$ concentrations are shown in Table 2-12.

	Final concentration	Volume per reaction	Final concentration	Volume per reaction	Final concentration	Volume per reaction
	2 mM $MgCl_2$		4 mM $MgCl_2$		6 mM $MgCl_2$	
dd H_2O		19.25 µl		18.25 µl		17.25 µl
10 x buffer $MgCl_2$ free	1 x	2.5 µl	1 x	2.5 µl	1 x	2.5 µl
50 mM $MgCl_2$	2 mM	1 µl	4 mM	2 µl	6 mM	3 µl
Primer F	1 µM	0.25 µl	1 µM	0.25 µl	1 µM	0.25 µl
Primer B	1 µM	0.25 µl	1 µM	0.25 µl	1 µM	0.25 µl
10 mM dNTP-Mix	200 µM	0.5 µl	200 µM	0.5 µl	200 µM	0.5 µl
DYNAzyme	0.5 U	0.25 µl	0.5 U	0.25 µl	0.5 U	0.25 µl
template DNA		1 µl		1 µl		1 µl

Table 2-12: Composition of the reaction mixture for the optimisation of the $MgCl_2$ concentrations (25 µl reactions)

The primers used for the screening of the bacteriophage loci with the optimal annealing temperatures and $MgCl_2$ concentrations determined in the optimisation experiments described above are shown in Table 2-13.

Primer Pair Name	**Sequence**	**Annealing Temperature**	**$MgCl_2$-Concentration**
SE10_5N_F	5'-TGCACATCATAGTAGTGGTGAA-3'	61.5 ° C	4 mM
SE10_5N_B	5'-TTATAGATAGCGTAAGCCACTTC-3'		
Target: SEN0908A / SEN0909 expected product size: 887 bp			
SE10_3_F	5'-CGGTCAAGATACCAGGTAATAT-3'	61.5 ° C	4 mM
SE10_3_B	5'-TATCACTATTCAAGCAGTTTGC-3'		
Target: SEN0921 expected product size: 984 bp			
SE10_SSEI_F	5'-TGTAAATTTATAAAGGTTTTTTGTT-3'	56.1 ° C	2 mM
SE10_SSEI_B	5'-TGCGCTTACATTTTACCTATTA-3'		
Target: SEN0916 (sseI) expected product size: 999 bp			
SE12_5N_F	5'-GCTTTGTGATCCATCCAATA-3'	58.8 ° C	4 mM
SE12_5N_B	5'-ACCCGGATACCAGAGATTAA-3'		
Target: SEN1131 expected product size: 986 bp			
SE12_3_F	5'-GTTAATACCCACCAGCAGTTC-3'	64 ° C	2 mM
SE12_3_B	5'-GTTACAGGATGCAGTGGATCT-3'		
Target: SEN1170 expected product size: 997 bp			
SE12_SOPE_N_F	5'-GGCTATTATTTTGATGGTTGA-3'	56.1 ° C	4 mM
SE12_SOPE_N_B	5'-TGTACATATAAAAGGAGCATTACC-3'		
Target: SEN1155 expected product size: 892 bp			
SE14_5N_F	5'-TTTCTTCGACGATTTTATATTCT-3'	56.1 ° C	2 mM
SE14_5N_B	5'-GAAGATGGCAAAACATTTATG-3'		
Target: SEN1378 expected product size: 980 bp			

Primer Pair Name	Sequence	Annealing Temperature	MgCl2-Concentration
SE14_3_F	5'-GAAAACACTGGACACACAGAAT-3'	64 ° C	2 mM
SE14_3_B	5'-GCAATACAATATCCGATGATAGT-3'		
Target: SEN1396A / 1398 expected product size: 976 bp			
SE14_CONSERVED_F	5'-CCATTAAGAAAGTTATGACAGTGA-3'	56.1 ° C	4 mM
SE14_CONSERVED_B	5'-ATTTCAACTAGAAGCAAGAATCA-3'		
Target: SEN1385 / SEN1386 expected product size: 991 bp			
SE20_5_F	5'-AGCTTGTGAGCTAAAGAAGATAA-3'	56.1 ° C	4 mM
SE20_5_B	5'-TACCTGATGAAGGCAGAGTAATA-3'		
Target: SEN1919A expected product size: 1,014 bp			
SE20_3_F	5'-GATGTATTGAAAATGAACTGGAA-3'	59 ° C	4 mM
SE20_3_B	5'-AGGTTTACCAGAAGAGGTATAGC-3'		
Target: SEN1966 / SEN1967 expected product size: 788 bp			
SE20_IMMC_F	5'-ACGTGCTGTAACGTATAACCA-3'	56.1 ° C	2 mM
SE20_IMMC-B	5'-GCTCTATGAGTGCAAATTACATT-3'		
Target: SEN1955 expected product size: 966 bp			

Table 2-13: Primers used for bacteriophage screening with optimal annealing temperatures and $MgCl_2$ concentrations

2.2.6.4 Application of the PCR for the screening of prophage presence

The primer-pairs shown in Table 2-13 were used to screen the strains from the strain collection described in 2.1.4 for the presence of the bacteriophages ΦSE10, ΦSE12/ΦSE12A, ΦSE14 and ΦSE20.

The reactions were performed in a volume of 25 µl in 0.2 ml eppendorf PCR tubes using a Biometra thermocycler T1. Depending on the optimised conditions evaluated as described in 2.2.6.3.2, the reaction mixtures for final $MgCl_2$ concentrations of 2 mM and 4 mM shown in

Table 2-12 were used. The respective PCR programmes used for the screening reactions are shown in Table 2-14.

Step	Temperature	Time	Cycles
Initial Denaturation	94 ° C	4 min	1
Denaturation	94 ° C	30 sec	30
Annealing	Depending on respective primer pair (see Table 2-13)	30 sec	
Elongation	72 ° C	1 min	
Final Elongation	72 ° C	7 min	1

Table 2-14: PCR programme for prophage screening

To ensure the reagents used did not contain any contaminations, one reaction without addition of any template DNA was used as a negative control. DNA from the sequenced strain SE125109 was used as a template in another reaction as a positive control to ensure the overall function of the PCR. This template should always give positive results in the PCR with all primer pairs used, as the sequence data used for the design of the respective primers was generated by the Sanger Institute using DNA from this strain.

2.2.6.5 Analysis of the PCR products

2.2.6.5.1 Agarose gel electrophoresis

Agarose gel electrophoresis was used to analyse the obtained PCR products. Gel electrophoresis is a commonly used technique for the analysis of proteins and nucleic acids. Molecules are separated under the influence of an electrical field on the basis of their movement trough a gel. The negatively charged DNA molecules migrate to the anode. The movement of the molecules is slowed down by the agarose gel, which allows their separation by size. The mobility of linear DNA through agarose gels is inversely proportional to the $\log_{10}$ of their molecular weight with shorter molecules moving faster. DNA moves slower in gels with higher agarose concentrations and faster at higher voltages. Corresponding to the

expected size of the PCR products, 1.0 % agarose gels were used for the analysis of the PCR products from the prophage screening experiments and 1.5 % agarose gels for the analysis of the products of the *S.* Enteritidis and *Salmonella spp*. PCR.

To prepare the agarose gels, agarose was mixed with 1 x TAE-buffer to the desired concentration and then heated in a microwave oven until the agarose was completely melted and a clear solution was obtained. After cooling the solution to a temperature of about 60 ° C, the gel was carefully poured into the gel tray equipped with a sample comb. The gel polymerised when it was cooled down to room temperature. After removing the casting gates, the tray was placed in the electrophoresis chamber (Agagel Standard, Whatman Biometra) and covered with 1 x TAE-buffer, which was used as running buffer. For the gel-electrophoretic analysis of the PCR products, 10 µl of each sample were mixed with 5 µl loading buffer. These samples were then loaded onto the gel after the sample comb had been carefully removed from the gel. A ready to use prestained mix of a lambda DNA *Hind* III digest and a φX174 DNA *Hae* III digest provided by Finnzymes was used as a DNA standard size marker. 5 µl of the DNA standard size marker were loaded onto the gel.

Depending on the expected PCR product size and the gel concentration, the following voltages and running times were used:

Bacteriophage screening PCR:

1.0 % agarose gel, expected PCR product size: 788-1,014 bp.
100 V for 30 min

***Salmonella spp.* PCR and *S.* Enteritidis PCR:**

1.5 % agarose gel, expected PCR product sizes: 293 bp for the Sdf I fragment and 412 bp for the PhoPBis fragment.
60 V for 10 min followed by 75 V for 60 min

2.2.6.5.2 Visualization and analysis of the separated DNA fragments

The agarose gels were stained for 30 min in a 0.0001 % ethidium bromide solution after the electrophoretic separation of the DNA fragments. Ethidium bromide is commonly used as a

nucleic acid stain. It intercalates between the bases of the DNA. When exposed to ultraviolet light, it will fluoresce with a red-orange colour, intensifying almost 20-fold after binding to DNA. The gel was placed on an ultraviolet transilluminator (TFX-20M UV-Transilluminator, Vilber Lourmat), which emits UV-light of 312 nm wavelength to visualize the ehtidiumbromide-stained DNA-fragments. The sizes of the PCR products were verified by comparing the products' position on the gel with those of the DNA size marker. Polaroid photographs of the gels were taken for documentation using a DS 34 Polaroid camera.

2.2.6.6 Buffers and solutions used for PCR and agarose gel electrophoresis

Mg^{2+}-free DyNAzyme buffer (10X)

100 mM Tris-HCl
500 mM KCl
1 % Triton X-100

dNTP mix

10 mM dATP
10 mM dCTP
10 mM dGTP
10 mM dTTP

Mg^{2+}-solution

50 mM $MgCl_2$

TAE-buffer (50X stock solution)

2 M Tris-acetate
50 mM EDTA

TAE-buffer (1X)

40 mM Tris-acetate
1 mM EDTA
pH 8.3

Gel loading buffer

40 % (w/v) saccharose
0.25 % (w/v) bromphenolblue
in 6X TAE-buffer

Ethidium bromide staining solution

(0.0001 % solution)

1000 ml 1 x TAE buffer
100 µl 1 % Ethidium bromide solution

DNA standard size marker

lambda DNA *Hind* φX174 DNA *Hae* III (Finnzymes)

Fragment size (in bp): 23.130, 9.416, 6.557, 4.361, 2.322, 2.027, 1.353, 1.078, 872, 603, 564, 310, 281, 271, 234, 194, 125, 118, and 72

2.2.7 Microarray experiments

Microarray technology was used in this study to detect the presence or absence of the genes located in the previously identified putative prophage locations and the genes in their direct vicinity. Additionally, the presence of the genes comprising the prophage locations in *S.* Typhi CT18 and *S.* Typhimurium LT2 in the eleven isolates tested was analysed as well.

2.2.7.1 Overview of application for microarray technology

Microarray technology can be used in gene expression analysis, gene discovery and gene mapping, diagnostics and drug discovery (Anon. 2002, Microarray Handbook).

In differential gene expression analysis, levels of specific transcripts in two or more RNA samples are compared to identify differences in the abundance and identity of the transcripts they contain (Anon. 2002, Microarray Handbook). While one of the samples is the control, the others are derived from cells whose response or status is being investigated. This gives information about the cell state and the activity of genes. Changes in mRNA levels are related to proteome changes as they are precursors of translated proteins (Anon. 2002, Microarray Handbook). The effects of treating cells with chemicals, the consequences of over-expression of regulatory factors in transfected cells and the comparison of mutant strains with parental strains for the discovery of functional pathways have been investigated through differential gene expression (Anon. 2002, Microarray Handbook). Differential gene expression analysis has been applied to all kinds of tissues, plants, yeast and bacteria (Baldwin *et al.*, 1999; Braxton and Bedilion, 1998; Mirnics *et al.*, 2001; Schulze and Downward, 2001; Berkum, van and Holstege, 2001). In gene discovery and gene mapping, microarrays have been utilized in the identification of new genes for the annotation of genomes and in the identification of functional regulatory elements leading to the understanding of gene regulation (Lieb *et al.*, 2001; Shoemaker *et al.*, 2001). In addition to that they have been applied to the analysis of genomic fragments derived from genomic analysis methods like genomic mismatch scanning

and representational difference analysis, and for the prediction of splice variants, the analysis of single nucleotide polymorphisms (SNPs) and mutations, and for sequencing (Drobyshev *et al.*, 1997; Hu *et al.*, 2001; Larsen *et al.*, 2001; Meltzer 2001; Sapolsky *et al.*, 1999). In the field of drug discovery, microarrays have been useful during different stages of the drug discovery process including the identification of potential drug targets and the analysis of their toxic properties and their function modes by examining the expression profiles they induce (Gray *et al.*, 1998; Jain 2000; Lockhardt and Winzeler, 2000; Meltzer, 2001; van Berkum, van *et al.*, 2001).

2.2.7.2 General principle of microarray technology

A DNA-microarray is an ordered collection of microspots, in which each spot contains a single defined species of a nucleic acid. The microarray technique evolved from Southern blotting and has been rapidly adopted as a flexible method for analysing large numbers of nucleic acid fragments in parallel. It is based on the hybridisation of two single-stranded nucleic acid molecules due to sequence complementarity (Anon. 2002, Microarray Handbook; Gabig and Wegrzyn, 2001; Southern *et al.*, 1999; Wikipedia contributors, 2007). In 1995 and 1996 the first papers, in which the term "microarray" was used, were published by the group of P.O. Brown at Stanford University (Schena *et al.*, 1995; Schena *et al.*, 1996). Despite the diversity of technical solutions that have been developed, all microarray systems share the following key components (Anon. 2002, Microarray Handbook):

- an array, which contains immobilized nucleic acid sequences (targets) on a matrix
- one or more labelled samples that are hybridised with the array (probes)
- a detection system to quantify the hybridisation signal.

There are two different types of DNA-microarrays: spotted microarrays and oligonucleotide arrays. Spotted microarrays are also known as two-channel or two-colour microarrays. In spotted microarrays, oligonucleotides, cDNA or small fragments corresponding to mRNAs are immobilised by high-speed robots on a solid surface e.g.: membranes, glass or silicon chips (Gabig and Wegrzyn, 2001; Lockhart *et al.*, 1996; Schena *et al.*, 1995; Schena *et al.*, 1996; Wikipedia contributors, 2007). Spotted microarrays are used for large-scale screening and expression studies in many cases. The sample DNAs used for the spotting of this type of array are amplified by PCR. In prokaryotes chromosomal DNA is amplified by gene-specific primers. The PCR products representing specific genes have a size of about 0.6-2.4 kb. After

purification of the PCR products by precipitation or gel filtration, they can be spotted onto a matrix to produce the array (Gabig and Wegrzyn, 2001).

Oligonucleotide arrays are fabricated either by *in situ* light-directed chemical synthesis or by conventional synthesis followed by immobilisation of the prefabricated oligonucleotides on a glass substrate (Gabig and Wegrzyn, 2001; McGall *et al.*, 1997; Wodicka *et al.*, 1997). These arrays with short nucleotides of up to 25 bp are useful for the detection of mutations and expression monitoring, gene discovery and mapping (Gabig and Wegrzyn, 2001). The oligonucleotides can be designed to distinguish between alternative splicing variants or different alleles of a gene (Anon. 2002, Microarray Handbook).

Membranes commonly used as matrixes are nitrocellulose and charged nylon membranes (Gingeras *et al.*, 1987). Glass-based arrays are usually printed on microscope slides that are coated with poly-lysine, amino silanes or amino-reactive silanes to enhance the hydrophobicity of the slide and the adherence of the deposited DNA (Gabig and Wegrzyn, 2001; Schena *et al.*, 1996). Amino-modified DNA can be attached to slides that have been modified with aldehyde groups (Anon. 2002, Microarray Handbook). In a final step, the deposited DNA is split single-stranded by a heat or alkali treatment (Gabig and Wegrzyn, 2001). The probes to be analyzed, which usually represent pools of cellular RNA or DNA are converted to a labelled population of nucleic acids consisting of several thousands of different labelled nucleic acid fragments that can be hybridized with a microarray and subsequently detected (Gabig and Wegrzyn, 2001; Anon. 2002, Microarray Handbook). Fluorescent dyes, especially the cyanine dyes Cy3 and Cy5 are predominantly used for labelling in microarray analysis. Fluorescent dyes offer a high sensitivity of detection and enable quantitative measurements. Choosing dyes with different pairs of excitation and detection wavelengths gives the opportunity to detect two or more different signals in one experiment to perform comparative analyses of two or more samples on one array. This reduces the experimental error because the hybridization conditions are the same for the samples on the same array (Anon. 2002, Microarray Handbook). The cyanine dyes Cy3 and Cy5 belong to a family of fluors that consist of a chemically-related group of fluorescent dyes whose emission spectra span the spectrum of visible light. They share a core structure, which consists of two heterocyclic indocyanine ring structures joined by a polymethine bridge. This bridge is different in each of the dyes, additional pairs of conjugated C atoms in the chain result in a wavelength shift of approximately 100 nm. The absorbance maximum is at 550 nm for Cy3 and at 649 nm for Cy5. Cy3 and Cy5 have become the most commonly used pair of Cy dyes because they give bright fluorescent signals and possess a high photostability. Their

fluorescence is only minimally affected by factors such as pH level or the presence of DMSO. Because of their good spectral separation, each can be excited at a different wavelength and their emissions can be detected separately (Anon. 2002, Microarray Handbook). The excitation efficiency is usually highest when the dye's absorption maximum correlates closely with the excitation wavelength of the imaging system (Anon. 2002, Microarray Handbook).

Accurate information can only be obtained from microarray experiments if the labelled nucleic acids hybridize to their complementary target efficiently and with high specificity. The length of the labelled fragments strongly influences the efficiency and specificity of the hybridization reaction with probes consisting of fragments of 200-500 bp length giving optimal results (Anon. 2002, Microarray Handbook). To label samples for gene expression microarray analysis, several strategies based on molecular biology or chemical reactions have been developed. They must neither be biased towards any nucleotide sequences nor label differently transcripts of different sizes or sequences that are expressed at different levels so that all information present in the original transcript population are still present in the labelled form (Anon. 2002, Microarray Handbook). Labelling strategies for expression analysis have in common that they start with an RNA population. Only a small proportion of about 1.5 - 2.5 % of the cellular RNA is mRNA. As most of the cellular RNA is ribosomal RNA, specific techniques are used in most cases to separate mRNA from ribosomal RNA prior to labelling and hybridisation (Anon. 2002, Microarray Handbook).

Labelling strategies based on molecular biology utilise enzymes to convert mRNA into new populations of RNA or DNA. One strategy is to convert an mRNA population into a labelled first-strand cDNA population by copying the transcripts into cDNA molecules in a reaction catalyzed by a reverse transcriptase. Modified CyDye nucleotides are incorporated into the newly synthesized cDNA. In an alternative strategy, mRNA is converted into first-strand cDNA containing aminoallyl-dUTP. After elimination of mRNA templates, the amine groups in the cDNA are reacted with CyDye-NHS esters to generate a fluorescently labelled cDNA. The smaller aminoallyl nucleotides used in this cDNA post labelling method are more efficiently incorporated than CyDye nucleotides resulting in a higher yield and longer fragments. A more random attachment of labels compared to the first-strand DNA labelling method is achieved with this method because the sequences of the cDNAs being labelled do not have a major impact on the labelling outcome. Besides that the labelling process is independent of the structure of different fluorescent dyes resulting in an equal labelling intensity for each dye when different dyes are used. The yield of the labelled probe, the optimal labelling density and an equal labelling with different flours are critical factors in the

preparation of labelled probes for microarray analysis. To degrade the RNA template after cDNA synthesis RNase H can be used to digest the RNA component of the RNA-DNA heterohybrid. Alternatively the RNA strands can be degraded by raising the pH of the probe solution. The degradation is necessary to prevent the labelled probe from hybridizing with the original template instead of the microarray target during the microarray hybridization (Anon. 2002, Microarray Handbook). Oligo(dT) primers can be used in labelling reactions to select mRNA from total RNA as they will hybridize with the poly-A-tail in the transcripts. This method will result in only one copy of cDNA that contains primarily 3' sequences synthesized from each transcript. It is not suitable for bacterial mRNA as it lacks poly-A tails. To produce probes that contain sequences that are derived from all parts of transcripts, random primers can be used. Both priming strategies can also be combined. Another option is the use of specific primers, but this is more cost intensive, as a new set of primers has to be prepared for each different microarray. However, an advantage of this method is that only those sequences analyzed on the microarray are labelled. When using a chemical labelling method, mRNA can be directly labelled by coupling of modified CyDye reagent to RNA molecules. No RNA modification is required before labelling (Anon. 2002, Microarray Handbook). To label DNA a modified random prime labelling method can be used. This is a practical solution for genomic microarrays, although direct chemical labelling methods can be used as well. The use of random prime labelling methods is not recommended because of the necessity of two different enzymes to convert mRNA into a labelled form (Anon. 2002, Microarray Handbook). After labelling it is necessary to purify the labelled nucleic acids regardless of the labelling strategy to remove unincorporated fluorescent dye. The labelled probes can than be hybridized with the microarray.

The hybridization process involves the annealing of a single-stranded nucleic acid to a complementary target strand. The binding of the labelled probe molecules to the sample on the slide highlights complementary sequences. The signal intensity is proportional to the amount of immobilized sample. The target molecules must be in excess of the corresponding labelled probe because the hybridization signal will be saturated otherwise (Anon. 2002, Microarray Handbook). The procedure usually starts with a pre-hybridization step, in which the spotted slide is incubated in a buffer in the absence of probe. During pre-hybridization, badly adhered target is removed. It might otherwise wash off during hybridization and then hybridize with probe in solution, which decreases the hybridization signal. The pre-treatment ensures that the target is available for hybridization through its denaturating condition. Additionally pre-hybridization is used to block all sites on the slide surface that could bind the

probe non-specific to lower the background (Anon. 2002, Microarray Handbook). The hybridization procedure can be carried out either using automated instruments or as a manually performed process. In this case usually the coverslip method is used. The hybridization buffer containing a known amount of labelled probe is placed on the slide surface and carefully covered with a coverslip. If two or more colours were used for the labelling of the probe, it is important to use exactly the same amount of probe labelled with each dye. This prevents the results from being in favour of one of the probes. Hybridization buffers normally vary in their composition but usually contain a buffering component to stabilize the pH level, a detergent, which allows the buffer to flow easily under the coverslip through a reduced surface tension, and compounds that act as rate enhancers and volume excluders or speed up the hybridisation and lower the T_m (Anon. 2002, Microarray Handbook). To prevent non-specific hybridization of the probe to common genetic elements, probe blocking should be performed. Usually blocking agents are added to the hybridization buffer containing the labelled probe prior to the application of the buffer to the slide. The solution should be heated to denature any double-stranded DNA. The blocking can then take place before the hybridization reaction is started. The slide is incubated in a humid environment to prevent the evaporation of the hybridization buffer for up to 16 h afterwards. The probe is in contact with the targets on the slide and will adhere to the target if the sequence homology is good. After completion of the hybridization, the slides are washed to remove probes with little or no homology and buffer. Normally SSC/SDS solutions of different concentrations are used for the post-hybridization washes. The primary wash solutions have high salt content and remove most of the hybridization buffer components. The consecutive wash solutions with a lower salt content should remove loosely bound probes and any remaining salt from the primary wash. Labelled probes with high homology will remain attached to the target and are available for detection (Anon. 2002, Microarray Handbook).

After hybridization and washing, the fluorescent signals of the labelled probes bound to individual spots on the array can be detected with a confocal laser scanner. To scan microarrays that were hybridized with probes labelled with dyes with different pairs of excitation and detection wavelengths like the cyanine dyes Cy3 and Cy5, dual-wavelength confocal laser scanners are used. Wavelengths of 532 nm and 635 nm are required for Cy3 and Cy5 respectively (Iida and Nishimura, 2002). The separately scanned images of each of the two probes are subsequently combined and coloured by means of a computer software. The scanned signal intensities of Cy3 and Cy5 should be at the same level to allow an accurate comparison of two samples. Due to in most cases different RNA or DNA starting

volumes of the labelled samples, the signal intensities of Cy3 and Cy5 must be adjusted to be as close as possible using sets of positive control genes.

To simplify the normalization of signal intensities between samples, an adjustment of scanning levels is usually made during the scanning process (Iida and Nishimura, 2002). The hybridized microarray should be scanned immediately after the washing because the fluorescent dyes loose signal intensity with time. Especially in the case of Cy5, the fluorescent signal intensity can also decrease due to the repeated scanning of the microarray (Hal, van *et al.*, 2000). To enable the identification of experimental errors in microarray experiments, the slides should contain replicate spots of each target. As stated above, microarray experiments should contain a series of controls to ensure the accuracy of the obtained data. DNA from organisms that are only distantly related to the organisms being studied is spotted onto the microarray slides as a negative control. This DNA should not hybridize with any labelled probe and therefore not produce any signal at all (Anon. 2002, Microarray Handbook). DNA labelled with CyDye flours is spotted onto the array as a positive control to verify the efficient binding of the target DNA to the slide surface during the hybridization and washing. Placing positive controls on different locations of the slides eases the spotfinding process by providing clearly detectable signals in known positions regardless of the probes used (Anon. 2002, Microarray Handbook). Housekeeping genes, which are expressed relatively consistently can be included in microarray experiments as controls to ensure proper hybridization. They can also be used as a normalization factor. Besides using different nucleic acid starting volumes in the labelling reaction, also relative incorporation levels of Cy3- and Cy5-labelld nucleotides during the labelling reaction and differences in the efficiency of detection of Cy3 and Cy5 by the detection system within the scanner can lead to differences between the relative Cy3 and C5 signals from one slide to another. To be able to compare ratio data between slides, the ratio data needs to be normalized to correct for experimental variations (Anon. 2002, Microarray Handbook). Two different approaches are usually used as normalization strategies (Duggan *et al.*, 1999). When a general normalization method is applied, all target genes are considered for normalization (Hardwick *et al.*, 1999; Ross *et al.*, 2000). When a large-scale microarray consisting of thousands of genes is used, the Cy3 / Cy5 ratios are very likely to show a “bell-shaped curve” A similar situation for the Cy3 / Cy5 ratio can be expected when two probes are derived from closely related samples. The transcriptional levels of many genes are expected to be unchanged in this case. When divergent samples are compared, the transcriptional level may become more varied possibly resulting in a deviated distribution of the Cy3 / Cy5 rations towards one

sample. The same can happen when a small-scale microarray with hundreds or fewer genes is used. In such cases, a second approach, which is based on sets of selected normalization control spots such as housekeeping genes should be used (Iida and Nishimura, 2002; Lashkari *et al.*, 1997; Loftus *et al.*, 1999; Stephan *et al.*, 2000).

2.2.7.3 Microarray based analysis of prophage content in different *S.* Enteritidis phagetypes

A *Salmonella* microarray as described below was used in this study to further investigate eleven isolates from the *Salmonella* strain collection. These were chosen based on the results of the PCR screening and are marked in bold in Table 2-4, which shows the strain collection. Strains that belonged to different phage types and showed varying patterns in the PCR analysis were selected. Additionally three different isolates belonging to the phage type 4 and including the vaccine strain AviPro® SALMONELLA VAC E and its parent strain Leipzig were included into the panel of the eleven strains to analyse if there is homogeneity of the prophage content within the same phage type and if there are differences between the vaccine strain and its parent strain in the putative prophage locations.

2.2.7.3.1 Salmonella Microarray

The PCR-product spotted *Salmonella* Microarray Generation IV constructed at the Sanger Institute, Cambridge, UK was used for the microarray analyses in this study. The *Salmonella* Microarray Generation IV is an extension of the previously described *Salmonella* Microarray Generation I and III (Anjum *et al.*, 2005; Bishop, *et al.*, 2005; Cooke *et al.*, 2007, Thomson *et al.*, 2004). The Generation IV array includes additional coding sequences from the *Salmonella* genomes being sequenced at The Wellcome Trust Sanger Institute (http://www.sanger.ac.uk/Projects/Salmonella/). Therefore it is an essentially non-redundant array containing features representing the following nine genomes: *S.* Typhi CT18, *S.* Typhi Ty2, *S.* Typhimurium LT2, *S.* Typhimurium DT104, *S.* Typhimurium SL1344, *S.* Enteritidis 125109, *S.* Gallinarum 287/91, *S.* Paratyphi A ATTC9150, and *S. bongori* 12419.

2.2.7.3.2 DNA labelling

The microarray experiments were performed as dye-swap experiments to prevent any bias in the data from uneven labelling. Each sample of interest and the reference DNA (SE125109) was labelled once with Cy3 and once with Cy5. The DNA used for the microarray experiments was isolated with the NucleoSpin® Tissue Kit as described in 2.2.4. A modified random-primed oligo-labeling method was used to label the DNA with Cy dyes as described below.

A total of 2 µg DNA were diluted with TE to a total volume of 21 µl. The samples were sonicated on ice for 10 sec at level 2 with a Virsonic 300 sonicator (Virtis) to fragmentize the DNA and then mixed with 20 µl of 2.5X random primer solution followed by an incubation of the reaction mixtures at 100 ° C for 5 min. The tubes with the reaction mixtures were then snap-chilled on ice for 5 min. After spinning down the reaction mixtures for 15 sec at 2000 rpm in a Micro Centaur centrifuge (Sanyo), they were placed back on ice where 5 µl of 10X dNTP mix were added. 3 µl of the respective Cy-labelled dCTP (dCTP-Cy3 or dCTP-Cy5) were added to each tube. The exposure of the Cy dyes to light was kept to a minimum. 1 µl of Klenow enzyme (from BioPrime® DNA Labeling System) was added to each reaction mixture. Klenow polymerase incorporates Cy3- or Cy5-labelled dCTP in a DNA synthesis reaction that is primed with random octamer primers. After spinning down for 15 seconds at 2000 rpm (Micro Centaur centrifuge), they were incubated at 37 ° C for 2 h protected from light. 5 µl of stop buffer (from BioPrime® DNA Labeling System) were added to each reaction mixture. To purify the labelled nucleic acids AutoSeq G-50 columns (GE Healthcare) were used. To prepare the columns the resin was resuspended by gentle vortexing. After snapping of the bottom closure and loosening of the cap by one-quarter turn, the columns were placed in collections tubes for support and then centrifuged for 1 minute at 2,000 x g (Micro Centaur centrifuge). The column was then placed in a 1.5 ml eppendorf reaction tube and the labelled nucleic acids to be purified were slowly applied to the centre of the angled surface of the compacted resin bed. Special care was taken not to disturb the resin and not to allow any of the liquid to flow around the sides of the bed. The purified sample was collected at the bottom of the tube after spinning of the column for 1 min at 2,000 x g (Micro Centaur centrifuge). The labelled experimental DNA was pooled with the labelled reference DNA. Afterwards 1/10 volume of 3M NaAc pH 5.2 (11 µl) and 3 volumes of ethanol (100 %, RT, 363 µl) were added, the components were mixed and then stored at -70 ° C for 30 min to precipitate the DNA. After centrifugation at RT for 10 min at 13,000 rpm (Micro Centaur

centrifuge), the supernatant was removed and 100 µl of ethanol (70 %, 4 ° C) were added to the purple pellet. Following a spin of 5 min at 13,000 rpm (Micro Centaur centrifuge), most of the supernatant was removed and a quick spin step of 5 sec was performed before the remaining supernatant was carefully removed with a pipette. The pellet was left at the air at room temperature to dry for 5 minutes. 30 µl of hybridization buffer and 6 µl of yeast t-RNA were added to each pellet, which was then resuspended with a pipette. The hybridization mixtures were placed in a temperature block at 100 ° C for 5 minutes and then left to cool down at RT for 10 minutes. After spinning the tubes for 15 sec to remove evaporated liquid from the lids they were gently vortexed.

2.2.7.3.3 Slide hybridization

The microarray slide and the LifterSlips to be used were cleaned immediately before use with pressurized air to remove dust. The hybridization mixture was transferred onto the middle of an inverted clean 60 x 25 mm LifterSlip. A microarray slide was then slowly lowered with the DNA side onto the LifterSlip carefully avoiding bubbles and misplacement.

A Whatman filter paper with the inside dimensions of a Genetix hybridization chamber was moistened with 1.5 ml of 15 x SSC and then placed in the chamber. The microarrays were then put into the hybridization chamber and incubated at 49 ° C for 16 h in a hybridization oven.

After the hybridization the microarrays were removed from the hybridization oven and immediately placed into a staining jar filled with wash solution 1 (2 x SSC, filter sterilized). The LifterSlip was left to fall off by itself. The microarray slides were then put into a slide rack and washed in a staining jar filled with 100 ml of solution 1 at room temperature for 5 min with gentle shaking. The microarray slides in the slide rack were then transferred to wash solution 2 to be washed for 5 min at 65 ° C with gentle shaking as before. After repeating this washing step once with fresh wash solution 2, the microarray slides in the slide rack were transferred to wash solution 3 to be washed for 5 min at 65 ° C with gentle shaking. This washing step was repeated once with fresh wash solution 3. The microarray slides were then dried by centrifugation in the slide rack at 1200 rpm for 3 min and stored protected from light to be scanned.

2.2.7.3.4 Microarray data acquisition

The microarray slides were scanned using an Axon 4000B scanner and the Genepix software was used to quantify the signal intensities. Quality control software features were routinely used. For each of the two fluorophores used, a separate scan was done and the images were then combined for analysis. A bounding box, fitted to the size of the DNA spots was placed over each array element. A scatter plot was visualized before normalization for the quick and easy comparison of slide replicates (1 forward and 1 reverse slide). Data from spots that were marred by dust particles or hybridization artefacts were excluded from further analysis. A gal file containing the feature name and any comment related to its synthesis (ID column, "A" for PCR failure") as well as its coordinates in the array was created by M. Fookes and A. Ivens at the Sanger Institute, Cambridge, UK and loaded into the software. For each hybridised slide, a set of two tif files (one for each channel), a settings file (gps) and a results file (gpr) were created.

2.2.7.3.5 Microarray data analysis and validation

The Genepix results file (gpr) for each slide was slightly modified. Those array features, for which the percentage of pixels greater than two standard deviations (2SD) were below 85 % in at least one of the channels were labelled as marginal "M" in the flags column.
The modified gpr files were imported into GeneSpring 7.2 (Silicon Genetics), a software package designed to display and analyze microarray data. For normalization, for each array feature the median pixel intensity for the local background was subtracted from the median pixel intensity of the feature independently of their status as being flagged "A" for bad or "M" for marginal. The slides were marked as "Forward" or "Reverse" with "Forward" representing cy5/cy3; test strain/control reference) for dye swap transformation. The intensities of the test strain per feature or spot were divided by those of the control strain and finally normalised per slide to the median. For all values of the control reference below 0.001 the value of 0.001 was used instead. Three commonly applied methods were used for the analysis of the normalized ratio data to determine the presence or absence of the respective genes (Cooke *et al.*, 2007; Witney *et al.*, 2005):

a) Twofold cut off:

An arbitrary cut off of twofold was used for the identification of those genes present or absent in the tested strain in relation to the reference strain. The twofold cut off is the default on the GeneSpring microarray analysis software V7.2 (Silicon Genetics). For all strains the upper cut off was set at a ratio of 2 and the lower ratio at a ratio of 0.5. All results with a ratio below 0.5 were deemed to be absent or divergent to the reference strain, for all other results the genes were deemed to be present.

b) 3SD:

Instead of using a fixed-value cut off for all arrays as described above, a cut off based on the variation in the ratio data of the core genes was determined for each strain. The subset of coregenes, defined as those genes being present in all strains analysed, was determined by identifying those genes with a ratio between 0.5 and 2 on every single array. A total of 3625 coregenes was determined for the strains analysed in this study. The standard deviation of ratios for genes within the subset of coregens was calculated for each strain to measure variation in the data. Then the ratio cut offs were set at 3 standard deviations (3SD) on either side of the median value for each strain. For each test strain, the standard deviation was calculated independently. The values that have been determined for the individual strains are shown in Table 2-15:

Strain	PT	M	3SD	M+3SD	M-3SD
125109	**4**	0.99700005	0.29561043	1.29261048	0.70138962
Leipzig	**4**	0.98568227	0.34385855	1.32954082	0.64182372
VAC E[1]	**4**	0.99820102	0.35932268	1.35752370	0.63887833
04-03158	**1**	0.99237950	0.32878321	1.32116271	0.66359629
03-01906	**8**	0.99450055	0.43643306	1.43093361	0.55806749
03-03059	**9b**	0.99874554	0.33747977	1.33622531	0.66126576
04-03092	**11**	1.01138960	0.32175189	1.33314149	0.68963771
03-03561	**13a**	0.99863550	0.34268979	1.34132529	0.65594571
02-00191	**20**	0.99136540	0.44272596	1.43409136	0.54863944
02-06391	**21**	0.99735770	0.34822164	1.34557934	0.64913606
04-03909	**21c**	0.99263288	0.39117163	1.38380450	0.60146125

Table 2-15: Values determined for median (M); 3 standard deviations (3SD), and 3 standard deviations on either side of the median (M+3SD and M-3SD). As these figures were used for actual calculations, 8 decimal places are displayed in the table.

[1]AviPro® SALMONELLA VAC E

For all ratios above the M-3SD values the genes were deemed to be present, while for the other ratios they were deemed to be absent.

c) GACK

The gene calling software GACK was used as the third method for the analysis of the normalized ratio data. The programme is based on $\log_2$ ratios with trinary analysis and uses the distribution of the ratio data for each strain to classify genes based on the probability that a gene is either present or absent/divergent (Kit *et al.*, 2002).

The gene calling status has been determined by these three methods and was then recorded for each of the methods in a binary way with 1 indicating a present gene and 0 indicating an absent or divergent gene.
For the analysis of the obtained data, the gene calling statuses for the putative prophage genes present in SE125109 and the genes in the direct vicinity of the putative prophage locations were extracted from the overall data set of almost 143,000 individual results for all strains analysed by microarray in this study. Additionally, the gene calling statuses for those genes comprising the prophage locations in *S.* Typhi CT18 and *S.* Typhimurium LT2, which were also used for the *in silico* analysis of the prophage content in SE125109, were also extracted from the overall data set for all strains analysed by microarray in this study.

2.2.7.4 Buffers and solutions used for the microarray experiments

TE-buffer pH 8.0

10 mM Tris-HCl
1 mM EDTA

2.5X random primer solution (component of invitrogen BioPrime® DNA Labeling System)

125 mM Tris-HCl pH 6.8
12.5 mM $MgCl_2$
25 mM 2-mercaptoethanol
750 µg/ml oligodeoxyribonucleotide primers (random octamers)

10X dNTP mix

1.2 mM dATP
1.2 mM dGTP
1.2 mM dTTP
0.6 mM dCTP
in
10 mM Tris pH 8.0
1 mM EDTA

Klenow Fragment (large fragment of DNA Polymerase I)
(component of invitrogen BioPrime® DNA Labeling System)

40 U/µl Klenow Fragment in
50 mM potassium phosphate pH 7.0
100 mM KCl
1 mM DTT
50 % Glycerol

Stop buffer (component of invitrogen BioPrime® DNA Labeling System)

0.5 m EDTA pH 8.0

Hybridization buffer

5 x SSC
6 x Denhardt's solution
60 mM TrisHCl pH 7.6
0.12 % sarkosyl
48 % formamide
sterile filtered

Wash solution 1	Wash solution 2	Wash solution 3
2 x SSC sterile filtered	0.1 x SSC 0.1 % SDS sterile filtered	0.1 x SSC sterile filtered

2.2.8 Phage release and induction experiments

2.2.8.1 Analysis of the inducibility of temperate bacteriophages

To investigate the inducibility of the temperate prophages identified in the genome comparisons, chemically and physically inducing agents were applied to duplicate samples of the sequenced strain SE125109 that was used for the genome comparisons in this study. As a control, a pair of samples without exposure to inducing agents was incubated accordingly.
A modified version of the soft-top agar overlay technique described by Adams (Adams, 1950) was used to detect bacteriophages released after induction of the temperate bacteriophages present in the strain investigated.

2.2.8.1.1 Culture conditions for strains used in the induction experiments

The strain used for the induction experiments (SE125109) and the indicator strain (STm 576) used for the detection of bacteriophages released after induction of the temperate bacteriophages present in the strain investigated were grown as overnight cultures at 37 ° C under permanent shaking. 9 ml of TPB were inoculated with one bead from the Cryobank system used for the long-term storage of bacteria. As a control for media sterility, TPB without inoculation was incubated similarly.

2.2.8.1.2 Induction of the temperate bacteriophages

2.2.8.1.2.1 Chemical induction using mitomycin C

Mitomycin C was used as a chemical inducer in the induction experiments according to the method described by Mirold *et al.* (Mirold *et al.*, 1990). A mitomycin C stock solution was prepared according to 2.2.8.1.3. 150 µl of the fresh overnight culture described in 2.2.8.1.1 were diluted in 1.5 ml TPB supplemented with 3 µl of the mitomycin C stock solution (final mitomycin C concentration: 2 µg/ml) and incubated at 37 ° C for 6 h. One duplicate pair of each sample was incubated under permanent shaking, while another duplicate pair was incubated without shaking.

2.2.8.1.2.2 Physical induction using UV light

UV light with a wavelength of 312 nm was used for the physical induction of the temperate bacteriophages present in the strain investigated. 150 µl of the fresh overnight culture described in 2.2.8.1.1 were diluted in 1.5 ml TPB and transferred to a 55 mm dish. The petri dishes were irradiated with UV light for 30 sec by placing them on the TFX-20M UV-Transilluminator. The samples were incubated at 37 ° C for 6 h afterwards.

2.2.8.1.3 Preparation of mitomycin C stock solution

Mitomycin C stock solution (2 µg/µl)
5 mg Mitomycin C were dissolved in 2.5 µl ddH_2O. 50 µl aliquots were stored at – 20 ° C.

2.2.8.1.4 Preparation of purified phage lysates

After incubation, the samples were processed by centrifugation at 10,000 x *g* for 5 min at 4 ° C. Filtration trough 0.45 µm membrane filters then purified the supernatant. The lysates were stored in 1.5 ml eppendorf tubes at 4 ° C. To test the sterility of the lysates after the membrane filtration, material was streaked out onto Columbia blood agar plates with 10 µl

inoculation loops. The blood agar plates were incubated for 48 hours at 37 ° C. If no growth could be detected on the plates after this incubation time, the lysates were considered to be sterile.

2.2.8.1.5 Preparation of the soft top test agar

A semi-solid Standard I Nutrient Agar was prepared according to 2.1.5. The molten agar was cooled down to a temperature of 45 ° C. 2 ml of the liquid agar were substituted with 100 µl of a fresh overnight culture of the *Salmonella* Typhimurium strain STm 576. The mixture was poured into 55 mm petri dishes to become solid.

2.2.8.1.6 Analysis of the phage lysates on soft top test agar

To test the purified phage lysates described in 2.2.8.1.4 for released phages, 5 µl each of the purified supernatants to be tested were spotted onto the soft top test agar plates described in 2.2.8.1.5 at four different locations as shown in Figure 2-1. The plates were incubated overnight at 37 ° C. They were then checked at each of the positions where phage lysates had been spotted onto the plates for the formation of plaques within the bacterial lawn that had grown during the overnight incubation.

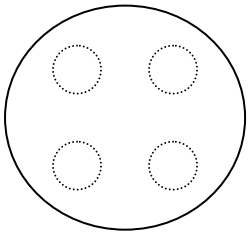


Figure 2-1: Positions of the phage lysate spots on the soft-top test agar.

2.2.8.2 Analysis of the spontaneous release of temperate bacteriophages

The spontaneous release of temperate bacteriophages from the strains analysed in the microarray experiments was assayed by a spot test method modified from Schickelmaier and

Schmieger based on the classic Fisk method (Fisk, R.T., 1942; Schickelmaier and Schmieger, 1995).

2.2.8.2.1 Culture conditions for the spontaneous release of temperate bacteriophages

All strains used for the assay were grown as overnight cultures at 37 ° C under permanent shaking. 9 ml of TPB were inoculated with one bead from the Cryobank system used for the long-term storage of bacteria. As a control for media sterility, TPB without inoculation was incubated similarly.

2.2.8.2.2 Preparation of purified phage lysates

To prepare purified phage lysates, 1.5 ml of the overnight cultures with the strains to be tested and the control without inoculation were each purified by filtration trough 0.45 µm membrane filters. The lysates were stored in 1.5 ml eppendorf tubes at 4 ° C. To test the sterility of the lysates after the membrane filtration, material was streaked out onto Columbia blood agar plates with 10 µl inoculation loops. The blood agar plates were incubated for 48 hours at 37 ° C. If no growth could be detected on the plates after this incubation time, the lysates were considered to be sterile.

2.2.8.2.3 Cross-screening for the susceptibility of strains for phage infection and detection of the released phages

To test strains for their susceptibility for phage infection, 1 ml of the overnight culture of the strain to be tested was transferred onto a 92 mm Standard-I agar plate and evenly distributed on the plate by carefully agitating the plate into all directions. After the overnight culture solution had permeated into the plate for 5 min, the excess volume of solution was removed from the plate. The plates were now given 20 min to dry on their surface.
To test the purified phage lysates described in 2.2.8.2.2 for spontaneously released phages that are capable of infecting other *Salmonella* strains, the lysates were applied onto the Standard-I-agar plates coated with the overnight cultures to be tested as described above. Up to 20 positions where the lysates to be tested could be spotted onto the plates were marked. At

each of these position 20 µl of the purified phage lysate to be tested or the control was spotted onto the dried Standard-1-agar plate as shown in Figure 2-2, and the lysate was given time to dry. The plates with the lysate spots were incubated overnight at 37 ° C. They were then checked at each of the positions where phage lysates had been spotted onto the plates for the formation of plaques within the bacterial lawn that had grown during the overnight incubation. After keeping the plates at room temperature for up to 72 hours, they were checked for the formation of plaques again. In accordance with Fisk (1942), the phage action can sometimes be seen following the initial incubation but is most often detectable more clearly after the plates have been kept at room temperature. The plates were checked for the formation of plaques with a colony counter (IUL Instruments) and a binocular (Zeiss). Photo documentation was made using a Nikon DS-5M digital camera.

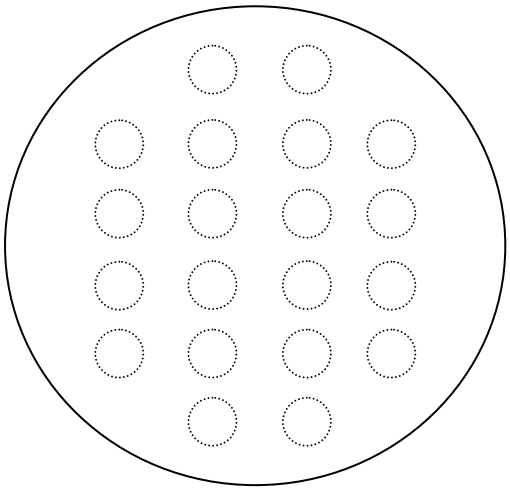


Figure 2-2: Positions of the phage lysate spots on the agar plate.

3 Results

3.1 Genome analysis

The genome data generated in this work was integrated into the full annotation of the *S.* Enteritidis PT4 genome done by "The Pathogen Sequencing Unit", The Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge, UK and submitted to EMBL under the accession number *S.* Enteritidis PT4 genome AM933172. The overall results of the full annotation of the *S.* Enteritidis PT4 genome and its comparison to *S.* Gallinarum 287/91 have been published in Genome Research (Thomson *et al.*, 2008) and the publication includes results from this study.

Four clusters of putative bacteriophage genes were detected in the genome of *S.* Enteritidis PT4. These led to the identification of 5 prophage-like elements as a result of the genome comparisons described above. The *S.* Enteritidis prophages were named ΦSE10, ΦSE12, ΦSE12A, ΦSE14 and ΦSE20 after their position in the genome and are described in detail below. The position of the identified prophage regions are displayed in the Circular representation of the *S.* Enteritidis PT4 chromosome published in Thomson *et al.*, 2008. Orthologues of the CDS present in the *S.* Enteritidis prophages described below that are present in non-prophage locations in *S.* Typhimurium or *S.* Typhi are not mentioned unless there is evidence for genes of importance being taken up by these prophage regions as they are often only single scattered remnants found isolated in the genome.

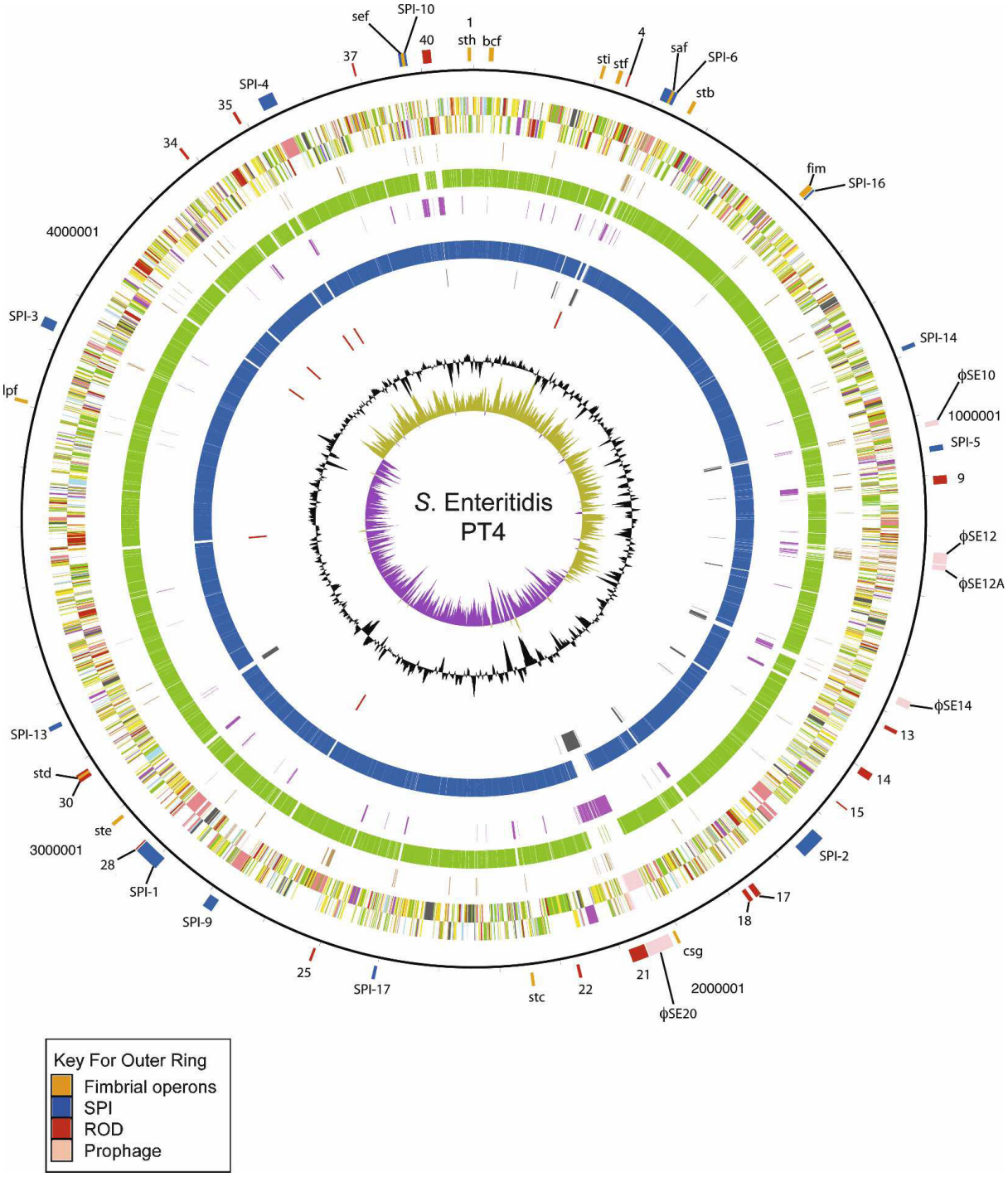


Figure 3-1: Circular representation of the *S.* Enteritidis PT4 chromosome (from Thomson *et al.*, 2008): From the outside in, the *outer* circle 1 marks the position of regions of difference. Circle 2 shows the size in base pairs. Circles 3 and 4 show the position of CDS transcribed in a clockwise and anti-clockwise direction, respectively (for colour codes see below); circle 5 shows the position of *S.* Enteritidis PT4 pseudogenes. Circles 6 and 8 show the position of *S.* Enteritidis PT4 genes that have orthologs (by reciprocal FASTA analysis) in *S.* Typhimurium strain LT2 (all CDS coloured green) and *S.* Gallinarum strain 287/91 (all CDS coloured blue), respectively. Circles 7 and 9 show the position of *S.* Enteritidis PT4 genes that lack orthologs (by reciprocal FASTA analysis) in *S.* Typhimurium strain LT2 (all CDS coloured pink) and *S.* Gallinarum strain 287/91 (all CDS coloured gray), respectively. Circle 10 shows the position of *S.* Enteritidis PT4 rRNA operons (red). Circle 11 shows a plot of G + C content (in a 10-kb window). Circle 12 shows a plot of GC skew ([G _ C]/[G + C]; in a 10-kb window). Genes in circles 3 and 4 are colour-coded according to the function of their gene products: dark green, membrane or surface structures; yellow, central or intermediary metabolism; cyan, degradation of macromolecules; red, information transfer/cell division; cerise, degradation of small molecules; pale blue, regulators; salmon pink, pathogenicity or adaptation; black, energy metabolism; orange, conserved hypothetical; pale green, unknown; and brown, pseudogenes.

3.1.1 ΦSE10

The prophage remnant ΦSE10 extends over a range of 8,186 bps (1013381-1021566) and is predicted to encode 8 intact CDS (coding sequences) and 5 pseudogenes (see Table 3-1). ΦSE10 is similar in parts to regions of the lambda-like phage Gifsy-2, but also harbours one pseudogene (SEN0914) with similarity to a gene encoding a putative tail fibre assembly protein (STM2705) in the P2-like phage Fels-2 from *S.* Typhimurium. Orthologues of some of the genes present in Gifsy-2 that are found in ΦSE10 are also carried by other prophages present in *S.* Typhimurium or *S.* Typhi. In these cases they are also listed in Table 3-1. Two genes in ΦSE10 (SEN0910 and SEN0912) encode hypothetical phage proteins for that no putative function could be assigned by the applied comparison methods.

CDS	genome location	putative gene function	orthologue genes	carried by
SEN0908A pseudogene	1013381..1013578	phage integrase (remnant) similar to *E. coli* putative transposase	STM1005 STY1011	Gifsy-2 ST10
SEN0909 pseudogene	1013579..1013812	prophage exodeoxyribonuclease (remnant) similar to enterohemolysin 1 in *E. coli*	STM1008.S STM2633.S STY2074	Gifsy-2 Gifsy-1 ST18
SEN0910	1013812..1014334	hypothetical phage protein		
SEN0912	1014335..1015051	hypothetical phage protein		
SEN0912A	1015048..1016757	chimeric prophage tail protein (the product of a deletion event)	STM0926 STM1049 STM2588	Fels-1 Gifsy-2 Gifsy-1
SEN0913	1016757..1017338	phage tail fibre assembly protein	STM0927 STM1050 STM2586 STM2704	Fels-1 Gifsy-2 Gifsy-1 Fels-2
SEN0914 pseudogene	1017342..1017557	putative tail fibre assembly protein (remnant)	STM2705 STY1073 STY2013	Fels-2 ST10 ST18

CDS	genome location	putative gene function	orthologue genes	carried by
SEN0916	1017816..1018784	*sseI* putative type III secreted protein	STM1051	Gifsy-2
SEN0916A pseudogene	1018882..1019300	putative insertion sequence protein (remnant)	STM1052	Gifsy-2
SEN0917	1019432..1020058	hypothetical phage protein	STM1053	Gifsy-2
SEN0918 pseudogene	1020127..1020366, 1020368..1020421	hypothetical phage protein	STM1054	Gifsy-2
SEN0920	1020418..1021104	*gtgE* prophage-encoded virulence factor	STM1055	Gifsy-2
SEN0921	1021375..1021566	*gtgF* prophage-encoded virulence protein	STM1056	Gifsy-2

Table 3-1: ΦSE10 gene content

3.1.2 ΦSE12/ΦSE12A

The prophage remnants ΦSE12 and ΦSE12A are located adjacent to each other. ΦSE12 is predicted to be 17,753 bps in size (1226471-1244223) and to encode 17 intact CDS and 8 pseudogenes (see Table 3-2). Like ΦSE10, ΦSE12 is similar in parts to regions of the lambda-like phage Gifsy-2. Orthologues of some of the genes present in Gifsy-2 are also carried by other prophages present in *S.* Typhimurium or *S.* Typhi. In these cases they are also listed in Table 3-2. The 5'-end of ΦSE12 seems to have a different origin as it shows more similarity to a lambda-like ST18 phage from *S.* Typhi. Two genes in ΦSE12 (SEN1134 and SEN1140) encode hypothetical phage proteins for that no putative function could be assigned by the applied comparison methods. One gene (SEN1136) shows a weak similarity to a putative phage protein from ST10, another gene (SEN1139) shows a weak similarity to the phage antitermination protein Q found in Gifsy-1. The very 3'-end of ΦSE12 harbours the

sopE gene found in the P2-like phage SopE in *S.* Typhimurium and a pseudogene encoding a fragment of a putative site-specific DNA invertase that has probably been truncated following the acquisition of the adjoining *sopE* gene locus. Only separated by one gene conserved between different *Salmonella* serovars that encodes a hypothetical protein (SEN1157), ΦSE12A lies directly adjacent to ΦSE12. ΦSE12A is predicted to extend over a range of 8040 bps (1246083-1254122) and to encode 4 intact CDS and 8 pseudogenes (see Table 3-2). The genes and pseudogenes identified in ΦSE12A display a mosaic of genes from related bacteriophages (ST10, ST18, Gifsy-1, Gifsy-2 and Fels-1). There is a strong similarity between ΦSE12A and the CS 40 island in *S.* Typhimurium. One gene in ΦSE12A (SEN1163) encodes a putative phage membrane protein for that no similarity in the phages used for the comparisons was found, and the two pseudogenes SEN1171 and SEN1171A do only show similarities to the CS 40 island in *S.* Typhimurium mentioned above but not to any of the phages used for the comparisons.

CDS	genome location	putative gene function	orthologue genes	carried by
SEN1131	1226471..1227550	putative integrase similar to many bacteriophage integrases e.g. bacteriophage HK022 integrase	STY2077	ST18
SEN1132	1227525..1227803	putative excisionase similar to phage excisionases e.g. *E. coli* excisionase xis, and bacteriophage lambda excisionase xis	STY2076	ST18
SEN1133	1228217..1230196	putative phage-encoded hydrolase	STY2004	adjacent to 5'-end of ST18
SEN1134	1230217..1230453	putative phage membrane protein		

CDS	genome location	putative gene function	orthologue genes	carried by
SEN1135	1230491..1230613	*hokW* phage-encoded Hok-like membrane protein	STY2054 (similar to cell-killing genes toxin/antitoxin system comprised of two overlapping transcriptional units (*hok/mok*); this CDS is equivalent to *hok* (host cell killing))	ST18
SEN1136	1230831..1231133	hypothetical phage protein	STY1033 (weak similarity)	ST10
SEN1137	1231197..1231796	hypothetical phage protein	STY2052 STM1020	ST18 Gifsy-2
SEN1138 pseudogene	1231868..1232020	ninG protein (remnant)	STY1035 STM1021 STM2619	ST10 Gifsy-2 Gifsy-1
SEN1139	1232150..1232839	phage antitermination protein Q	STM2617 (weak similarity)	Gifsy-1
SEN1140	1232930..1233460	hypothetical phage protein		
SEN1141 pseudogene	1233694..1233744	hypothetical phage protein	STM1024 STY1038 STY2045	Gifsy-2 ST10 ST18
SEN1142	1233834..1234283	putative phage lipoprotein	STM1025 STY1039	Gifsy-2 ST10

CDS	genome location	putative gene function	orthologue genes	carried by
SEN1143	1234644..1235330	*pipA* putative bacteriophage-encoded virulence protein	STM1026 (gtgA) STM2614 (gogA) (*pipA* present on SPI-5 in many *Salmonella* serovars)	Gifsy-2 Gifsy-1
SEN1144	1235591..1235935	putative bacteriophage holin	STM1027 STM2613	Gifsy-2 Gifsy-1
SEN1145	1235919..1236371	*nucD* putative phage lysozyme	STM1028 STM2612	Gifsy-2 Gifsy-1
SEN1146	1236389..1236868	putative phage lysozyme	STM1029 STM2611.S STM0908	Gifsy-2 Gifsy-1 Fels-1
SEN1147 pseudogene	1237076..1237387	putative phage terminase, small subunit (remnant)	STM1030	Gifsy-2
SEN1148 pseudogene	1237389..1237646	*ompX* phage attachment and invasion protein (remnant)	STM1043 STM0920	Gifsy-2 Fels-1
SEN1149	1237763..1238296	*sodCI* phage-encoded superoxide dismutase [Cu-Zn] precursor	STM1044	Gifsy-2
SEN1150	1238386..1239081	phage minor tail protein	STM1045 STM2592 STM0921	Gifsy-2 Gifsy-1 Fels-1
SEN1151 pseudogene	1239091..1239351	phage tail assembly protein (remnant)	STM1046 STM2591 STM0922	Gifsy-2 Gifsy-1 Fels-1
SEN1152 pseudogene	1239351..1241717, 1241719..1242585	phage tail fibre protein involved in host recognition (remnant)	STM1048/1048.1N STM2589 STM0925.S	Gifsy-2 Gifsy-1 Fels-1

CDS	genome location	putative gene function	orthologue genes	carried by
SEN1154 pseudogene	1242641..1242934	*ycdD* tail fibre assembly protein (remnant)	STM1050 STM2586 STM0927 STM2704 STY1075	Gifsy-2 Gifsy-1 Fels-1 Fels-2 ST10
SEN1155	1243136..1243858	*sopE* type III secretion system, secreted effector protein	STY4609	SopE
SEN1156 pseudogene	1244065..1244223	putative site-specific DNA invertase (fragment)	STY1075 STY1643 STY4608	ST10 ST15 SopE
End of ΦSE12				
SEN1157	1244338..1245138	conserved hypothetical protein		
Beginning of ΦSE12A				
SEN1158 pseudogene	1246083..1246978	*intE* putative phage integrase (remnant)	STY2077	ST18
SEN1160 pseudogene	1247057..1247556	*recE* exodeoxyribonuclease VIII (remnant)	STM1009 STY2632 STY2073	Gifsy-2 Gifsy-1 ST18
SEN1161 pseudogene	1247719..1247907, 1247911..1247967	phage-encoded chitinase protein (remnant)	STM0907 STY1042	Fels-1 ST10
SEN1162	1247964..1248497	exported phage protein	STM0908 STY1043	Fels-1 ST10
SEN1163	1248754..1248921	putative phage membrane protein		
SEN1164	1249229..1249720	putative phage terminase; small subunit	STM0909	Fels-1
SEN1165 pseudogene	1249707..1249863, 1249865..1250274	putative phage terminase; large subunit (remnant)	STM0910 STM1031	Fels-1 Gifsy-2

CDS	genome location	putative gene function	orthologue genes	carried by
SEN1167 pseudogene	1250279..1252325	*mig-3* this CDS has been subject to multiple deletion events and contains sequence resembling phage tail assembly protein (remnant) and phage tail collar protein (remnant)	STM1049 STM1050 STM2586 STM2587 STM2588 STM0926 STM0927 STM2706 STY3691	Gifsy-2 Gifsy-2 Gifsy-1 Gifsy-1 Gifsy-1 Fels-1 Fels-1 Fels-2 ST35
SEN1170	1252422..1252622	*pagK* phage-encoded *pagK* (*phoPQ*-activated protein)	STM2585A	Gifsy-1
SEN1171 pseudogene	1253079..1253201	transposase (remnant)		
SEN1171A pseudogene	1253530..1253696	*pagM* phage-encoded *pagM* (*phoPQ*-activated protein)		
SEN1171B pseudogene	1253979..1254122	putative DNA invertase (remnant)	STY1075	ST10

Table 3-2: ΦSE12 gene content

3.1.3 ΦSE14

ΦSE14, the third prophage-like region in the *S.* Enteritidis genome, is predicted to extend over a distance of 12,642 bps (1469390-1482031) and to encode 18 intact CDS and 3 pseudogenes (see Table 3-3). While most of the genes code for putative phage proteins and show similarities to genes of the lambda-like phage ST18 from *S.* Typhi, there are also some genes harboured in ΦSE14, for which no significant database hits revealing similarities to known genes were found. Additionally, one gene (SEN1394) with some similarity to a gene found in ST10 is present in ΦSE14. A homologue of the *lygE* gene was found in ΦSE14 but

not included into the final annotation of SE125109 by the Sanger Institute. It has been included into Table 3-3 for information.

CDS	genome location	putative gene function	orthologue genes	carried by
SEN1378 (pseudogene)	1469415..1469723	putative phage integrase (remnant)		
SEN1379	1469680..1470303	putative phage-encoded exodeoxyribonuclease (*lygA*)		
SEN1380	1470325..1470642	predicted phage protein (*lygB*)		
SEN1381	1470727..1470948	FtsZ inhibitor protein (*kil/ydaD*)	STY2070	ST18
SEN1382	1471371..1471907	putative phage membrane protein (*lygC*)		
SEN1383	1472555..1473022	predicted phage protein (*lygD*)		
SENXXXX*	1473126..1473479	(*lygE*)		
SEN1384	1473295..1473624	putative phage-encoded DNA-binding protein (*lygF*)	STY2060	ST18
SEN1385	1473786..1474340	putative phage membrane protein	STY2058	ST18
SEN1386	1474337..1475269	predicted phage protein	STY2057	ST18

CDS	genome location	putative gene function	orthologue genes	carried by
SEN1387	1475696..1475851	regulatory peptide whose translation enables *hokC* expression; small toxic peptide (*hokC*)	STY2054 (similar to cell-killing genes toxin/antitoxin system comprised of two overlapping transcriptional units (*hok/mok*); this CDS is equivalent to *hok* (host cell killing))	ST18
	1475639..1475851	regulatory peptide whose translation enables *hokC* expression (MokW); regulator of *hokC*	STY2054A (host cell-killing modulation protein, similar to cell-killing genes toxin/antitoxin system comprised of two overlapping transcriptional units (*mok/hok*); this CDS is equivalent to *mok* (modulation of host cell killing))	ST18
SEN1388	1476127..1476312	predicted phage protein		

CDS	genome location	putative gene function	orthologue genes	carried by
SEN1389	1476375..1476974	hypothetical protein	STY2052	ST18
SEN1390	1476974..1477264	putative bacteriophage protein	STY2051	ST18
SEN1391	1477141..1477797	hypothetical protein	STY2050	ST18
SEN1392	1479296..1479544	predicted phage protein		
SEN1393	1479968..1480363	putative bacteriophage protein		
SEN1394	1480457..1480744	putative prophage membrane protein	STY1041	ST10
SEN1395	1480741..1481286	conserved phage protein		
SEN1396	1481283..1481492	putative phage-encoded exported protein		
SEN1396A pseudogene	1481726..1481776	part of a duplicated sequence		
SEN1398 pseudogene	1481828..1482031	putative lambdoid prophage *rac* integrase		

***(not included into annotation by Sanger Institute)**

Table 3-3: ΦSE14 gene content

3.1.4 ΦSE20

The prophage ΦSE20 is predicted to be 40,664 bps in length (2018460-2059123) and to encode 51 CDS (see Table 3-4). It is highly similar in sequence and gene order to the *S.* Typhimurium DT64 lambda-like phage ST64B. An intact version of the SER t-RNA present at the 3'-end of ΦSE20 is repeated as a fragment at the 5'-end of ΦSE20 probably as a consequence of the insertion of the phage. While the 5'-end of ΦSE20 shows almost no differences to ST64B, some genes different to ST64B but within the same gene order were identified in the 3'-part of ΦSE20. Some of the genes in ΦSE20 show similarities to genes harboured by the *S.* Typhimurium phage ST10. Single genes in ΦSE20 also show similarities to genes from bacteriophages Gifsy-1, P22, P27, ΦK02 and SfV. Orthologues of some of the genes present in ST64B that are found in ΦSE20 are also carried by other prophages present in *S.* Typhimurium or *S.* Typhi. In these cases they are also listed in Table 3-4. Five genes in

ΦSE20 (SEN1945, SEN1946, SEN1957, SEN1957A and SEN1959) encode hypothetical phage proteins for that no putative function could be assigned by the applied comparison methods. Two additional putative prophage genes which were identified in ΦSE20 by their high similarity in sequence and gene order to ST64B were not included into the final annotation of SE125109 by the Sanger Institute. These putative genes have been included into Table 3-4 for information.

A transposase of probable phage origin which is carried by the *S.* Gallinarum strain 287/91 as well, is present close to the 5'-end of ΦSE20 (SEN1915). The 3'-end of ΦSE20 is flanked by a pseudogene coding for a phage integrase remnant (SEN1968) also carried by the *S.* Gallinarum strain 287/91. Another gene present at this location (SEN1970) codes for a phage integrase which is again also present in the *S.* Gallinarum strain 287/91. It shows partial similarity to a gene (STY4821) carried by the *S.* Typhi phage ST64 and another gene carried by *S.* Typhi outside a phage location (STY4680).

CDS	genome location	putative gene function	orthologue genes (putative function in ST64B)	carried by
SEN1919A	2018787..2019008	phage-encoded DNA-binding protein	**sb27** (DNA invertase pin protein) STY1075	ST64B ST10
SEN1920	2019221..2020228	phage protein	*sseK3* **sb26** (hypothetical protein)	ST64B
SEN1921	2020513..2021082	putative phage tail protein	**sb25** (probable tail fibre assembly protein) STM1050 STM2704 STM2705	ST64B Gifsy-2 Fels-2 Fels-2

CDS	genome location	putative gene function	orthologue genes (putative function in ST64B)	carried by
SEN1922	2021082..2022644	putative phage tail fibre protein	sb24 (tail protein) STM1049 STM2706 STY1072 STY2014	ST64B Gifsy-2 Fels-2 ST10 ST18
SEN1923	2022631..2023218	putative phage tail protein	**sb23** (putative tail protein)	ST64B
SEN1924	2023221..2024300	phage protein	2 genes in ST64B: sb22 (putative tail protein) sb21 (putative head assembly protein)	 ST64B ST64B
SEN1925	2024293..2024706	phage protein	**sb20** (putative tail protein)	ST64B
SEN1926	2024711..2025244	putative phage baseplate protein	sb19 (putative base plate assembly protein)	ST64B
SEN1927	2025244..2026302	phage tail protein	sb18 (tail protein)	ST64B
SEN1928	2026299..2027639	hypothetical protein	sb17 (tail/DNA circulation protein)	ST64B
SEN1929	2027673..2029601	phage tape-measure protein	sb16 (tail protein)	ST64B
SEN1930	2029686..2030012	phage protein	sb15 (hypothetical protein)	ST64B
SEN1931	2030009..2030365	phage tail tube protein	**sb14** (tail tube protein)	ST64B

CDS	genome location	putative gene function	orthologue genes (putative function in ST64B)	carried by
SEN1932	2030365..2031861	phage tail sheath protein	sb13 (tail sheath protein)	ST64B
SEN1932A	2031851..2032015	bacteriophage SfV hypothetical protein	**sb12** (hypothetical protein)	ST64B
SEN1933	2032019..2032579	phage protein	sb11 (hypothetical protein)	ST64B
SEN1934	2032576..2033088	phage protein	sb10 (hypothetical protein)	ST64B
SEN1935	2033060..2033464	phage protein	sb9 (hypothetical protein)	ST64B
SEN1935A	2033461..2033784	phage protein	sb8 (hypothetical protein)	ST64B
SEN1936	2033864..2035093	bacteriophage SfV phage major capsid protein	gene size and order similar to sb6 (major capsid protein precursor)	ST64B
SEN1937	2035103..2035705	phage pro-head protease	gene size and order similar to sb5 (pro-head protease)	ST64B
SEN1938	2035698..2036948	phage portal protein	gene size and order similar to sb4 (portal protein)	ST64B
SEN1939	2037064..2038794	bacteriophage P27 phage terminase (large subunit)	gene size and order similar to sb2 (terminase large subunit)	ST64B
SEN1940	2038794..2039234	putative phage terminase (small subunit)	gene size and order similar to sb1 (terminase small subunit)	ST64B
SEN1941	2039378..2039728	phage protein	sb56 (hypothetical protein)	ST64B

CDS	genome location	putative gene function	orthologue genes (putative function in ST64B)	carried by
SEN1942	2039752..2040291	putative exported phage protein	STY1043	ST10
SEN1943	2040288..2040905	putative phage-encoded lysozyme	sb52 (lytic enzyme [putative glycohydrolase]) STM0907 STY1042	ST64B Fels-1 ST10
SENXXXX*	2040905..2041186	putative prophage membrane protein	gene size and order similar to sb51 (lysis protein [holin]) STY1041	ST64B ST10
SEN1944	2041173..2041562	putative prophage membrane protein	STY1040	ST10
SEN1945	2041651..2042223	phage membrane protein		
SEN1946	2042236..2043327	exported phage protein		
SEN1947	2043359..2044111	putative prophage antitermination protein	STY1036	ST10
SEN1948	2044125..2045114	phage protein	two genes in ST64B: sb47 (hypothetical protein) sb48 (hypothetical protein)	 ST64B ST64B
SEN1949	2045122..2045982	phage protein (similar to KilA in the N-terminal region)	sb46 (hypothetical protein)	ST64B
SEN1949A	2045999..2046388	*rusA* (crossover junction endodeoxyribonuclease *rusA*)	sb45 (holiday-junction resolvase)	ST64B

CDS	genome location	putative gene function	orthologue genes (putative function in ST64B)	carried by
SEN1950	2046397..2047278	putative DNA methylase	STY1014 gene size and order similar to sb44 (putative DNA methyltransferase)	ST10 ST64B
SEN1951	2047275..2047748	phage protein	gene size and order similar to sb43 (putative transcriptional activator)	ST64B
SEN1952	2047745..2048719	phage protein	gene order similar to sb42 (putative replication protein)	ST64B
SEN1953	2048937..2050094	phage immunity protein	sb41 (putative antirepressor)	ST64B
SEN1954	2050091..2050645	putative phage-encoded DNA-binding protein	sb40 (hypothetical protein)	ST64B
SEN1955	2050996..2051691	phage-encoded transcriptional regulator	sb38 (regulatory protein)	ST64B
SEN1957	2052158..2052409	phage protein		
SEN1957A	2052419..2052880	phage protein		
SEN1959	2052979..2053896	phage-encoded recombination associated protein		
SEN1960	2053991..2054530	phage protein	**sb35** (hypothetical protein)	ST64B
SENXXXX*	2054601..2054831	putative phage protein	**sb34** (hypothetical protein)	ST64B
SEN1961	2054828..2055343	phage protein	sb33 (hypothetical protein)	ST64B

CDS	genome location	putative gene function	orthologue genes (putative function in ST64B)	carried by
SEN1962	2055340..2055699	bacteriophage P22, *Salmonella* phage epsilon34, phage protein	STM2623 at 3'-end of gene also short region with similarity to: STM1017 STY1024 STY1025	Gifsy-1 Gifsy-2 ST10 ST10
SEN1963	2055971..2056264	bacteriophage ΦKO2, phage protein		
SEN1964	2056261..2057124	phage protein	STY1027 gene size and order similar to sb31 (hypothetical protein)	ST10 ST64B
SEN1965	2057121..2057690	phage protein	sb30 (endodeoxyribonuclease)	ST64B
SEN1965A	2057715..2057957	phage protein	**sb29** (hypothetical protein)	ST64B
SEN1966	2057959..2058948	phage integrase	**sb28** (integrase protein)	ST64B

***(not included into annotation by Sanger Institute)**

Table 3-4: ΦSE20 gene content; orthologues in ST64B printed in bold represent a 100 % identity in amino acid sequence.

3.1.5 G + C content

Results of the G + C content analysis of the putative prophage regions identified in *S.* Enteritidis 125109 in comparison to that of the prophage regions in *S.* Typhi CT18 and *S.* Typhimurium LT2 are displayed in Table 3-5:

***S.* Enteritidis 125109**	
prophage	G + C content
ΦSE10	45.81 %
ΦSE12	48.86 %
ΦSE12A	48.74 %
ΦSE14	45.15 %
ΦSE20	50.59 %
host G + C content	52.17 %
***S.* Typhi CT18** (Parkhill *et al.*, 2001)	
ST10	50.65 %
ST15	54.75 %
ST18	50.67 %
ST27	52.62 %
ST35	51.84 %
sopE	51.57 %
ST46	48.88 %
host G + C content	52.09 %
***S.* Typhimurium LT2** (McClelland *et al.,* 2001)	
Gifsy-1	51.22 %
Gifsy-2	51.18 %
Fels-1	52.68 %
Fels-2	52.57 %
host G + C content	52.22 %

Table 3-5: G + C content of the putative prophage regions identified in *S.* Enteritidis 125109 in comparison to that of the prophage regions in *S.* Typhi CT18 and *S.* Typhimurium LT2.

3.2 PCR results

3.2.1 *Salmonella spp.* and *Salmonella* Enteritidis PCR

Two PCRs were used for the characterization of the isolates in the strain collection. The *Salmonella spp.* PCR for the specific detection of *Salmonella spp.* targets the *phoP/phoQ* locus. The *Salmonella* Enteritidis PCR for the detection of *S.* Enteritidis targets the *lygD* gene. Results for the visualization and analysis of the separated DNA fragments from the *Salmonella spp.* and *S.* Enteritidis PCR by agarose gel electrophoresis are shown in Table 3-6.

Group	Serovar	Strain	PT	*Salmonella spp.* PCR	*S.* Enteritidis PCR
D1	***S.* Enteritidis**	**125109**	**4**	**+**	**+**
	***S.* Enteritidis**	**Leipzig**	**4**	**+**	**+**
	***S.* Enteritidis**	**VAC E[1]**	**4**	**+**	**+**
	S. Enteritidis	FUR Working Seed	4	+	+
	S. Enteritidis	05-00229	4	+	+
	S. Enteritidis	04-01518	4	+	+
	S. Enteritidis	05-00213	4	+	+
	S. Enteritidis	05-00264	4	+	+
	S. Enteritidis	04-00319	4	+	+
	S. Enteritidis	03-01771-1	4	+	+
	S. Enteritidis	1004	4	+	+
	S. Enteritidis	1007	4	+	+
	S. Enteritidis	03-03058	4	+	+
	S. Enteritidis	02-02864	4	+	+
	S. Enteritidis	125589	4	+	+
	S. Enteritidis	1135	4	+	+
	S. Enteritidis	Salmovac SE	4	+	+
	S. Enteritidis	K482/91	4	+	+
	***S.* Enteritidis**	**04-03158**	**1**	**+**	**+**
	S. Enteritidis	02-07368	1	+	+
	S. Enteritidis	02-07381	1	+	+
	S. Enteritidis	02-07396	1	+	+
	S. Enteritidis	02-00900	4b	+	+
	S. Enteritidis	451/02	6a	+	+
	S. Enteritidis	809/02	6a	+	+
	***S.* Enteritidis**	**05-01906**	**8**	**+**	**+**
	S. Enteritidis	03-01087	8	+	+
	S. Enteritidis	K1298/05	8	+	+
	***S.* Enteritidis**	**03-03059**	**9b**	**+**	**+**
	***S.* Enteritidis**	**04-03092**	**11**	**+**	**-**
	***S.* Enteritidis**	**03-03561**	**13a**	**+**	**+**
	***S.* Enteritidis**	**02-00191**	**20**	**+**	**-**
	***S.* Enteritidis**	**02-06391**	**21**	**+**	**+**
	S. Enteritidis	05-01372	21	+	+
	S. Enteritidis	518/02	21	+	+
	***S.* Enteritidis**	**04-03909**	**21c**	**+**	**+**
	S. Enteritidis	86/360	34	+	+
	S. Enteritidis	1005	na*	+	+
	S. Enteritidis	1006	na*	+	+
	S. Enteritidis	7497	na	+	+
	S. Enteritidis	7499	na	+	+
	S. Enteritidis	7661	na	+	+
	S. Enteritidis	1607	na	+	+
	S. Enteritidis	K229/63	na	+	+
	S. Enteritidis	F971/82 (669)	na	+	+
	S. Gallinarum	K517/94-5	na	+	-
	S. Eastbourne	S2 (R22)	na	+	-

Group	Serovar	Strain	PT	*Salmonella spp.* PCR	*S.* Enteritidis PCR
B	*S.* Typhimurium	576	na	+	-
	S. Indiana	R1	na	+	-
	S. Saint Paul	898/1	na	+	-
	S. Agona	533-4	na	+	-
	S. Paratyphi B	B 1086/00	na	+	-
	S. Stanley	R20	na	+	-
C1	*S.* Virchow	V1	na	+	-
	S. Infantis	6633	na	+	-
C2-3	*S.* Hadar	18UM	na	+	-
	S. Albany	2713	na	+	-
E1	*S.* Anatum	4279	na	+	-
E4	*S.* Senftenberg	1331/7	na	+	-
I	*S.* Yoruba	322 SII	na	+	-

Table 3-6: PCR results for the *Salmonella spp.* and the *Salmonella* Enteritidis PCR.

[1]AviPro® SALMONELLA VAC E

*** serologically rough; na: information not available**

The *Salmonella spp.* PCR was positive for all samples tested and produced products of the expected length of 412 bp. The *Salmonella* Enteritidis PCR produced products of the expected length of 293 bp for all *S.* Enteritidis isolates except those of phage type 11 and phage type 20. For all non-Enteritidis samples tested in this study no amplification of the target sequence was detected in the *S.* Enteritidis PCR.

3.2.2 PCRs for screening of prophage presence

The presence of the previously identified prophage loci ΦSE10, ΦSE12/ΦSE12A, ΦSE14 and ΦSE20 in the strains of the strain collection was analyzed in a PCR based screening.

3.2.2.1 ΦSE10

The isolates of the strain collection described in 2.1.4 were screened for the presence of the previously identified prophage locus ΦSE10 with three PCRs targeting the 5'-end, the 3'-end and the central part of the prophage region respectively. The primer sequences and their target genes are displayed in Table 2-13. Results for the visualization and analysis of the separated DNA fragments from the PCR-based screening are shown in Table 3-7.

Group	Serovar	Strain	PT	SE10_5N	SE10_SSEI	SE10_3
D1	***S.* Enteritidis**	**125109**	**4**	**+**	**+**	**+**
	***S.* Enteritidis**	**Leipzig**	**4**	**+**	**+**	**+**
	***S.* Enteritidis**	**VAC E[1]**	**4**	**+**	**+**	**+**
	S. Enteritidis	FUR Working Seed	4	+	+	+
	S. Enteritidis	05-00229	4	+	+	+
	S. Enteritidis	04-01518	4	+	+	+
	S. Enteritidis	05-00213	4	+	+	+
	S. Enteritidis	05-00264	4	+	+	+
	S. Enteritidis	04-00319	4	+	+	+
	S. Enteritidis	03-01771-1	4	+	+	+
	S. Enteritidis	1004	4	+	+	+
	S. Enteritidis	1007	4	+	+	+
	S. Enteritidis	03-03058	4	+	+	+
	S. Enteritidis	02-02864	4	+	+	+
	S. Enteritidis	125589	4	+	+	+
	S. Enteritidis	1135	4	+	+	+
	S. Enteritidis	Salmovac SE	4	+	+	+
	S. Enteritidis	K482/91	4	+	+	+
	***S.* Enteritidis**	**04-03158**	**1**	**+**	**+**	**+**
	S. Enteritidis	02-07368	1	+	+	+
	S. Enteritidis	02-07381	1	+	+	+
	S. Enteritidis	02-07396	1	+	+	+
	S. Enteritidis	02-00900	4b	+	+	+
	S. Enteritidis	451/02	6a	+	+	+
	S. Enteritidis	809/02	6a	+	+	+
	***S.* Enteritidis**	**05-01906**	**8**	**+**	**+**	**+**
	S. Enteritidis	03-01087	8	+	+	+
	S. Enteritidis	K1298/05	8	+	+	+
	***S.* Enteritidis**	**03-03059**	**9b**	**-**	**+**	**+**
	***S.* Enteritidis**	**04-03092**	**11**	**-**	**+**	**+**
	***S.* Enteritidis**	**03-03561**	**13a**	**+**	**+**	**+**
	***S.* Enteritidis**	**02-00191**	**20**	**-**	**+**	**+**
	***S.* Enteritidis**	**02-06391**	**21**	**+**	**+**	**+**
	S. Enteritidis	05-01372	21	+	+	+
	S. Enteritidis	518/02	21	+	+	+
	***S.* Enteritidis**	**04-03909**	**21c**	**+**	**+**	**+**
	S. Enteritidis	86/360	34	+	+	+
	S. Enteritidis	1005	na*	+	+	+
	S. Enteritidis	1006	na*	+	+	+
	S. Enteritidis	7497	na	+	+	+
	S. Enteritidis	7499	na	+	+	+
	S. Enteritidis	7661	na	+	+	+
	S. Enteritidis	1607	na	+	+	+
	S. Enteritidis	K229/63	na	+	+	+
	S. Enteritidis	F971/82 (669)	na	+	+	+
	S. Gallinarum	K517/94-5	na	-	-	-
	S. Eastbourne	S2 (R22)	na	-	-	-

Group	Serovar	Strain	PT	SE10_5N	SE10_SSEI	SE10_3
B	*S.* Typhimurium	576	na	-	+	+
	S. Indiana	R1	na	-	-	-
	S. Saint Paul	898/1	na	-	-	-
	S. Agona	533-4	na	-	-	-
	S. Paratyphi B	B 1086/00	na	-	-	-
	S. Stanley	R20	na	-	-	+
C1	*S.* Virchow	V1	na	-	-	-
	S. Infantis	6633	na	-	-	+
C2-3	*S.* Hadar	18UM	na	-	-	+
	S. Albany	2713	na	-	-	-
E1	*S.* Anatum	4279	na	-	-	-
E4	*S.* Senftenberg	1331/7	na	-	-	+
I	*S.* Yoruba	322 SII	na	-	-	-

Table 3-7: PCR results for the screening analysis of the putative prophage location ΦSE10 in the strains of the strain collection.

[1]AviPro® SALMONELLA VAC E

*** serologically rough; na: information not available**

The PCR targeting the *sseI* gene and the PCR targeting the 3'-end of the putative prophage region ΦSE10 were positive for all *S.* Enteritidis isolates tested and produced products of the expected length of 984 bp for the SE10_3 primer pair and 999 bp for the SE10_SSEI primer pair. The PCR targeting the 5'-end of the putative prophage region ΦSE10 produced products of the expected length of 887 bp for the SE10_5 primer pair for all *S.* Enteritidis isolates tested except for those belonging to the phage types 9b, 11 and 20. For the non-Enteritidis isolates tested, amplification of the target sequence was detected in case of the SE10_SSEI for *S.* Typhimurium only, and in case of the SE10_3 primer pair for the serovars Typhimurium, Stanley, Infantis, Hadar and Senftenberg. The SE10_5 primer pair did not amplify the target region in any of non-Enteritidis isolates tested.

3.2.2.2 ΦSE12/ΦSE12A

The isolates of the strain collection described in 2.1.4 were screened for the presence of the previously identified prophage locus ΦSE12/ΦSE12A with three PCRs targeting the 5'-end, the 3'-end and the central part of the prophage region respectively. The primer sequences and their target genes are displayed in Table 2-13. Results for the visualization and analysis of the separated DNA fragments from the PCR-based screening are shown in Table 3-8.

Group	Serovar	Strain	PT	SE12_5N	SE12_SOPE_N	SE12_3
D1	***S.* Enteritidis**	**125109**	**4**	**+**	**+**	**+**
	***S.* Enteritidis**	**Leipzig**	**4**	**+**	**+**	**+**
	***S.* Enteritidis**	**VAC E[1]**	**4**	**+**	**+**	**+**
	S. Enteritidis	FUR Working Seed	4	+	+	+
	S. Enteritidis	05-00229	4	+	+	+
	S. Enteritidis	04-01518	4	+	+	+
	S. Enteritidis	05-00213	4	+	+	+
	S. Enteritidis	05-00264	4	+	+	+
	S. Enteritidis	04-00319	4	+	+	+
	S. Enteritidis	03-01771-1	4	+	+	+
	S. Enteritidis	1004	4	+	+	+
	S. Enteritidis	1007	4	+	+	+
	S. Enteritidis	03-03058	4	+	+	+
	S. Enteritidis	02-02864	4	+	+	+
	S. Enteritidis	125589	4	+	+	+
	S. Enteritidis	1135	4	+	+	+
	S. Enteritidis	Salmovac SE	4	+	+	+
	S. Enteritidis	K482/91	4	+	+	+
	***S.* Enteritidis**	**04-03158**	**1**	**+**	**+**	**+**
	S. Enteritidis	02-07368	1	+	+	+
	S. Enteritidis	02-07381	1	+	+	+
	S. Enteritidis	02-07396	1	+	+	+
	S. Enteritidis	02-00900	4b	+	+	+
	S. Enteritidis	451/02	6a	+	+	+
	S. Enteritidis	809/02	6a	+	+	+
	***S.* Enteritidis**	**05-01906**	**8**	**+**	**+**	**+**
	S. Enteritidis	03-01087	8	+	+	+
	S. Enteritidis	K1298/05	8	+	+	+
	***S.* Enteritidis**	**03-03059**	**9b**	**+**	**+**	**+**
	***S.* Enteritidis**	**04-03092**	**11**	**+**	**+**	**+**
	***S.* Enteritidis**	**03-03561**	**13a**	**+**	**+**	**+**
	***S.* Enteritidis**	**02-00191**	**20**	**+**	**+**	**-**
	***S.* Enteritidis**	**02-06391**	**21**	**+**	**+**	**+**
	S. Enteritidis	05-01372	21	+	+	+
	S. Enteritidis	518/02	21	+	+	+
	***S.* Enteritidis**	**04-03909**	**21c**	**+**	**+**	**+**
	S. Enteritidis	86/360	34	+	+	+
	S. Enteritidis	1005	na*	+	+	+
	S. Enteritidis	1006	na*	+	+	+
	S. Enteritidis	7497	na	+	+	+
	S. Enteritidis	7499	na	+	+	+
	S. Enteritidis	7661	na	+	+	+
	S. Enteritidis	1607	na	+	+	+
	S. Enteritidis	K229/63	na	+	+	+
	S. Enteritidis	F971/82 (669)	na	+	+	+
	S. Gallinarum	K517/94-5	na	+	+	+
	S. Eastbourne	S2 (R22)	na	+	-	+

Group	Serovar	Strain	PT	SE12_5N	SE12_SOPE_N	SE12_3
B	*S.* Typhimurium	576	na	+	-	+
	S. Indiana	R1	na	-	-	-
	S. Saint Paul	898/1	na	+	-	+
	S. Agona	533-4	na	-	-	-
	S. Paratyphi B	B 1086/00	na	-	-	-
	S. Stanley	R20	na	+	-	+
C1	*S.* Virchow	V1	na	-	+	+
	S. Infantis	6633	na	+	+	+
C2-3	*S.* Hadar	18UM	na	-	+	+
	S. Albany	2713	na	-	-	-
E1	*S.* Anatum	4279	na	+	-	+
E4	*S.* Senftenberg	1331/7	na	-	-	-
I	*S.* Yoruba	322 SII	na	-	-	-

Table 3-8: PCR results for the screening analysis of the putative prophage location ΦSE12/ΦSE12A in the strains of the strain collection.

[1]AviPro® SALMONELLA VAC E

*** serologically rough; na: information not available**

The three PCRs targeting the 5'-end, the *sopE* gene and the 3'-end of the putative prophage region ΦSE12/ΦSE12A were positive for all *S.* Enteritidis isolates tested with the exception of the isolate belonging to phage type 20, for which the PCR was negative with the SE12_3 primer pair. The PCR products had the expected length of 986 bp for the SE12_5N primer pair, 892 bp for the SE12_SOPE_N primer pair and 997 bp for the SE12_3 primer pair.

For the non-Enteritidis isolates tested, amplification with all three primer pairs was detected for *S.* Gallinarum and *S.* Infantis. For five isolates the PCR gave positive results with the SE12_5N primer pair and the SE12_3 primer pair only (serovars Typhimurium, Eastbourne, Saint Paul, Stanley and Anatum), while for *S.* Virchow and *S.* Hadar positive PCR results were obtained with the SE12_SOPE_N primer pair and the SE12_3 primer pair.

3.2.2.3 ΦSE14

The isolates of the strain collection described in 2.1.4 were screened for the presence of the previously identified prophage locus ΦSE14 with three PCRs targeting the 5'-end, the 3'-end and the central part of the prophage region respectively. The primer sequences and their target genes are displayed in Table 2-13. Results for the visualization and analysis of the separated DNA fragments from the PCR-based screening are shown in Table 3-9.

Group	Serovar	Strain	PT	SE14_5N	SE14_CONSERVED	SE14_3	*S.* Enteritidis PCR
D1	***S.* Enteritidis**	**125109**	**4**	**+**	**+**	**+**	**+**
	***S.* Enteritidis**	**Leipzig**	**4**	**+**	**+**	**+**	**+**
	***S.* Enteritidis**	**VAC E[1]**	**4**	**+**	**+**	**+**	**+**
	S. Enteritidis	FUR Working Seed	4	+	+	+	+
	S. Enteritidis	05-00229	4	+	+	+	+
	S. Enteritidis	04-01518	4	+	+	+	+
	S. Enteritidis	05-00213	4	+	+	+	+
	S. Enteritidis	05-00264	4	+	+	+	+
	S. Enteritidis	04-00319	4	+	+	+	+
	S. Enteritidis	03-01771-1	4	+	+	+	+
	S. Enteritidis	1004	4	+	+	+	+
	S. Enteritidis	1007	4	+	+	+	+
	S. Enteritidis	03-03058	4	+	+	+	+
	S. Enteritidis	02-02864	4	+	+	+	+
	S. Enteritidis	125589	4	+	+	+	+
	S. Enteritidis	1135	4	+	+	+	+
	S. Enteritidis	Salmovac SE	4	+	+	+	+
	S. Enteritidis	K482/91	4	+	+	+	+
	***S.* Enteritidis**	**04-03158**	**1**	**+**	**+**	**+**	**+**
	S. Enteritidis	02-07368	1	+	+	+	+
	S. Enteritidis	02-07381	1	+	+	+	+
	S. Enteritidis	02-07396	1	+	+	+	+
	S. Enteritidis	02-00900	4b	+	+	+	+
	S. Enteritidis	451/02	6a	+	+	+	+
	S. Enteritidis	809/02	6a	+	+	+	+
	***S.* Enteritidis**	**05-01906**	**8**	**+**	**+**	**+**	**+**
	S. Enteritidis	03-01087	8	+	+	+	+
	S. Enteritidis	K1298/05	8	+	+	+	+
	***S.* Enteritidis**	**03-03059**	**9b**	**+**	**+**	**-**	**+**
	***S.* Enteritidis**	**04-03092**	**11**	**-**	**-**	**-**	**-**
	***S.* Enteritidis**	**03-03561**	**13a**	**+**	**+**	**+**	**+**
	***S.* Enteritidis**	**02-00191**	**20**	**-**	**-**	**-**	**-**
	***S.* Enteritidis**	**02-06391**	**21**	**+**	**+**	**+**	**+**
	S. Enteritidis	05-01372	21	+	+	+	+
	S. Enteritidis	518/02	21	+	+	+	+
	***S.* Enteritidis**	**04-03909**	**21c**	**+**	**+**	**+**	**+**
	S. Enteritidis	86/360	34	+	+	+	+
	S. Enteritidis	1005	na*	+	+	+	+
	S. Enteritidis	1006	na*	+	+	+	+
	S. Enteritidis	7497	na	+	+	+	+
	S. Enteritidis	7499	na	+	+	+	+
	S. Enteritidis	7661	na	+	+	+	+
	S. Enteritidis	1607	na	+	+	+	+
	S. Enteritidis	K229/63	na	+	+	+	+
	S. Enteritidis	F971/82 (669)	na	+	+	+	+
	S. Gallinarum	K517/94-5	na	-	-	-	-
	S. Eastbourne	S2 (R22)	na	-	-	-	-

Group	Serovar	Strain	PT	SE14_5N	SE14_CONSERVED	SE14_3	*S.* Enteritidis PCR
B	*S.* Typhimurium	576	na	-	-	-	-
	S. Indiana	R1	na	-	-	-	-
	S. Saint Paul	898/1	na	-	-	-	-
	S. Agona	533-4	na	-	-	-	-
	S. Paratyphi B	B 1086/00	na	-	-	-	-
	S. Stanley	R20	na	-	-	-	-
C1	*S.* Virchow	V1	na	-	-	-	-
	S. Infantis	6633	na	-	-	-	-
C2-3	*S.* Hadar	18UM	na	-	-	-	-
	S. Albany	2713	na	-	-	-	-
E1	*S.* Anatum	4279	na	-	-	-	-
E4	*S.* Senftenberg	1331/7	na	-	-	-	-
I	*S.* Yoruba	322 SII	na	-	-	-	-

Table 3-9: PCR results for the screening analysis of the putative prophage location ΦSE14 in the strains of the strain collection. The results of the *Salmonella* Enteritidis PCR are shown for comparison.

[1]AviPro® SALMONELLA VAC E

*** serologically rough; na: information not available**

The three PCRs used for the analysis of the putative prophage region ΦSE14 target the 5'-end, a region highly conserved in relation to the *S.* Typhimurium prophage ST18 and the 3'-end of ΦSE14. The PCR products had the expected length of 980 bp for the SE14_5N primer pair, 991 bp for the SE14_CONSERVED primer pair and 976 bp for the SE14_3 primer pair. All three PCRs were positive positive for all *S.* Enteritidis isolates tested with the exception of the isolates belonging to phage types 11 and 20 that did not produce PCR products with any of the three primer pairs. For the phage type 9b isolate, no positive PCR result was obtained with the primer pair targeting the 3'-end of ΦSE14.

For the non-Enteritidis isolates tested, amplification was not detected with any of the three primer pairs. The results from the *Salmonella* Enteritidis PCR discussed above are included into the result table for comparison.

3.2.2.4 ΦSE20

The isolates of the strain collection described in 2.1.4 were screened for the presence of the previously identified prophage locus ΦSE20 with three PCRs targeting the 5'-end, the 3'-end and the central part of the prophage region respectively. The primer sequences and their target genes are displayed in Table 2-13. Results for the visualization and analysis of the separated DNA fragments from the PCR-based screening are shown in Table 3-10.

Group	Serovar	Strain	PT	SE20_5	SE20_IMMC	SE20_3
D1	***S.* Enteritidis**	**125109**	**4**	**+**	**+**	**+**
	***S.* Enteritidis**	**Leipzig**	**4**	**+**	**+**	**+**
	***S.* Enteritidis**	**VAC E[1]**	**4**	**+**	**+**	**+**
	S. Enteritidis	FUR Working Seed	4	+	+	+
	S. Enteritidis	05-00229	4	+	+	+
	S. Enteritidis	04-01518	4	+	+	+
	S. Enteritidis	05-00213	4	+	+	+
	S. Enteritidis	05-00264	4	+	+	+
	S. Enteritidis	04-00319	4	+	+	+
	S. Enteritidis	03-01771-1	4	+	+	+
	S. Enteritidis	1004	4	+	+	+
	S. Enteritidis	1007	4	+	+	+
	S. Enteritidis	03-03058	4	+	+	+
	S. Enteritidis	02-02864	4	+	+	+
	S. Enteritidis	125589	4	+	+	+
	S. Enteritidis	1135	4	+	+	+
	S. Enteritidis	Salmovac SE	4	+	+	+
	S. Enteritidis	K482/91	4	+	+	+
	***S.* Enteritidis**	**04-03158**	**1**	**+**	**+**	**+**
	S. Enteritidis	02-07368	1	+	+	+
	S. Enteritidis	02-07381	1	+	+	+
	S. Enteritidis	02-07396	1	+	+	+
	S. Enteritidis	02-00900	4b	+	+	+
	S. Enteritidis	451/02	6a	+	+	+
	S. Enteritidis	809/02	6a	+	+	+
	***S.* Enteritidis**	**05-01906**	**8**	**-**	**-**	**-**
	S. Enteritidis	03-01087	8	-	-	-
	S. Enteritidis	K1298/05	8	-	-	-
	***S.* Enteritidis**	**03-03059**	**9b**	**-**	**-**	**-**
	***S.* Enteritidis**	**04-03092**	**11**	**-**	**-**	**+**
	***S.* Enteritidis**	**03-03561**	**13a**	**-**	**-**	**-**
	***S.* Enteritidis**	**02-00191**	**20**	**+**	**-**	**-**
	***S.* Enteritidis**	**02-06391**	**21**	**+**	**+**	**+**
	S. Enteritidis	05-01372	21	+	+	+
	S. Enteritidis	518/02	21	+	+	+
	***S.* Enteritidis**	**04-03909**	**21c**	**(+)**	**(+)**	**-**
	S. Enteritidis	86/360	34	+	+	+
	S. Enteritidis	1005	na*	+	+	+
	S. Enteritidis	1006	na*	+	+	+
	S. Enteritidis	7497	na	+	+	+
	S. Enteritidis	7499	na	+	+	+
	S. Enteritidis	7661	na	+	+	+
	S. Enteritidis	1607	na	+	+	+
	S. Enteritidis	K229/63	na	-	-	-
	S. Enteritidis	F971/82 (669)	na	-	-	-
	S. Gallinarum	K517/94-5	na	-	-	-
	S. Eastbourne	S2 (R22)	na	-	-	-

Group	Serovar	Strain	PT	SE20_5	SE20_IMMC	SE20_3
B	*S.* Typhimurium	576	na	-	+	+
	S. Indiana	R1	na	-	-	-
	S. Saint Paul	898/1	na	-	-	-
	S. Agona	533-4	na	-	-	-
	S. Paratyphi B	B 1086/00	na	-	-	-
	S. Stanley	R20	na	-	-	-
C1	*S.* Virchow	V1	na	-	-	-
	S. Infantis	6633	na	-	-	-
C2-3	*S.* Hadar	18UM	na	-	-	-
	S. Albany	2713	na	-	-	-
E1	*S.* Anatum	4279	na	-	+	-
E4	*S.* Senftenberg	1331/7	na	-	-	-
I	*S.* Yoruba	322 SII	na	-	-	-

Table 3-10: PCR results for the screening analysis of the putative prophage location ΦSE20 in the strains of the strain collection.

[1]AviPro® SALMONELLA VAC E

*** serologically rough; na: information not available; nr: not relevant**

The three PCRs used for the analysis of the putative prophage region ΦSE20 were positive for most of the *S.* Enteritidis isolates from the strain collection analysed. The PCR products had the expected length of 1,014 bp for the SE20_5 primer pair targeting the 5'-end, 966 bp for the SE20_IMMC primer pair targeting the *immC* region, a superinfection immunity system highly conserved in relation to the *S.* Typhimurium phage ST64B and 788 bp for the SE20_3 primer pair targeting the 3'-end of ΦSE20. No PCR products were generated with any of the primer pairs for isolates belonging to the phage types 8, 9b and 13a and for two isolates of unknown phage type. For the phage type 11 isolate, production of PCR products was observed with the SE20_3 primer pair only, while for the phage type 20 isolate, this was the case with the SE20_5 primer pair only. The results for the phage type 21c isolates were unclear, no amplification was seen for the SE20_3 primer pair, and the two other PCRs seemed not to work properly as only weak bands were obtained when the products were analyzed by gel electrophoresis. In case of the non-Enteritidis isolates, the SE20_3 primer pair led to the amplification of the target sequence for *S.* Typhimurium and *S.* Anatum, and with *S.* Typhimurium a PCR product was also obtained with the SE20_IMMC primer pair. All other serovars did not give positive PCR results with any of the three primer pairs.

3.3 Microarray experiments

Based on the results of the PCR screening of the strain collection, eleven isolates were chosen to be analyzed in a microarray analysis for the presence of the individual genes located in the putative prophage locations previously identified and the genes in their direct vicinity. Additionally, the presence of the genes comprising the prophage locations in *S.* Typhi CT18 and *S.* Typhimurium LT2 in these isolates was analysed as well.

A total of almost 143,000 individual results was generated in these experiments (4,331 screened and validated PCR products spotted onto the array x 11 isolates x analysis by three statistical methods). It would of course go far beyond the scope of this study to individually analyse this tremendous amount of data. The data relevant for this study was therefore analysed in a stringently condensed way.

3.3.1 ΦSE10

Results for the analysis of the gene calling statuses of the genes present in the putative prophage ΦSE10 and the genes in the direct vicinity of ΦSE10 are shown in Table 3-11 for those of these genes represented on the microarray together with the PCR results for the PCR based screening of ΦSE10 for the selected isolates.

		Microarray results		PCR results		
Strain	**PT**	**Prophage**	**adjacent region***	**SE10_5N**	**SE10_SSEI**	**SE10_3**
125109	**4**	+	+	+	+	+
Leipzig	**4**	+	+	+	+	+
VAC E[1]	**4**	+	+	+	+	+
04-03158	**1**	+	+	+	+	+
05-01906	**8**	+	+	+	+	+
03-03059	**9b**	+	+	-	+	+
04-03092	**11**	**5':-**	+	-	+	+
03-03561	**13a**	+	+	+	+	+
02-00191	**20**	+	+	-	+	+
02-06391	**21**	+	+	+	+	+
04-03909	**21c**	+	+	+	+	+

Table 3-11: Microarray results in comparison to the PCR results for the putative prophage location ΦSE10 in selected isolates.

[1]AviPro® SALMONELLA VAC E

*** comprising the four neighbouring genes on the array at the 5'- and the 3'-end respectively**

The genes of the putative prophage region ΦSE10 represented on the microarray were present in all isolates analysed except for the phage type 11 isolate according to the microarray data. In the phage type 11 isolate the genes SEN0910 – SEN0912A at the 5'-end of the putative prophage seem to be absent. The genes in the direct vicinity of the putative prophage location were present in all isolates tested including the phage type 11 isolate at the 5'- and the 3'-end.

3.3.2 ΦSE12/ΦSE12A

Results for the analysis of the gene calling statuses of the genes present in the putative prophage ΦSE12/ΦSE12A and the genes in the direct vicinity of ΦSE12/ΦSE12A are shown in Table 3-12 for those of these genes represented on the microarray together with the PCR results for the PCR based screening of ΦSE12/ΦSE12A for the selected isolates.

		Microarray results		PCR results		
Strain	**PT**	**Prophage**	**adjacent region***	**SE12_5N**	**SE12_SOPE_N**	**SE12_3**
125109	**4**	+	+	+	+	+
Leipzig	**4**	+	+	+	+	+
VAC E[1]	**4**	+	+	+	+	+
04-03158	**1**	+	+	+	+	+
05-01906	**8**	+	+	+	+	+
03-03059	**9b**	+	+	+	+	+
04-03092	**11**	+	+	+	+	+
03-03561	**13a**	+	+	+	+	+
02-00191	**20**	+	**3':-**	+	+	-
02-06391	**21**	+	+	+	+	+
04-03909	**21c**	+	+	+	+	+

Table 3-12: Microarray results in comparison to the PCR results for the putative prophage location ΦSE12/ΦSE12A in selected isolates.

[1]AviPro® SALMONELLA VAC E

*** comprising the four neighbouring genes on the array at the 5'- and the 3'-end respectively**

The genes of the putative prophage region ΦSE12/ΦSE12A represented on the microarray could be detected in all tested isolates according to the microarray data. Only in the phage type 9b isolate the microarray data did not indicate the presence of the putative pseudogene SEN1152. The genes in the direct vicinity of the putative prophage location ΦSE12/ΦSE12A were present in all isolates tested except for the phage type 20 isolate, where the genes SEN1174, SEN1176 and SEN 1178 adjacent to the 3'-end of the putative phage locus seemed to be missing.

3.3.3 ΦSE14

Results for the analysis of the gene calling statuses of the genes present in the putative prophage ΦSE14 and the genes in the direct vicinity of ΦSE14 are shown in Table 3-13 for those of these genes represented on the microarray together with the PCR results for the PCR based screening of ΦSE14 for the selected isolates.

		Microarray results		PCR results		
Strain	**PT**	**Prophage**	**adjacent region***	**SE14_5N**	**SE14_CONSERVED**	**SE14_3**
125109	**4**	+	+	+	+	+
Leipzig	**4**	+	+	+	+	+
VAC E[1]	**4**	+	+	+	+	+
04-03158	**1**	+	+	+	+	+
05-01906	**8**	+	+	+	+	+
03-03059	**9b**	**3': -**	+	+	+	-
04-03092	**11**	-	+	-	-	-
03-03561	**13a**	+	+	+	+	+
02-00191	**20**	-	+	-	-	-
02-06391	**21**	+	+	+	+	+
04-03909	**21c**	+	+	+	+	+

Table 3-13: Microarray results in comparison to the PCR results for the putative prophage location ΦSE14 in selected isolates.

[1]AviPro® SALMONELLA VAC E

*** comprising the four neighbouring genes on the array at the 5'- and the 3'-end respectively**

According to the microarray data, the genes of the putative prophage region ΦSE14 present on the microarray could be detected in most of the strains tested. For the isolates belonging to the phage types 11 and 20 however, all genes except for one gene seemed to be absent. Interestingly, there was evidence for a gene similar to the STY2007 gene being present in these two isolates which in return was absent from the isolates belonging to all other phage types tested. The STY2007 gene comprises the gene bordering the 5'-end of the *S.* Typhi CT18 phage ST18. The isolate belonging to phage type 9b seemed to be lacking the genes SEN1393, SEN1394 and SEN1395 at the 3'-end of the putative prophage region ΦSE14 and the gene SEN1384 according to the microarray data. The genes in the direct vicinity of the putative prophage location ΦSE14 were present in all isolates tested.

3.3.4 ΦSE20

Results for the analysis of the gene calling statuses of the genes present in the putative prophage ΦSE20 and the genes in the direct vicinity of ΦSE20 are shown in Table 3-14 for those of these genes represented on the microarray together with the PCR results for the PCR based screening of ΦSE20 for the selected isolates.

		Microarray results		PCR results		
Strain	**PT**	**Prophage**	**adjacent region***	**SE20_5**	**SE20_IMMC**	**SE20_3**
125109	**4**	**+**	**+**	**+**	**+**	**+**
Leipzig	**4**	**+**	**+**	**+**	**+**	**+**
VAC E[1]	**4**	**+**	**+**	**+**	**+**	**+**
04-03158	**1**	**+**	**+**	**+**	**+**	**+**
05-01906	**8**	**-**	**+**	**-**	**-**	**-**
03-03059	**9b**	**-**	**5': +**	**-**	**-**	**-**
04-03092	**11**	**50 %; 3':+**	**+**	**-**	**-**	**+**
03-03561	**13a**	**67 %; PCR -**	**+**	**-**	**-**	**-**
02-00191	**20**	**33 %; 5': +**	**+**	**+**	**-**	**-**
02-06391	**21**	**+**	**+**	**+**	**+**	**+**
04-03909	**21c**	**-**	**+**	**(+)**	**(+)**	**-**

Table 3-14: Microarray results in comparison to the PCR results for the putative prophage location ΦSE20 in selected isolates.

[1]AviPro® SALMONELLA VAC E

*** comprising the four neighbouring genes on the array at the 5'- and the 3'-end respectively**

The results obtained in the analysis of the microarray data for the genes of the putative prophage region ΦSE20 present on the microarray were more diverse than those obtained for the other putative prophage regions of SE125109. ΦSE20 seemed to be present in the isolates belonging to the phage types 1, 4 and 21, with some indication that the gene SEN1937 was absent in the phage type 1 isolate and the gene 1949A was absent in the phage type 4 VAC E isolate. The absence of ΦSE20 was indicated by the microarray data for the isolates belonging to the phage types 8, 9b and 21c, with the genes SEN 1921, SEN1943 and SEN1944 probably being present in the phage type 9b isolate and the genes SEN1921, SEN1922, SEN1944, SEN1950 and SEN1964 probably being present in the phage type 21c isolate. The phage type 11, 13a and 20 isolates showed a scattered distribution of genes being present and absent in small blocks in the analysis of the microarray data. In the phage type 11 isolate, about 50 % of the genes of the putative prophage region ΦSE20 present on the array were detected including those located at the 3'-end (SEN1921 - SEN1932, SEN1943 – SEN1946, SEN1948 – SEN1950, SEN1953, SEN1954, SEN1957A, SEN1960 and SEN1964 – SEN1966). In the

phage type 13a isolate, two thirds of the genes were detected with all target genes for the PCR-based screening being absent or not present on the array (SEN1921 – SEN1935 and SEN1941). In the phage type 20 isolate about one third of the genes being present mainly located at the 5'-end (SEN1920 - SEN1935, SEN1944 and SEN1953). The genes in the direct vicinity of the putative prophage location ΦSE20 were present in all isolates tested except for the phage type 9b isolate where there was evidence for absence of the genes (SEN1967 – SEN1971) adjacent to the 3'-end of the putative prophage region ΦSE20. However, the phage type 20 isolate was missing the SEN1986 gene.

3.3.5 Presence of prophage genes from *S.* Typhimurium LT2S and *S.* Typhi CT18

Summarized results for the analysis of the gene calling statuses of the genes comprising the prophage locations in *S.* Typhi CT18 and *S.* Typhimurium LT2 for the *S.* Enteritidis isolates included into the microarray experiments are shown in Table 3-15 and Table 3-16. The number of genes present in the respective isolates according to the microarray data are given in relation to the total number of genes of the respective prophages represented on the array.

Strain	PT	Gifsy-1	Gifsy-2	Fels-1	Fels-2
125109	4	2/23	10/22	2/6	3/66
Leipzig	4	2/23	10/22	1/6	5/66
VAC E[1]	4	3/23	9/22	2/6	5/66
04-03158	1	3/23	10/22	3/6	23/66
05-01906	8	3/23	10/22	3/6	57/66
03-03059	9b	9/23	16/22	6/6	58/66
04-03092	11	22/23	22/22	3/6	58/66
03-03561	13a	2/23	10/22	0/6	60/66
02-00191	20	9/23	21/22	1/6	7/66
02-06391	21	2/23	9/22	1/6	4/66
04-03909	21c	16/23	9/22	0/6	5/66

Table 3-15: Summarized results for the presence of the genes comprising the prophage locations in *S.* Typhimurium LT2 in the *S.* Enteritidis isolates according to microarray data.

[1]AviPro® SALMONELLA VAC E

Strain	PT	ST10	ST15	ST18	ST27	ST35	sopE	ST48
125109	4	7/24	5/5	8/27	1/1	0/30	2/29	0/1
Leipzig	4	6/24	4/5	9/27	1/1	0/30	2/29	0/1
VAC E[1]	4	6/24	4/5	8/27	1/1	0/30	2/29	0/1
04-03158	1	8/24	4/5	12/27	1/1	6/30	9/29	0/1
05-01906	8	4/24	5/5	10/27	1/1	29/30	22/29	1/1
03-03059	9b	12/24	5/5	18/27	1/1	27/30	29/29	1/1
04-03092	11	18/24	4/5	4/27	1/1	28/30	26/29	1/1
03-03561	13a	2/24	4/5	11/27	1/1	28/30	22/29	1/1
02-00191	20	12/24	4/5	7/27	1/1	0/30	4/29	0/1
02-06391	21	6/24	4/5	8/27	1/1	0/30	2/29	0/1
04-03909	21c	6/24	4/5	12/27	1/1	0/30	0/29	0/1

Table 3-16: Summarized results for the presence of the genes comprising the prophage locations in *S.* Typhi CT18 in the *S.* Enteritidis isolates according to microarray data.

[1]AviPro® SALMONELLA VAC E

According to the microarray data, all *S.* Enteritidis isolates tested harboured genes from the genes comprising the prophage locations in *S.* Typhi CT18 and *S.* Typhimurium LT2. At the same time it could also be seen from the microarray data that genes from all prophage locations in *S.* Typhi CT18 and *S.* Typhimurium LT2 were harboured in at least some of the *S.* Enteritidis isolates tested. Certain patterns and tendencies in the correlation between the prophage content of *S.* Typhi CT18 and *S.* Typhimurium LT2 and the putative prophage content in the Enteritidis isolates tested could be seen from the analysis of the microarray data. While the isolates belonging to the phage types 4 and 21 seemed to harbour only few of the prophage genes from *S.* Typhi CT18 and *S.* Typhimurium LT2, a much higher proportion of these genes seemed to be present in the isolates belonging to the phage types 8, 9b, 11, and 13a. The content of *S.* Typhi CT18 and *S.* Typhimurium LT2 prophage genes for the isolates belonging to the phage types 1, 20 and 21c seemed to be somewhere in between these two groups. For some of the prophages present in *S.* Typhi CT18 and *S.* Typhimurium LT2, only very few genes of the respective prophage were present on the array, while for other prophages the proportion of these genes was much higher. Due to this inhomogenicity no statement could be made in relation to a preference for any of these prophages to be harboured in *S.* Enteritidis with the available microarray.

3.4 Prophage release and induction experiments

3.4.1 Prophage induction experiments

The inducibility of the temperate prophages in SE125109 by chemically and physically inducing agents was tested. A smooth bacterial lawn had grown on the soft top test agar after the overnight incubation. The dried drops were still clearly visible where the phage lysates to be tested were spotted onto the agar. The formation of plaques within these spots as a result of lysis by previously induced prophages could not be detected in any case. There was now difference at all visible between the control samples and the samples that had been exposed to mitomycin C or those that had been exposed to UV light. Detailed results for all samples tested can be seen in Table 3-17.

Sample	**Plaque Formation**
Mitomycin without shaking 1	-
Mitomycin without shaking 2	-
Mitomycin permanent shaking 1	-
Mitomycin permanent shaking 2	-
UV exposure 1	-
UV exposure 2	-
Sample without inducing agents 1	-
Sample without inducing agents 2	-
Control sample (non-inoculated media only) 1	-
Control sample (non-inoculated media only) 2	-

Table 3-17: Analysis of plaque formation on the soft top test agar with lysates from the phage induction experiments

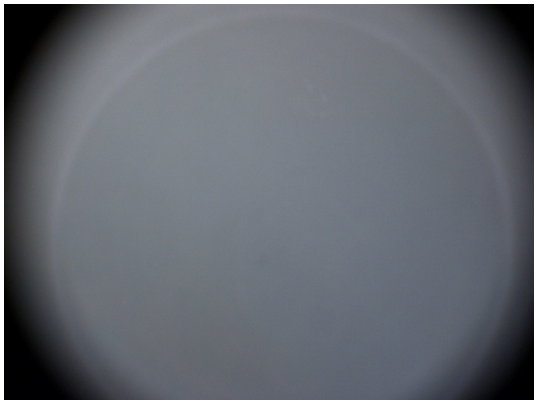
The bacteria in the control group as well as in the different induction groups continued to multiply during the 6 h incubation time of the induction experiments. This was noticeable by the increasing turbidity of the samples. Any quantitative effect of the inducing agents on the

bacterial growth rate was not investigated. All supernatants were sterile in the sterility tests performed.

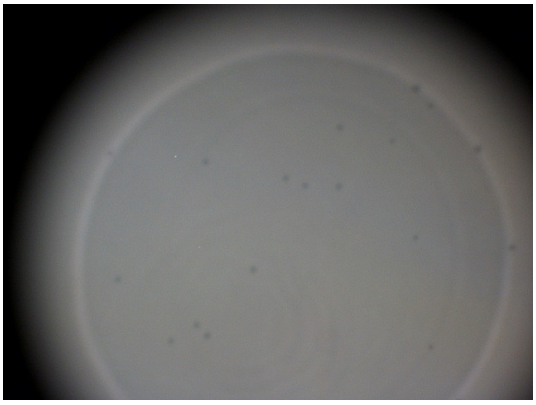
3.4.2 Spontaneous prophage release experiments

The spontaneous release of temperate bacteriophages and the susceptibility of these strains to infection by spontaneously released phages were investigated in a cross-screening experiment by a spot test method using the strains analysed in the microarray experiments as described in paragraph 2.2.8.2 of the methods section.

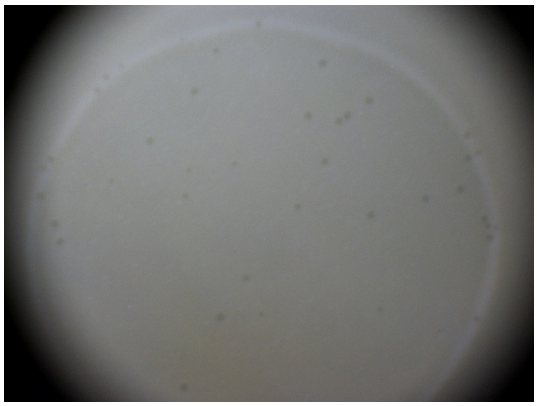
Sample photos of the obtained results with and without plaque formation in the tester strains are shown in Figure 3-2. A smooth bacterial lawn had grown on the agar plates used for the bacteriophage detection after the overnight incubation. The boundaries of the dried drops with the supernatants were still clearly visible where the phage lysates to be tested were spotted onto the agar. At all locations where the control samples containing only media without inoculation were applied onto the test agar, no plaque formation could be detected. At the other locations, where the supernatants of the overnight cultures were applied as processed phage lysates, plaque formation could be detected in some cases. Detailed results for all samples tested can be seen in Table 3-18. All supernatants were sterile in the sterility tests performed.



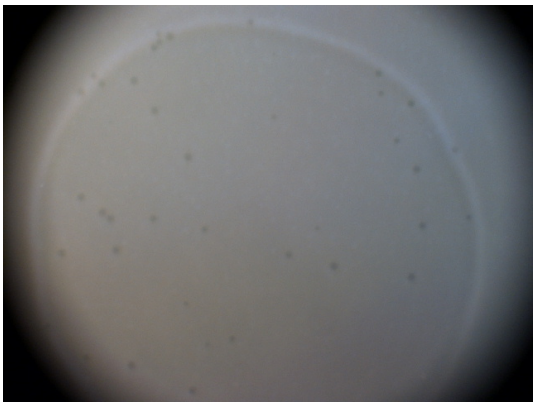
a)



b)



c)



d)

Figure 3-2: Sample pictures for the results of the spontaneous phage induction experiments: The boundaries of the dried drops with the supernatants are clearly visible. Picture a) shows the result for a sample that did not induce plaque formation, pictures b) – d) are examples for cases in which plaque formation was induced by the supernatant applied to the tester strain.

		supernatant applied to the tester strain											
		medium	125109	Leipzig	VAC E[1]	04-03158	05-01906	03-03059	04-03092	03-03561	02-00191	02-06391	04-03909
tester strain	phage type		PT4	PT4	PT4	PT1	PT8	PT9b	PT11	PT13a	PT20	PT21	PT21c
125109	PT4	-	-	-	-	-	-	-	+	+	-	-	-
Leipzig	PT4	-	-	-	-	-	-	-	+	+	-	-	-
VAC E[1]	PT4	-	-	-	-	-	-	-	+	+	-	-	-
04-03158	PT1	-	-	-	-	-	-	-	+	+	-	-	+
05-01906	PT8	-	+	+	+	-	-	-	+	+	-	-	-
03-03059	PT9b	-	-	-	-	-	-	-	+	-	-	-	+
04-03092	PT11	-	-	-	-	-	-	-	-	+	-	-	-
03-03561	PT13a	-	-	-	-	-	-	-	-	-	-	-	-
02-00191	PT20	-	-	-	-	-	-	-	-	-	-	-	-
02-06391	PT21	-	-	-	-	-	-	-	+	+	-	-	+
04-03909	PT21c	-	+	+	+	+	-	-	+	+	-	+	-

Table 3-18: The formation of plaques in the tester strains is shown in relation to the respective supernatants applied for the experiments based on the classic Fisk method.

[1]AviPro® SALMONELLA VAC E

4 Discussion

4.1 Genome analysis

The number of 5 prophages found in *S.* Enteritidis is in accordance with the 5 lysogenic phages Gifsy-1, -2 and -3, Fels-2 and the P2-like phage SopE identified in the genome of *S.* Typhimurium (Figueroa-Bossi and Bossi, 1999; Figueroa-Bossi *et al.*, 1997; Hardt *et al.*, 1998a; Miao and Miller 1999; Mirold *et al.*, 1999; Thomson *et al.*, 2004), and the seven prophages or prophage-like elements which were identified in the *S.* Typhi genome: ST10, ST15, ST18, ST27, ST35, SopEST (ST44) and ST46 (Thomson *et al.*, 2004).

4.1.1 ΦSE10

The putative prophage ΦSE10 is similar in parts to regions of the lambda-like phage Gifsy-2, which has been shown to significantly contribute to pathogenesis in *S.* Typhimurium (Figueroa-Bossi and Bossi, 1999; Ho *et al.*, 2002). Gifsy-2 is probably defective in the *S.* Typhimurium strain LT2 but active in ACTT14028s (Bossi and Figueroa-Bossi, 2005). *S.* Typhimurium strains cured of Gifsy-2 were over 100-fold attenuated in their virulence in mice indicating that Gifsy-2 contributes virulence factors to its host (Figueroa-Bossi and Bossi, 1999; Ho *et al.*, 2002). Like Gifsy-2, ΦSE10 was found to encode the highly conserved virulence determinants *sse*I, *gtg*E and *gtg*F (Ho *et al.*, 2002). Gifsy-2 genes with homology to known virulence factors individually had no significant effect on the *S.* Typhimurium virulence in intra-peritoneal competition assays. This could be explained by the fact that some genes had been acquired only recently by the Gifsy-2 phage and have not evolved or adapted to the appropriate regulatory circuitry or that the products of these genes might perform redundant functions. In case of the *gtgA* gene for example, a second nearly identical copy of the gene of Gifsy-2 is present in the Gifsy-1 prophage (Ho *et al.*, 2002).

The expression of the *S.* Typhimurium *sseI*-gene is dependent on the SPI2-encoded two-component regulatory system SsrA/SsrB, and is transcriptionally induced in macrophages (Miao and Miller, 2000; Uzzau *et al.* 2001; Worley *et al.*, 2000). The SseI protein has been described as a Salmonella-translocated effector (TSE), which is translocated via the SPI-2

T3SS into host cells (Ehrbar and Hardt, 2005, Miao and Miller, 2000; Uzzau *et al.* 2001; Worley *et al.*, 2000). It is present in most *S.* Typhimurium strains and co-localizes with the polymerizing actin cytoskeleton through interactions of its N-terminal domain with the actin cross-linking protein filamin (Miao *et al.*, 2003). The first 142 amino acid residues of SseI and SspH2 (another *S.* Typhimurium virulence factor; see ΦSE20 for more information) are 89 % identical suggesting that several TSEs might use a common amino acid sequence to direct translocation (Miao *et al.*, 1999; Miao and Miller, 2000). The role of *sseI* for *Salmonella* virulence is unclear, as mutations in this gene had very little effect on virulence (Ho *et al.*, 2002; Ruiz-Albert *et al.*, 2002).

As mentioned before, the *gtgE*-gene encodes a putative protein of 228 amino acids in *Salmonella* for which no significant homologues in other bacteria have been detected so far. The biochemical function of GtgE is still unknown, but *gtgE* has been shown to be the major virulence determinant carried by Gifsy-2 besides *sodCI* (Ho *et al.*, 2002).

The *gtgF*-gene is predicted to code for a small protein of only 63 amino acids in *S.* Typhimurium which shows 76 % identity to the *Salmonella* virulence protein MsgA (Ho *et al.*, 2002). Mutations of the *msgA*-gene were found to have an effect on mouse virulence and macrophage survival (which has been shown to be essential for virulence in mice by Fields *et al.*, 1986) in *S.* Typhimurium (Gunn *et al.*, 1995), while deletion of the *gtgF*-gene had no significant effect on virulence (Ho *et al.*, 2002). Another homologue which is 48 % identical to GtgF is SrfE, which has been identified as the product of a gene regulated by the SsrA/SsrB regulatory system like the *sseI*-gene (Worley *et al.*, 2000). Interestingly, the second major virulence determinant which is carried by Gifsy-2, *sodCI* (Ho *et al.*, 2002) and the virulence determinant *gtgA* (Ho *et al.*, 2002) are not conserved in ΦSE10. Probably, ΦSE10 is a phage remnant only because many of the genes required for phage proliferation are either missing from ΦSE10 or are degenerated to pseudogenes by frameshifts and insertions.

4.1.2 ΦSE12/ΦSE12A

ΦSE12 is similar in parts to regions of Gifsy-2 like ΦSE10, while the 5'-end of ΦSE12 seems to have a different origin as it shows more similarity to ST18.

ΦSE12 harbours the well-conserved *sodCI* gene. The *sodCI* gene encodes a periplasmatic Cu/Zn superoxide dismutase ([Cu,Zn]-SOD), which is likely to be important for the survival of *S.* Enteritidis in the host.

Superoxide dismutases (SODs) catalyse the conversion of superoxide anions into hydrogen peroxide and oxygen (McCord and Fridovich, 1969). The fact that bacterial [Cu,Zn]-SODs are located in the periplasm while superoxide is not able to cross the cytoplasmic membrane suggests that they provide protection to periplasmatic or membrane constituents from superoxide generated outside the cell or conceivably in the periplasm by phagocytic cells (Battistoni, A., 2003; Benov *et al.*, 1995; Farrant *et al.*, 1997; Hassan and Fridovich, 1979; Lynch and Kuramitsu, 2000). Studies in cultured macrophages and mice have shown a consistent contribution of SodCI to virulence for *Salmonella* (*S.* Typhimurium, *S.* Dublin, *S.* Choleraesuis and *S.* Enteritidis) and *E. coli* (Ammendola *et al.*, 2005; Battistoni *et al.*, 2000; De Groote *et al.*, 1997; Fang *et al.*, 1999; Farrant *et al.*, 1997; Figueroa-Bossi *et al.*, 2006; Krishnakumar *et al.*, 2004; Pacello *et al.*, 2008; Sansone *et al.*, 2002; Sly *et al.*, 2002; Uzzau *et al.*, 2002). SodCI is believed to be essential for the intracellular survival of virulent *Salmonella* within host cells (Ammendola *et al.*, 2008). *S.* Typhimurium *sodCI* mutants showed reduced survival in macrophages and attenuated virulence in mice (De Groote *et al.*, 1997; Farrant *et al.*, 1997). SodCI is known to protect the bacterium from phagocytic superoxide during infection, and factors enhancing bacterial resistance to the oxidative burst of phagocytes are believed to play an important role in influencing the outcome of infection in normal hosts (De Groote *et al.*, 1997; Farrant *et al.*, 1997; Krishnakumar *et al.*, 2007). In *S.* Typhimurium *sodCI* is carried by Gifsy-2, and its presence in genomic regions containing sequences derived from bacteriophages or phage remnants indicates a selective pressure to maintain *sodCI* in *Salmonella* (Ammendola *et al.*, 2005; Ammendola *et al.*, 2008; Figueroa-Bossi *et al.*, 2006).

In Gifsy-2, a gene encoding the integral outer membrane protein X (OmpX) is located next to the *sodCI*-gene. In general, outer membrane proteins (Omps) promote adherence to and invasion of host cells, resistance to complement-mediated killing, survival in macrophages, and internalization in epithelial cells (Cirillo *et al.*, 1996; Otto and Hermansson, 2004). Gram-negative bacteria causing invasive disease must resist the bactericidal action of complement, and the presence of specific Omps has been shown to be critical for this resistance (Joiner, 1988; Vogt and Schulz, 1999). OmpX was first described in *Enterobacter cloacae*, but homologues like PagC, Lom, Rck and Ail were identified in other Gram-negative bacteria (Barondess and Beckwith, 1990; De Kort *et al.*, 1994; Dupont *et al.*, 2004; Heffernan *et al.*, 1992; Mecsas *et al.*, 1995; Miller *et al.*, 1990; Miller *et al.*, 2001; Pulkkinen and Miller, 1991; Stoorvogel *et al.*, 1991). Is has been shown that the surface loops of these proteins are involved in virulence (Beer and Miller, 1992). Cloning of the *ompX* gene on a multicopy

plasmid into *Enterobacter cloacae* and *E. coli* conferred decreased susceptibility to several β-lactams and quinolones (Stoorvogel *et al.*, 1987). The exact role of OmpX remains to be investigated although it has been suggested that it binds to foreign proteins on the cell surface, which is possibly part of a cellular defence mechanism. Cell adhesion and invasion might be achieved through this binding affinity (De Kort *et al.*, 1994; Stoorvogel *et al.*, 1987; Stoorvogel *et al.*, 1991; Vogt and Schulz, 1999). In *Enterobacter* OmpX contributed to invasion of host cells, its role in *E. coli*, however, remains unclear: the deletion of *ompX* led to increased cell-surface contact in fimbriated strains and decreased contact in a non-fimbriated strain (Otto and Hermansson, 2004). In ΦSE12 there is evidence for a CDS encoding OmpX, but the CDS has degenerated and is thought to be a pseudogene. The complete gene is present at a non-phage location in *S.* Enteritidis (SEN0778) which might explains why it probably could have become degenerated in ΦSE12 without negative effect on *S.* Enteritidis.

ΦSE12 also contains a gene (SEN1143) with similarity to the *gogA*-gene carried by Gifsy-1 and the *gtgA*-gene carried by Gifsy-2 (Figueroa-Bossi *et al.*, 2001; Wood *et al.*, 1998). These genes show a high similarity to the *pipA*-gene (Figueroa-Bossi *et al.*, 2001; Wood *et al.*, 1998). Mutations in the *pipA*-gene have been shown to affect the enteropathogenicity of *S.* Dublin in a model using bovine ligated ileal loops (Wood *et al.*, 1998). Only minor effects on the ability of *S.* Dublin to cause systemic disease in mice were seen, although in other experiments *pipA* has been shown to be preferentially expressed under conditions known to induce SPI-2 genes to contribute to the development of systemic disease in mice (Knodler *et al.*, 2002; Wood *et al.*, 1998). PipA is encoded on SPI-5 in many *Salmonella* serovars including *S.* Dublin, *S.* Typhimurium; *S.* Typhi and *S.* Enteritidis (Figueroa-Bossi *et al.*, 2001; Wood *et al.*, 1998). In *S.* Enteritidis the putative *pipA* gene is flanked by short fragments with similarity to regions present in the ST10 and ST18 phages carried by *S.* Typhimurium which is in accordance with the fact that the 5'-end of ΦSE12 seems to have a different origin as it shows more similarity to a lambda-like ST18 phage from *S.* Typhi. Overlapping SEN1154 a small fragment with similarity to a putative DNA invertase present at the very 3'-end of ST10 (STY1075) is present in ΦSE12. This is an indicator for multiple recombination events that have happened in ΦSE12 during *S.* Enteritidis evolution. The virulence determinants *sodCI* and *gtgA* carried by Gifsy-2 which were not conserved in ΦSE10 as mentioned above are present in ΦSE12 completing the set of virulence genes found in Gifsy-2 for *S.* Enteritidis PT4.

ΦSE12 also carries the *sopE*-gene which is another T3SS secreted effector protein (Hardt *et al.*, 1998a). As mentioned earlier, it is known to activate the Jun N-terminal kinase (JNK) in a

CDC42- and Rac-1-dependent manner, to stimulate cytosceletal reorganisation and to modulate host-cell RhoGTPase function via a non-covalent interaction (Hardt *et al.*, 1998b; Rudolph *et al.*, 1999). In *S.* Typhimurium and *S.* Typhi, SopE is encoded on a moron which is widespread in the *Salmonellae* and carried by several different bacteriophage families (Mirold *et al.*, 2001). As already mentioned before, it is encoded in the P2-like phage SopEΦ in *S.* Typhimurium and *S.* Typhi, and in a lambda-like bacteriophage in the *Salmonella* serovars Hadar, Gallinarum, Enteritidis and Dublin (Mirold *et al.*, 2001). SopE was the first type III effector protein that has been identified in the genome of different non-related bacteriophages (Mirold *et al.*, 2001). Most of the *S.* Typhimurium isolates harbouring the SopEΦ-phage belong to the small group of epidemic strains that have been responsible for a large percentage of human and animal *Salmonellosis* and have persisted for a long period of time (Mirold *et al.*, 1999). *S.* Typhimurium LT2 does not possess the *sopE* gene (McClelland *et al.*, 2001).

In *S.* Enteritidis carriage of *sopE* may have contributed to its epidemiological success. The *sopE*-gene was identified in all isolates of phage types 1, 4, 6, 8, 11 and 13 but in only a few isolates of PT14b and 21 by Prager *et al.* (2000). The PTs 1,4,6,8 and 13 were epidemiologically important in the UK at the time of their investigations while PT14b was relatively uncommon at that time (Communicable Disease Surveillance Centre, 2002). In an investigation by Hopkins and Threlfall (2004) using real-time PCR and sequencing, all *S.* Enteritidis isolates that came from humans in England and Wales in 2001 were positive for *sopE*. In addition to the improved interaction between *Salmonella* and the host due to carriage of the *sopE*-encoding phage, lysogeny by this phage may result in a population of *sopE*-positive strains through killing of sensitive bacteria. This may be an additional advantage over *sopE*-negative strains (Bossi, 2003; Hopkins and Threlfall, 2004).

The *hokW* gene, which is part of the *hok-sok* postsegregational killing system, is conserved in the 5-region region of ΦSE12 for which another origin is supposed due to its similarity to ST18. The *hok-sok* system belongs to a group of "addiction modules" that have been described mainly in a number of prokaryotic extrachromosomal elements responsible for the postsegregational killing effect (Aizenman *et al.*, 1996). Usually the addiction modules consist of two genes: the product of one is long lived and toxic, while the product of the second is short lived and antagonizes the toxic effect (Aizenman *et al.*, 1996). In case of the *hok-sok* system, the Hok mRNA is very stable and can be translated into Hok killer protein. The translation of the Hok mRNA is inhibited by the small unstable Sok antisense RNA, which is subject to rapid degradation by nucleases. The translation of *hok* is coupled to an

overlapping reading frame termed *mok*, whose translation is tightly regulated by Sok RNA. Through this mechanism Sok RNA regulates *hok* translation indirectly through *mok*. The system mediates plasmid stabilization by killing of plasmid-free cells (Gerdes *et al.*, 1986a, b; Thisted *et al.*, 1995). The first described regulatable chromosomal addiction module is the *mazEF* system in *E. coli* which consists of the two genes *mazE* and *mazF* located in the *rel* operon (Aizenman *et al.*, 1996; Hazan *et al.*, 2001; Metzger *et al.*, 1988). It is believed to be responsible for programmed cell death in *E. coli* regulated by 3',5'-bispyrophosphate and may serve as a mechanism for altruistic cell death: during extreme conditions of starvation part of the starved cells lyse, thereby enabling the survival of the rest of the cell population (Aizenman *et al.*, 1996). In *E. coli* a coupling between the chromosomal *mazEF* system and the extrachromosomal *phd-doc*-System has been described (Hazan *et al.*, 2001). The role of the *hokW* gene present in the *Salmonella* prophage ΦSE12 remains unclear.

ΦSE12A displays a mosaic of genes from related bacteriophages (ST10, ST18, Gifsy-1, Gifsy-2 and Fels-1) and the locus of its integration resembles the CS 40 island which in *S.* Typhimurium contains various loci linked to pathogenicity, such as *mig-3, pagK-pagO* and *sopE2* interspersed with putative phage genes (Bakshi *et al.*, 2000; Balbontín *et al.*, 2008; Gunn *et al.*, 1998; Stender *et al.*, 2000; Valdivia and Falkow, 1997). The CS 40 island has been described as being made of two separate islets lying side by side, one carrying *mig-3* and *pagK-pagO* and the other containing the *sopE2* gene (Balbontín *et al.*, 2008). There is a strong similarity between the CS 40 island in *S.* Typhimurium and ΦSE12A in *S.* Enteritidis with homologues of the *mig-3, pagK* and *pagM* genes being present in the ΦSE12A prophage as putative genes or pseudogenes, and *pagO* and *sopE2* being present in the direct vicinity of the 3'-end of ΦSE12A. While in *S.* Typhimurium the lack of a recognizable attachment site at the right end of the insert and the apparent defective nature of the presumptive integrase gene STM1871 suggests that the *sopE2* islet was acquired earlier and has since suffered extensive decay, this might also be true in the case of *S.* Enteritidis with the ΦSE12A prophage containing the *mig-3, pagK* and *pagM* genes being the most recent acquisition (Balbontín *et al.*, 2008).

When promoters with intracellular-dependent activity were identified in *S.* Typhimurium using differential fluorescence induction, *mig-3* was identified as one macrophage-inducible gene downstream of one of these promoters (Valdivia and Falkow, 1997). The *mig-3* gene is present within an ORF with homology to phage tail-fibre assembly proteins and likely represents a promoter within an integrated phage in *S.* Typhimurium (Valdivia and Falkow,

1997). It is regulated by the PhoP/PhoQ two-component regulatory system in *S.* Typhimurium (Valdivia and Falkow, 1997). In ΦSE12A the *mig-3*-gene is present as a pseudogene.

pagK and *pagM* were identified as PhoP/PhoQ-activated genes by Tn*phoA* mutagenesis in *S.* Typhimurium (Belden and Miller, 1994). *pag*-gene products are involved in a late stage of bacterial parasitism of macrophages as transcriptional activation occurs 3 to 5 hours after phagocytosis when the phagosom pH drops below 5.0 (Alpuche Aranda *et al.*, 1992). Database analysis showed no similarities at the DNA level or with the predicted protein translation to any publicly available database sequences for *pagK* and *pagM* (Carnell *et al.*, 2007; Gunn *et al.*, 1998). The analysis by Gunn *et al.* showed 90 % identity between *pagK* and *pagJ* which are located within duplicated regions of the chromosome and were only found in *S.* Typhimurium and *S.* Enteritidis (Gunn *et al.*, 1998). Both serotypes have a broad host range, while those serotypes that did not hybridize to their *pagK* specific probe are strictly human pathogens, which made Gunn and co-workers suggest a possible correlation of these genes and host range (Gunn *et al.*, 1998). Analysis of the DNA close to the *pagK, pagM* and *pagJ* genes revealed a strong similarity of non-coding segments to a variety of proteins including transposases, DNA invertases phage proteins and proteins encoded on plasmids which appear to be left behind from previous recombination events (Gunn *et al.*, 1998). Therefore this region was believed to be a dynamic region of the chromosome in which sections were obtained by S*almonella* through inter-species transmissions and that mobility of a functional transposon or phage may have been responsible for the duplication of *pagK* and *pagJ* (Gunn *et al.*, 1998). Tn*phoA* insertions in *pagK, pagM* and *pagJ* have been shown to result in a virulence defect in the BALB7c mouse model and significantly reduced survival within macrophages (Belden and Miller, 1994). Strains with mutations in the *pagK* gene were also attenuated in their ability to colonize the intestinal tract in a porcine model of infection (Carnell *et al.*, 2007). Belden and Miller speculate that a cumulative effect of expression of several *pag*-encoded proteins could result in resistance to defensins. An altered membrane charge, electrical potential or lipid content due to an aggregate change in a large number of bacterial proteins could change defensin interaction with bacterial membranes (Belden and Miller, 1994). Deletions of the *pagK, pagM* and *pagJ* genes individually or in any combination however, did not attenuate strain virulence (Gunn *et al.*, 1998). One possible explanation for these surprising findings is that the Pag-PhoA fusion exhibits a dominant-negative effect on virulence through interaction with virulence factors which may include other PAG. A similar protein may substitute upon a deleted *pag* loci resulting in a functional interaction with the virulence factor (Gunn *et al.*, 1998). Redundancies and the level of

complexity will make it difficult to identify those PAG essential for virulence (Gunn *et al.*, 1998). In *S.* Typhimurium a gene encoding a PagK-like protein is also carried in phage Gifsy-1. In ΦSE12A, the *pagK* gene lies adjacent to the pseudogene with similarity to the *mig-3* gene, and a pseudogene with similarity to *pagM* lies in close vicinity to the *pagK* gene.

ΦSE12 and ΦSE12A are putative phage remnants with many of the genes required for phage proliferation being absent or degenerated. The presence of many pseudogenes and genes also present in a combination of related bacteriophages indicates a number of recombination events that have happened during *S*. Enteritidis evolution. Remnants of ΦSE12A are also present at the same location in *S*. Typhimurium LT2 and probably represent the most ancient phage insertion that has been maintained in these two *Salmonella* lineages.

4.1.3 ΦSE14

ΦSE14 seems to be a remnant of a bacteriophage with most of the genes encoding putative phage proteins with similarities to genes of the lambda-like phage ST18 from *S.* Typhi. For some of the genes harboured in ΦSE14 no significant database hits revealing similarities to known genes were found. The overlapping reading frames putatively encoding *hok/mok* from the *hok/sok* post segregational killing system are conserved in SEN1387 in ΦSE14 in relation to ST18. The function of this post segregational killing system has been explained above. Adjacent to this gene, several more genes are highly conserved in relation to ST18. These encode putative phage proteins. Database searches revealed no further information about a probable function of the encoded proteins, but their high conservation indicates that these genes are important for *S.* Enteritidis. Besides many phage structural genes, ST18 encodes an orthologue of lambda replication protein GpO, the cell division inhibitor and phage maintenance protein Kil, CII, which regulates lysogeny, an orthologue of the enterohemolysin-associated protein Ehly-1 and the exodeoxyribonuclease RecA involved in recombination (Thomson *et al.*, 2004). Genes similar to the primary and secondary lambda repressors Cro and CI, genes that encode products involved in DNA restriction and modification, an orthologue of the *lar*-gene involved in restriction allevation and modification enhancement in *E. coli* are also harboured in ST18. Interestingly, there is only one CDS (SEN1381) in ΦSE14 with similarity to the gene encoding Kil in ST18, while CDS with similarity to all other genes encoding the above-mentioned proteins in ST18 are missing in ΦSE14. In the defective lambdoid *E. coli* prophage Rac, the *kil* gene encodes a small protein

which is an inhibitor of bacterial septation (Conter *et al.*, 1996). Differently from previously described inhibitors of septation, *kil* is able to abolish rod shape when strongly expressed (Conter *et al.*, 1996). The inhibition of septation is relieved by excess of FtsZ, a protein responsible for the formation of a cytoskeletal ring at the leading edge of the bacterial division septum (Bi and Lutkenhaus, 1991; Conter *et al.*, 1996). In addition the CRP-cAMP boosts the efficiency of the inhibitor suggesting that either the target or a coinhibitor operating together with Kil is CRP-cAMP regulated (Conter *et al.*, 1996). The role of the *kil* gene conserved in ΦSE14 remains unclear.

Agron *et al.* identified a region unique to serovar Enteritidis strains using suppression subtractive hybridization (Agron *et al.*, 2001). The region harbours six ORFs (*lygA* to *lygF*; "linked to the *ydaO* gene") and was named Sdf I (Agron *et al.*, 2001). On the 5'-end of the Sdf I region a copy of a gene with near-perfect identity to the *E. coli* gene *ydaO* encoding a tRNA-thiolase was identified (Agron *et al.*, 2001). Nucleotide sequence comparisons with database sequences showed a near-perfect match at each end of the Sdf I region to two widely separated regions of the *S.* Typhi genome suggesting that this region is the site of a major rearrangement with respect to *S.* Enteritidis (Agron *et al.*, 2001). Similarity to database sequences was not high enough to provide sufficient evidence to ascribe functions to the putative proteins encoded by this region (Agron *et al.*, 2001). LygA shows similarity to exonuclease VIII of *S.* Typhimurium, LygC exhibits weak similarity to the *E. coli* phage superinfection exclusion protein B, while LygD shows even weaker similarity to phage lambda repressor cI (Agron *et al.*, 2001). LygF shows some similarity to a hypothetical protein of *E. coli* O157:H7 prophage CP-933R (Agron *et al.*, 2001). LygE and F overlap to a large extent, which may indicate that one, the other, or both are not genes (Agron *et al.*, 2001). The deduced amino acid sequences of *lygB* and *lygE* did not show any similarity to database sequences with a protein BLAST search in the analysis done by Agron and co-workers (Agron *et al.*, 2001).

The Sdf I region was identified in all clinical *S.* Enteritidis samples tested by PCR, but lacking from some phage type reference strains (Agron *et al.*, 2001). Agron *et al.* therefore claimed Sdf I to be a robust marker for pathogenic *S.* Enteritidis strains (Agron *et al.*, 2001). This claim will be discussed in detail below as part of the discussion of the results obtained in this study using a *S.* Enteritidis PCR based on the findings of Agron *et al.* and the PCRs used for the screening of the strains for the ΦSE14 locus.

The Sdf I region constitutes the 5'-region of ΦSE14 and contains the genes *lygA-lygF*. A homologue of the *ydaO* gene (SEN1377) is present adjacent to the 5'-end of ΦSE14. There is

evidence for the presence of the *lygE* gene in ΦSE14, but this gene was not included into the annotation of SE125109 by the Sanger Institute. The unique presence of this region in *S.* Enteritidis, and its existence in all clinical samples tested might indicate that it was acquired by integration of an active phage during the evolution of *S.* Enteritidis and influences *S.* Enteritidis pathogenicity. The findings of this study confirm the assumption by Agron and co-workers that the Sdf I region comprises a phage remnant due to the similarity of the *lygF* deduced amino acid sequence to a hypothetical protein of an *E. coli* cryptic phage.

4.1.4 ΦSE20

ΦSE20 is highly similar in sequence and gene order to the *S.* Typhimurium DT64 lambda-like phage ST64B. The phage ST64B was isolated from *S.* Typhimurium DT64 strain 2558 together with phage ST64T (Mmolawa *et al.*, 2002). Crude and CsCl-purified phages ST64B and ST64T could not be distinguished by electron microscopy (Mmolawa *et al.*, 2002). ST64B is inducible by mitomycin C but could not be propagated on any of the strains tested and failed to produce plaques on many diverse *Salmonella* and *E. coli* strains (Mmolawa *et al.*, 2002; 2003a). Although ST64B has a genomic architecture similar to that of phage lambda, its genome is clearly a mosaic composed of genes from phages of diverse bacterial groups including Gram-positive organisms (Mmolawa *et al.*, 2003b). ST64B is unlikely to be able to mediate transduction and is predicted not to have a tail as the tail genes are probably non-functional due to the insertion of virulence gene fragments (Mmolawa *et al.*, 2003b). The complete phage ST64T which propagates autonomously in the above mentioned *S.* Typhimurium strain might *trans*-activate the defective genome of ST64B and compensates for some ST64B deficiencies other than the interrupted tail genes allowing partially complete virions to be produced (Mmolawa *et al.*, 2003b). An analysis of the ST64B genome revealed that two putative genes (sb 21 and sb22) laying adjacent to each other in different reading frames in the tail operon are similar to the two halves of an uninterrupted ORF in other phages, including P27 and Mu (Figueroa-Bossi and Bossi, 2004). An analysis of ST64B revertants arising spontaneously in cultures revealed a fusion of the sb21 and sb22 ORFs into a single reading frame. This suggests that in ST64B a +1 frameshift mutation has occurred in ST64B leading to its inability to produce infectious virions. The apparent lack of visible tails in the ST64B phage preparations by Mmolawa *et al.*, is consistent with this theory (Figueroa-Bossi and Bossi, 2004; Mmolawa *et al.*, 2003b). The reactivation of the phage can be achieved by a reversion of the +1 frameshift mutation (Figueroa-Bossi and Bossi, 2004).

Interestingly in ΦSE20 sb21 and sb22 are present as one consecutive ORF. This might indicate that in *S.* Enteritidis the introduction of ST64B happened before it occurred in *S.* Typhimurium, introducing the region without the mutation into *S.* Enteritidis while the same region was introduced into *S.* Typhimurium after occurrence of the +1 frameshift mutation. Alternatively, *S.* Enteritidis and *S.* Typhimurium both acquired the unmutated gene and the mutation happened in *S.* Typhimurium at a later time point while it did not occur in *S.* Enteritidis. It is even possible that a transfer of the unmutated ST64B from *S.* Typhimurium to *S.* Enteritidis happened and the mutation occurred in *S.* Typhimurium then sometime afterwards. The effect of these findings on ΦSE20 induction will be discussed below.

Fragments of the virulence-associated genes *sopE, sspH2* and *orgA* are present in putative tail and tail fibre genes in ST64B (Mmolawa *et al.*, 2003b). In ΦSE20 fragments of these genes are also present in putative tail and tail fibre genes. In the SopEΦ phage harboured by *S.* Typhimurium the *sopE* gene is flanked by sequences resembling tail and tail fibre genes of P2-like phages (Hardt *et al.*, 1998a). The role of *sopE* for *Salmonella* virulence has been extensively discussed above already. Interestingly, besides the fragment present in ΦSE20 the complete *sopE* gene is also harboured in ΦSE12 indicating its putative importance for *S.* Enteritidis.

SspH2 and SseI both belong to a group of TSEs that are translocated into host cells via the SPI-2 T3SS (Ehrbar and Hardt, 2005, Miao *et al.*, 1999; Miao and Miller, 2000; Uzzau *et al.* 2001; Worley *et al.*, 2000). As mentioned earlier, the first 142 amino acid residues of SseI and SspH2 are 89 % identical suggesting that several TSEs might use a common amino acid sequence to direct translocation (Miao *et al.*, 1999; Miao and Miller, 2000). Because *sseI* is believed to have evolved more recently than *sspH2*, it is hypothesized that *sseI* was generated by recombination of the N-terminal domain of *sspH2* with novel DNA encoding the C-terminal domain (Miao and Miller, 2000). Like SseI, SspH2 also co-localizes with the polymerizing actin cytoskeleton through interactions of its N-terminal domain with the actin cross-linking protein filamin (Miao *et al.*, 2003). SspH2 was also found to interact with profilin and to alter the rate of actin polymerization *in vitro* (Miao *et al.*, 2003). It co-localized with vacuole-associated actin polymerizations (VAP) induced by intracellular bacteria through the SPI 2 T3SS suggesting that it functions to reduce or remodel VAP (Miao *et al.*, 2003). In spite of the effect of SspH2 on actin polymerization *in vitro*, no alteration in VAP morphology was observed for ΔsspH2 *Salmonella* mutants. This lack of phenotype may be explained by functional redundancy among effectors which is believed to be a common theme in SPI 1 and SPI 2 T3SS effectors (Miao *et al.*, 2003). Like *sseI*, *sspH2* transcription is

induced in the intracellular environment dependent upon the SPI 2-encoded two-component regulatory system SsrA/SsrB (Miao *et al.*, 1999; Miao and Miller, 2000). In *S.* Typhimurium Ssph2 has been shown to contribute to virulence in calves probably being important for permitting the bacteria to persist in the host rather than causing efflux and diarrhoea (Miao *et al.*, 1999). Recently SspH2 has been attributed to a growing class of bacterial effector proteins that harness and subvert the eukaryotic ubiquitination pathway representing a remarkable example of biochemical mimicry of host cell biology. The actual targets of the SspH2 ligase activity have yet to be identified (Quezada *et al.*, 2009).

When the p60 protein of *Listeria* was fused to Ssph2, the overexpression of this hybrid protein from a medium-copy-number vector led to simultaneous p60-specific CD4 and CD8 T-cell priming. Therefore SspH2 might be an attractive carrier molecule for antigen delivery when T-cell immune responses against complex microbes (or tumours) are needed (Panthel *et al.*, 2005). Putative homologues of SspH2 are present in other pathogens including *Shigella* and *Yersinia* and sequences similar to *sspH2* were found in most of the *Salmonella* serotypes tested indicating a central role of this gene in the virulence strategies of *Salmonella* (Miao *et al.*, 1999). *sspH2* appears to be located within a lysogenic bacteriophage in *S.* Typhimurium (Miao and Miller, 2000). As described for *sopE* above, besides the fragment present in ΦSE20 the complete *sspH2* gene is also harboured in *S.* Enteritidis 125109 (SEN2224) flanked by pseudogenes of probable phage origin. The conservation of the gene indicates its putative importance for *S.* Enteritidis.

The *orgA* gene has been first identified in a screening of oxygen-regulated genes that were required for bacterial invasion into Hep-2 cells by *S.* Typhimurium (Jones and Falkow, 1994). *orgA* seems to be required for passage through the intestinal epithelium and the Peyer's patches (Jones and Falkow, 1994). *S.* Typhimurium *orgA* mutants were non-invasive and showed reduced virulence after oral infection in mice (Jones and Falkow, 1994). The mutation prevented the invasion and destruction of M cells and the mutants had a general defect in secretion of invasion effector proteins (Penheiter el al., 1997). *orgA* encodes a type III secretory component and the phenotype of the *orgA* mutants indicated that OrgA is an integral component of the invasion secretion apparatus that transports specific effector proteins into the host cell to induce the uptake of the pathogen into the cell (Klein *et al.*, 2000; Penheiter *et al.,* 1997). *orgA* is similar to the *mxiK* gene in *Shigella*, which is a putative component of the type III secretion system in that pathogen (Allaoui *et al.*, 1992). Besides the fragment present in ΦSE20 the complete *orgA* gene is also present in SPI 1 in *S.* Enteritidis 125109 (SEN2712). The fact that all three genes (*sopE, sspH2* and *orgA*) are present as complete

genes in *S.* Enteritidis 125109 underlines their putative importance for *Salmonella* virulence and explains why they probably could have become degenerated to fragments in ΦSE20 without negative effect on *S.* Enteritidis.

S. Typhimurium mutants lacking the entire ST64B prophage behaved like the virulent wild-type strain in invasion and intracellular proliferation and survival assays performed in cultures macrophage, epithelial, and fibroblast cell lines (Alonso *et al.*, 2005). The mutants were also able to compete with the wild-type virulent strain for colonization of target organs as liver and spleen and penetrated the intestinal epithelium efficiently. When administered orally they produced systemic disease (Alonso *et al.*, 2005). The results indicate that none of the genes encoded in ST64B are required for *S.* Typhimurium pathogenesis in the murine typhoid model (Alonso *et al.*, 2005). The presence of fragments of the virulence-associated genes *sopE*, *sspH2* and *orgA* in ST64B nevertheless suggests that ST64B might have played a role in the transfer of virulence determinants, and it is obvious that the gene fragments can not contribute to virulence in the murine model.

By comparison to known secreted proteins from enterohemorrhagic *E. coli* and *Citrobacter rodentium* SseK1 and SseK2 were identified in *S.* Typhimurium (Kujat Choy *et al.*, 2004). SseK1 and SseK2 were found to be translocated into host cells (Kujat Choy *et al.*, 2004). During *in vitro* growth SseK1 was a substrate for secretion by both the SPI 1 and the SPI 2-encoded T3SS while the pattern of SseK2 protein expression suggested that it is co-ordinately regulated with the SPI 2 T3SS (Kujat Choy *et al.*, 2004). The *sb26* gene from ST64B was found to be a homologue of *sseK1* and *sseK2* and is therefore proposed to be named *sseK3* (Kujat Choy *et al.*, 2004). The possibility that *sseK1* and *sseK2* were acquired by horizontal gene transfer is strengthened by this finding (Kujat Choy *et al.*, 2004). Effects of SseK1 and SseK2 on the virulence of *S.* Typhimurium were not evident using *ΔssK1*, *ΔssK2* and *ΔssK1/ΔssK2* mutants during infection of tissue culture cells or susceptible mice (Kujat Choy *et al.*, 2004). The absence of SseK1 and/or SseK2 possibly may have been complemented by *sb26* (Kujat Choy *et al.*, 2004). These findings do not allow a statement to be made on the function of the *sb26* gene in ST64B or its homologue in ΦSE20.

ST64B harbours a region likely to be the immunity C region (*immC*) (Mmolawa *et al.*, 2003b). It consists of the putative genes for a CI repressor, a Cro-like protein and a CII-like gene product. The *immC* region is involved in the movement of phage into either the lytic or lysogenic cycles in bacteriophage lambda (Ptashne *et al.*, 1980).

When a bacterial population is infected by a temperate bacteriophage, most of the cells display a lytic response in which the virus multiplies and lyses the cells killing them, while a

fraction of the cells enters the lysogenic state in which the viral DNA has integrated into the bacterial chromosome and is transmitted to the bacterial progeny (Thieffry and Thomas, 1995). In the lysogenic state the product of the *cI* gene acts as a repressor blocking the expression of all other phage genes und conferring immunity towards infection with the same type of virus to the bacterium (Thieffry and Thomas, 1995). Concentrations of CI circulating at low levels in the cytoplasm of the cell enables it to bind to corresponding operator sites on any superinfecting phage DNA having a similar *immC* region which prevents the expression of lytic genes by the superinfecting phage (Poteete, 1988). The decision of whether or not the *cI* gene will be expressed and immunity will be established is subject to a precise control in which four phage genes (*cI, cro, cII and N*) interact (Echols, H. 1986; Eisen *et al.*, 1970; Herskowitz and Hagen, 1980; Oppenheim *et al.*, 1970; Ptashne *et al.*, 1986; Reichardt, 1975). The *cI* gene is normally switched on by the product of gene *cII* which acts as a trigger (Thieffry and Thomas, 1995). The *cI* gene will remain on as its product activates its own synthesis while the other bacteriophage genes including the *cII* gene which had just been switched on will be switched off (Thieffry and Thomas, 1995). The *cro* gene exerts a negative control on *cI*, directly and indirectly by impairing the expression of *cII* (Thieffry and Thomas, 1995).

Despite its inability to infect a host, it is possible that the *immC* region of ST64B can influence phage-type designation in strains in which it is present as a prophage (Tucker and Heuzenroeder, 2004). It has been demonstrated that the *immC* region can mediate phage-type conversion when present on a high-copy number plasmid which confirms the likelihood of this region being functional in ST64B (Tucker and Heuzenroeder, 2004).

Generally speaking, phage-type conversion or an altered sensitivity to the typing panel can be the result of the acquisition of a temperate phage which resides in the host strain as a prophage (Tucker and Heuzenroeder, 2004). Although classical phage typing has been used in epidemiology for many years, it is a highly empirical method requiring an experienced operator (Tucker and Heuzenroeder, 2004). The obtained results and how the determined phage types relate to each other genetically are currently not rationally explained (Tucker and Heuzenroeder, 2004). In case of the Anderson typing system it has been suggested that phage typing system might not be working properly anymore when the original stocks of phages are exhausted (Schmieger, 1999). This is caused by the fact that recombination of typing phages with endogenous phages within the strains used for propagation of the panel could yield new phages with different plaquing characteristics (Schmieger, 1999). This, together with the fact that phage-type conversion or an altered sensitivity to the typing panel can be the result of the

acquisition of a temperate phage which resides in the host strain as a prophage, demonstrate the advantages of developing a rational typing system based on the genetic properties of bacterial strains belonging to different phage types (Tucker and Heuzenroeder, 2004). The knowledge of phage-encoded genes as resulting from this work is one important factor for the development of such a system which at a later stage could be improved by utilizing microarray technology. Data generated with such a system should always be seen in the context of the epidemiological data generated with the classical system of phage typing. Ideally, a connection between the classical phage-types and the molecular types should be drawn.

4.1.5 G + C content

The G + C content of bacterial genomes varies between 25 and 75 % for unclear reasons (Sueoka, 1962). It is considered to be a phylogenetic characteristic of a species, which in the case of *S. enterica* averages 52 % (Aoyama *et al.*, 1994; Bäumler *et al.*, 1997). This is in accordance with the G + C content percentages previously published for *S.* Typhi CT18 of 52.09 % and *S.* Typhimurium LT2 of 52.22 % and the G + C content of 52.17 % determined for the *S.* Enteritidis strain 125109 (McClelland *et al.,* 2001; Parkhill *et al.*, 2001). Interestingly, all putative prophage regions identified in *S.* Enteritidis 125109 have lower G + C contents of between 45.15 and 50.59 % than their host strain while the G + C content of the prophages found in *S.* Typhi CT18 and *S.* Typhimurium LT2 average around the G + C content of their host strains with some prophages having lower and some having higher G + C contents than the host strains. Although the G + C contents of the putative *S.* Enteritidis prophages are not especially low, a differing G + C content might indicate that these segments were obtained horizontally from other organisms probably with a lower G + C content.

The genomes of bacteria that like obligatory pathogens or symbionts rely on their host for survival have been found to have a low G + C content (Rocha and Danchin, 2002). Genetic elements like chromosomes, plasmids, phages and insertion sequences using the cell's replication machinery might be expected to have the same G +C content as the host (Rocha and Danchin, 2002). Exceptions to this rule are thought to indicate recent horizontal transfer, and unusual G + C contents have been utilized for the identification of such elements in bacterial genomes (Karlin, 2001; Moszer *et al.*, 1999). The horizontally acquired elements are progressively altered towards the average nucleotide composition of the host genome (Lawrence and Ochman, 1998). An analysis of bacteriophages, plasmids and insertion

sequences which might also be regarded as 'intracellular pathogens', showed that like the obligatory pathogens and symbionts, these elements also have a significantly lower G + C content than their hosts (Rocha and Danchin, 2002). Temperate phages have smaller biases towards low G + C content than filamentous phages [-1.4 % versus -4.2 %] (Rocha and Danchin, 2002). Prophages replicate vertically with the bacterial chromosome and hence are subject to alteration towards the host G + C content resulting in smaller G + C deviations, while filamentous phages are not integrated into the host's genome (Rocha and Danchin, 2002). Therefore they are not subject to alteration towards the host G + C content, but because they replicate in a replicating bacterial cell, the evolution of higher A + T content will be beneficial due to the higher energy cost and limited availability of G and C over A and T/U (Rocha and Danchin, 2002). Additionally, when resources get depleted in the bacterial cell, the relative availability of A and T increases, and mis-incorporation of these nucleotides might produce a bias towards higher A + T content (Rocha and Danchin, 2002).
In general, in bacterial genomes the individual increase in fitness of each C/G to A/T mutation is unlikely to carry a sufficient advantage to allow frequent fixation (Sueoka, 1993). If a mutational bias however, has a selective advantage or if it hitchhikes with a selective mutation it can take place (Sueoka, 1993). The lower G + C content of the putative prophages found in *S.* Enteritidis 125109 could possibly be explained in the context of the results obtained by Rocha and Danchin.

4.2 PCR results

4.2.1 *Salmonella spp.* and *Salmonella* Enteritidis PCR

Amplification of the target sequence could be detected for all samples tested with the *Salmonella spp.* PCR in this study. This was one minimum inclusion criteria for the strains to be used in this study besides the results obtained in the slide agglutination.
The *Salmonella* Enteritidis PCR was positive for all *S.* Enteritidis isolates tested except for those of phage type 11 and phage type 20. These findings did not come unexpected and were also mentioned by Agron and co-workers in their initial publication of the PCR (Agron *et al.*, 2001). The Sdf I region was identified by suppression subtractive hybridization and found to be unique to *S.* Enteritidis including a wide range of clinical and environmental samples (Agron *et al.*, 2001). While no PCR products were amplified from 73 non-Enteritidis isolates,

clear positive results were obtained when 33 *S.* Enteritidis strains from various environmental sources comprising 11 phage types were tested (Agron *et al.*, 2001). When the primer pair targeting the Sdf I region was used to test 37 phage type reference strains of *S.* Enteritidis from the National Veterinary Services Laboratory (NVSL), the phage types 6A, 9A, 11, 16, 20 and 27 did not lead to the amplification of a PCR product (Agron *et al.*, 2001). Two clinical phage type 9A strains and four clinical phage type 6A strains were nevertheless identified by PCR with the Sdf I primer pair (Agron *et al.*, 2001). Based on these results they claim Sdf I to be a robust marker for pathogenic *S.* Enteritidis strains. A clear relationship between phage typing and the presence of Sdf I was lacking (Agron *et al.*, 2001). This shows the limitations of conventional phage typing and once again demonstrates the necessity of developing a rational typing system based on the genetic properties of bacterial strains belonging to different phage types.

A PCR with a primer pair internal to Sdf I showed the same results regarding the incoherent detection of certain phage types seen with the original primer pair (Agron *et al.*, 2001). This makes point mutations in the primer binding sites an unlikely explanation, and Agron and co-workers speculate that in some of the reference strains they tested the Sdf I region might have been lost during laboratory passage while selection maintained this region in environmental isolates tested (Agron *et al.*, 2001).

Clinical samples for phage types 11, 16, 20 and 27 were not available from the Centers for Disease Control and Prevention (CDC) to Agron and co-workers. They therefore suggest that infections from these phage types are extremely rare (Agron *et al.*, 2001). Contrary to this assumption, clinical samples for a phage type 11 strain (diagnostic sample originating from a cat) and a phage type 20 strain (originating from a duck) were available for this study from the National Reference Laboratories at the *Bundesinstitut für Risikobewertung* (BfR) and the *Robert Koch-Institut* (RKI). This together with findings from this study which indicate that the Sdf I region is localized in the putative prophage region ΦSE14 make it appear more likely that negative PCR results are linked to the absence of the putative prophage ΦSE14 which harbours the Sdf I regions as the target of the PCR. All non-Enteritidis samples tested in this study did not produce positive PCR results in the *S.* Enteritidis PCR confirming the uniqueness of the Sdf I region for this serovar.

A growing number of DNA based detection methods for *Salmonella* have been published currently. Many of those are real-time PCR methods (e.g. Hadjinicolaou *et al.*, 2009; Malorny *et al.*, 2007; Pan and Liu, 2002; Woodward and Kirwan, 1996), but there has also a method based on loop-mediated isothermal amplification (LAMP) capable of rapid, sensitive, and

specific detection of the O9 group of *Salmonella* been published recently (Okamura *et al.*, 2008).

Most of the PCR assays target the *sefA* gene encoding a fimbrial antigen termed SEF14, which has been found in some 7,500 *S.* Enteritidis isolates representing all phage types, according to Woodward and Kirwan (e.g. Hadjinicolaou *et al.*, 2009; Pan and Liu, 2002; Woodward and Kirwan, 1996). However, it is not specific enough to identify only *S.* Enteritidis as the gene is unique to S*almonella* serogroup D serovars which include amongst others *S.* Typhi, *S.* Gallinarum or *S.* Pullorum (Malorny *et al.*, 2007). The assay published by Malorny and co-workers targets the Prot6e gene located on the *S.* Enteritidis specific 60-kb virulence plasmid (Malorny *et al.*, 2007). 75 out of the 79 *S.* Enteritidis isolates tested in that study gave positive PCR results, but interestingly the only phage type 11 isolate in that study gave a negative PCR result and harboured a plasmid that differed in size (Malorny *et al.*, 2007). Therefore it would be interesting to test other phage type 11 (and phage type 20 isolates that were not included into the study by Malorny and co-workers) to see if these all give negative PCR results. In this case their PCR could not compensate for the lack of specificity seen in the PCR published by Agron and co-workers (Agron *et al.*, 2001; Malorny *et al.* 2007). The published results for the LAMP assay included 119 *S.* Enteritidis isolates, but no phage types were specified, so that no statement can be made about any influence of the phage type on the results obtained with that method (Okamura *et al.*, 2008). The findings from this study show the importance of including isolates representing all phage types into assay validation procedures to ensure that the assay which is being validated really possesses the specificity in the detection of *S.* Enteritidis that is being claimed. Probably this is true for assays specifically detecting other serovars as well.

4.2.2 PCRs for screening of prophage presence

4.2.2.1 ΦSE10

The putative prophage region ΦSE10 seemed to be conserved within *S.* Enteritidis across almost all phage types tested, with the 3'-end and the target region containing the conserved effector protein gene *sseI* being present in all *S.* Enteritidis isolates of the strain collection similar to their presence in SE125109. The negative PCR results that were obtained with the SE10_5 primer pair for those *S.* Enteritidis isolates belonging to the phage types 9b, 11 and 20

could be explained by point mutations in this region preventing one or both of the SE10_5 primers from binding to their target region or by a different integration of the phage or assortment of the phage genes in the 5'-region. The pseudogenes SEN0908A and SEN0909 present at the 5'-end of the ΦSE10 region are an indicator for this region not being essential for the *Salmonella* host allowing for mutations in this region while the effector protein gene *sseI* seems to be well conserved.

Since the ΦSE10 region has been described as being closely related to the Gifsy-2 region present in *S.* Typhimurium, it is not surprising to see positive PCR results for the SE10_SSEI primer pair and the SE10_3 primer pair in this serovar as well. Maybe for the same reasons mentioned for the *S.* Enteritidis phage types 9b, 11 and 20, the SE10_5 primer pair did not amplify the target region in *S.* Typhimurium. Positive PCR results in case of the SE10_3 primer pair for the serovars Stanley, Infantis, Hadar and Senftenberg might as well indicate the frequent rearrangements of fragments present in ΦSE10 or Gifsy-2 in these serovars as well. Interestingly, no positive PCR results were obtained for the two other group D serovars Gallinarum and Eastbourne tested in this study. This is in accordance with results from the *S.* Gallinarum sequence analysis that indicated the absence of the putative prophage ΦSE10 in *S.* Gallinarum (Thomson *et al.*, 2008).

4.2.2.2 ΦSE12/ΦSE12A

Like ΦSE10, the putative prophage region ΦSE12/ΦSE12A seems to be conserved within *S.* Enteritidis across all phage types tested as indicated by the positive PCR results obtained for all three primers pairs: SE12_5N; SE12_SOPE_N and SE12_3. The negative PCR result seen for the phage type 20 isolate with the SE12_3 primer pair as the only exception can probably also be explained by point mutations in this region or by a different integration of the phage or assortment of the phage genes at the 3'-end.

Confirming the results from the *S.* Gallinarum sequence analysis that indicated the presence of the putative prophage locus ΦSE12/ΦSE12A in this serovar, all three PCRs were positive when the *S.* Gallinarum DNA was used as a template (Thomson *et al.*, 2008). The same PCR results for *S.* Infantis suggest presence of the prophage locus ΦSE12/ΦSE12A also in this serovar. The finding that for five non-Enteritidis isolates (serovars Typhimurium, Eastbourne, Saint Paul, Stanley and Anatum) the PCR gave positive results with the SE12_5N primer pair and the SE12_3 primer pair but not with the SE12_SOPE_N primer pair is somewhat surprising because one would assume a conservation of this probable virulence determinant.

A possible explanation are the pseudogenes flanking the *sopE* gene in *S.* Enteritidis which might be mutated in other serovars in a way that prevent one or both SE12_SOPE_N primers from binding to the target DNA. SopE is known to be encoded on a moron widespread in the *Salmonellae* and carried by several different bacteriophage families (Mirold *et al.*, 2001). Positive PCR results for *S.* Virchow and *S.* Hadar with the SE12_SOPE_N primer pair and the SE12_3 primer pair but not with the SE12_5N primer pair might result from a different 5'-end in these two serovars. In *S.* Enteritidis 125109 the 5'-end of the putative prophage locus ΦSE12/ΦSE12A seems to have a different origin than the rest of the locus. In *S.* Virchow and *S.* Hadar this 5'-end might come from another source than it does in *S.* Enteritidis.

4.2.2.3 ΦSE14

The results obtained with the PCRs targeting the putative prophage region ΦSE14 perfectly match with the results obtained in the *Salmonella* Enteritidis PCR. The ΦSE14 region is conserved among almost all *S.* Enteritidis tested in this study with the exception of phage types 11 and 20 which were also negative in the *Salmonella* Enteritidis PCR. This is an additional strong indication for the absence of the ΦSE14 region including the Sdf I region in these *S.* Enteritidis phage types as discussed for the *Salmonella* Enteritidis PCR. More detailed studies looking at a higher number of isolates comprising those phage types that were negative in the *Salmonella* Enteritidis PCR will be a valuable tool to gain more information on the relationship between the phage type and the presence or absence of the ΦSE14 region, and a possible connection with the pathogenicity of strains as indicated by Agron and co-workers (Agron *et al.*, 2001). The lack of amplification with the SE14_3 primer pair in the phage type 9b isolate might be the result of a mutation that prevents one or both of the SE14_3 primers from binding to the target DNA or by a different integration of the phage or assortment of the phage genes at the 3'-end. As supposed by the presence of the pseudogenes SEN1396A and SEN1398 at the 3'-end of the ΦSE14 this region might not be essential for the *Salmonella* host allowing for mutations.

The lack of amplification for all three PCRs with all non-Enteritidis isolates tested confirms the results obtained with the *Salmonella* Enteritidis PCR and underlines the uniqueness of the putative prophage region ΦSE14 for *S.* Enteritidis. The absence of this region is in accordance with findings from the *S.* Gallinarum genome analysis which were also not able to locate this region in the genome of *S.* Gallinarum (Thomson *et al.*, 2008). Interestingly, no amplification with the SE14_CONSERVED primer pair was seen with the *S.* Typhimurium isolate although

the primers were designed to target a region in *S.* Enteritidis which is believed to be highly conserved in relation to the *S.* Typhimurium prophage ST18 by the genome comparisons performed in this study.

4.2.2.4 ΦSE20

The putative prophage region ΦSE20 seems to be present in most of the *S.* Enteritidis phage types. Interestingly it appears to be absent from the phage types 8, 9b and 13a. The phage type 8 and 13a isolates did not pop up in the screening for the presence of the other putative prophage locations in *S.* Enteritidis in this study. The two isolates of unknown phage type which were also PCR negative for all three ΦSE20 targeted PCRs might as well belong to one of these phage types. Phage ST64B, to which ΦSE20 is highly similar in sequence and gene order has been found to be present in only some *S.* Typhimurium isolates as well (Alonso *et al.*, 2005). The positive PCR result with the SE20_3 primer pair with a phage type 11 isolate and with the SE20_5 primer pair with a phage type 20 isolate might indicate that in these phage types only fragments of the putative prophage region ΦSE20 are present or that significant mutations or phage rearrangements have happened in these phage types. The unclear results seen in the phage type 21c isolate cannot be explained from the available PCR data, but one possible explanation might also be mutations that have affected the binding of the primers to their target sequences resulting in no or incomplete amplification of the target sequence.

The putative prophage region ΦSE20 seems to be quite unique to *S.* Enteritidis. Although the genome analysis that was part of this study revealed a high similarity to phage ST64B from *S.* Typhimurium DT64, positive PCR results were obtained only with the SE20_IMMC primer pair and the SE20_3 primer. So at least between the *S.* Typhimurium isolate used in this study and SE125109 there seems to be a relevant difference in genome sequence. The positive PCR with the SE20_IMMC primer pair using *S.* Anatum DNA as the target might be caused by fragments of the *immC* region which is also present in this serovar. The PCR results confirm the assumed absence of ΦSE20 in *S.* Gallinarum that was based on the genome comparisons (Thomson *et al.*, 2008).

4.2.3 General discussion of the PCR based screening

The PCRs designed in this study turned out to be a valuable tool for the screening of both the *S.* Enteritidis and non-Enteritidis isolates in the strain collection for the presence of the putative prophage regions identified in the genome analysis. The prophage content was quite conserved between the *S.* Enteritidis isolates. No differences were seen within the same phage type, and the large number of phage type 4 isolates from different sources strongly indicates the representative character of the sequenced *S.* Enteritidis strain 125109 for phage type 4 isolates in terms of prophage content. Based on the PCR data, no effect could be seen for isolates from different sources and different countries of isolation. Also between the attenuated vaccine strain AviPro® SALMONELLA VAC E and its parent strain Leipzig no differences could be detected in the prophage content based on the PCR screening.

Isolates belonging to the phage types 11 and 20, and to some extent also the isolate belonging to phage type 9b showed the most differences in the pattern of positive PCR reactions compared to the sequenced strain. The PCR results obtained for the non-Enteritidis isolates showed that the prophage content of *S.* Enteritidis comprises a characteristic pattern for this serovar, but the bacteriophages seem to form a pool that is rearranged in different patterns in other serovars which all display a different mosaic of genes from this pool. This becomes obvious through the scattered pattern of positive PCR reactions in the non-Enteritidis isolates. Remarkably, all PCR products that were obtained when DNA from the non-Enteritidis isolates was used as the template had the expected lengths that were calculated based on the sequence of SE125109. This seems to indicate that in those cases when amplification of the target sequence took place, the target seems to be relatively conserved as the length is always maintained. Stronger mutations or rearrangements might have interfered with the PCR to a larger extent inhibiting the formation of any PCR products.

To get a deeper insight into the conservation and the differences in bacteriophage content in the *S.* Enteritidis isolates belonging to different phage types, isolates were selected for a microarray analysis based on the results of the PCR screening.

4.3 Microarray experiments

4.3.1 ΦSE10

The analysis of the microarray data obtained for the genes present in the putative prophage region ΦSE10 confirm the conservation of this region within *S.* Enteritidis across almost all phage types tested which has already been seen in the PCR-based screening. The PCR results match quite well with the microarray data. The microarray data indicates the absence of the genes SEN0910 – SEN 0912A at the 5'-end of ΦSE10 in the phage type 11 isolate, which also gave negative PCR results with the SE10_5 primer pair targeting this region. Interestingly, the genes flanking the 5'-end of ΦSE10 are conserved in the phage type 11 isolate making no difference to all other isolates tested. Probably there seems to be a different integration of the phage in the phage type 11 isolate or some of the genes at the 5'-end might have been lost. While there was also no amplification obtained with the SE10_5 primer pair for the phage types 9b and 20 isolates, the microarray data indicates the presence of the complete putative prophage region ΦSE10 in these isolates. However, the pseudogenes SEN0908A and SEN0909 located at the 5'-end of ΦSE10, which are the target for the SE10_5 primer pair, are not present on the microarray used for the analysis, so that no direct statement on the presence of SEN0908A and SEN0909 can be made based on the microarray results. As already mentioned in the context of the PCR results, the presence of two pseudogenes (SEN0908A and SEN0909) at the 5'-end of the ΦSE10 region indicates this region not to be essential for the *Salmonella* host allowing for mutations in this region while the effector protein gene *sseI* seems to be well conserved. These mutations or even the loss of the whole pseudogene might explain the negative PCR results seen in the phage type 9b and 20 isolates despite conservation of the rest of the 5'-end of the ΦSE10 region according to the microarray data.

4.3.2 ΦSE12/ΦSE12A

Results from the PCR-based screening which had already indicated that like ΦSE10, the putative prophage region ΦSE12/ΦSE12A seems to be conserved within *S.* Enteritidis across all phage types tested, could be confirmed by the analysis of the microarray data obtained for

the genes present in ΦSE12/ΦSE12A. The ΦSE12/ΦSE12A genes represented on the microarray could be detected in all tested isolates according to the microarray data. The observation that from the microarray data no indication for the presence of the pseudogene SEN1152 in the phage type 9b isolate could be seen can probably be explained by the pseudogene character of SEN1152. It seems not to be important for the *Salmonella* host as it has mutated to a pseudogene in SE125109 and therefore further mutations or even the complete loss of the pseudogene would probably have no effect on the host. The negative PCR result seen for the phage type 20 isolate with the SE12_3 primer pair was not reflected in the microarray results at first view, because this isolate did not show differing results in the microarray for the genes of the putative prophage region ΦSE12/ΦSE12A. A closer look however, points out to a probable explanation for these findings. Only one gene from the very 5'-end of ΦSE12A is present on the microarray while all other genes are allocated to ΦSE12. The absence of the region targeted by the SE12_3 primer pair would therefore not have been visible from the microarray data. Nevertheless, the phage type 20 isolate was the only isolate showing divergent results in the analysis of the genes in the vicinity of ΦSE12/ΦSE12A with the genes SEN1174, SEN1176 and SEN 1178 adjacent to the 3'-end of ΦSE12/ΦSE12A seemed to be missing, which is a strong indication for a different integration of the phage or assortment of the phage genes at the 3'-end.

4.3.3 ΦSE14

The microarray data for the genes of the putative prophage region ΦSE14 present on the microarray completely matches with the results from the PCR based screening. According to PCR results and microarray results ΦSE14 is present in the isolates of most of the phage types tested. The absence of ΦSE14 from the isolates belonging to phage types 11 and 20 as indicated by the PCR results was also seen in the microarray data. The observation of a gene similar to the STY2007 gene from *S.* Typhi CT18 being present in these two isolates which in return was absent from the isolates belonging to all other phage types tested suggests that this gene was lost during the integration of ΦSE14. The location of the STY2007 gene in *S.* Typhi right at the 5'-end of the ST18 phage indicates that this gene might be left over from previous phage integration events in the *S.* Enteritidis isolates belonging to phage types 11 and 20 at a location possibly being a hot spot for phage integrations.

In the PCR based screening no amplification was seen with the SE14_3 primer pair targeting the 3'-end of the putative prophage region ΦSE14 in the phage type 9b isolate. This result is

also very well reproduced in the analysis of the microarray data, where there is evidence that of those genes represented on the array the genes SEN1393, SEN1394 and SEN1395 at the 3'-end of the phage type 9b isolate are missing probably due to different integration of the phage or assortment of the phage genes at the 3'-end. As discussed in the context of the PCR results already, the pseudogene-character SEN1396A and SEN1398 present at the 3'-end of the ΦSE14 might indicate that this region is not essential for the *Salmonella* host allowing for mutations. The absence of the SEN1384 gene in the same phage type 9b can not be explained with the available data.

The region neighbouring both ends of the putative prophage region ΦSE14 seems to be unaffected by the presence or absence of ΦSE14 as the genes in the direct vicinity were present in all isolates tested independent of the ΦSE14 presence.

4.3.4 ΦSE20

In case of the putative prophage region ΦSE20 the situation is much more complicated than for the other putative prophage regions seen in SE125109. However, the results of the analysis of the microarray data are in good correlation with the results of the PCR-based screening. The microarray data for the isolates belonging to the phage types 1, 4 and 21 indicated presence for all genes of the putative prophage region ΦSE20 that are present on the array. This was also the result of the respective PCR reactions for these phage types. For the isolates belonging to the phage types 8, 9b and 21c the microarray data suggested absence of ΦSE20 which was also reflected by the PCR results for these phage types. The unclear PCR results seen for the phage type 21c isolate should be regarded as negative in the context of the microarray results. The microarray data revealed a scattered distribution of genes being present and absent in small blocks for the remaining isolates belonging to the phage types 11, 13a and 20. While this was already assumed from the PCR results received for the phage type 11 and 20 isolates, the PCR results for the phage type 13a isolate suggested absence of ΦSE20 in this isolate. The microarray data however, gave strong evidence for approximately two thirds of the genes being present in this isolate. An obvious explanation for this at first sight contrary results can be found when a detailed look at the target genes of the PCR reactions is taken. The individual genes were either missing in the phage type 13a isolate (SEN1955) or in some cases not even present on the array (5'-end target). However, the SEN1920 gene, which contains the binding site for the reverse primer of the PCR targeting the 5'-end of ΦSE20 is present on the array and its absence in the isolates belonging to the

phage types 8, 9b, 11, 13a, and 21c matches very good with the negative PCR results obtained with these isolates in the respective PCR. In case of the target genes of the 3'-end PCR, the SEN1966 gene is absent in the isolates belonging to the phage types 8, 9b, 13a, 20 and 21c, which is in accordance with the obtained PCR results. Interestingly, the microarray data indicates absence of the SEN1967 gene, which contains the binding site for the reverse primer targeting the 3'-end of ΦSE20 in the phage type 21 isolate, but the PCR result was nevertheless positive for this isolate. A possible explanation for this observation might be a mutation in this gene, which prevents binding to the target sequence on the microarray while the PCR primer still seemed to be able to bind to its target. Therefore it is important to keep in mind that the PCR reactions are only valid for a screening analysis while the microarray was able to give more definite results on the presence or absence of the putative phage locations in SE125109. The absence or presence of isolated single genes in the putative phage locations or in their direct vicinity as seen in the microarray data show that the prophages are dynamic regions where mutations and rearrangements take place so that these regions should not be considered to be of static nature.

4.3.5 Presence of prophage genes from *S.* Typhimurium LT2S and *S.* Typhi CT18

The genome analysis of SE125109 had already shown the presence of various *S.* Typhi CT18 and *S.* Typhimurium LT2 prophage genes in the 4 putative prophage regions identified in the *S.* Enteritidis PT4 strain. The analysis of microarray data for the presence of genes from the *S.* Typhi CT18 and *S.* Typhimurium LT2 prophages in the *S.* Enteritidis isolates tested was able to confirm these findings and to illustrate the frequent exchange and rearrangement of genes belonging to what could be seen as a pool of phage genes. An individual set of these genes seemed to be present in each serovar and also each phage type. This shows why all *S.* Enteritidis isolates tested harboured genes from *S.* Typhi CT18 and *S.* Typhimurium LT2 prophages, as well as genes from all prophage locations in *S.* Typhi CT18 and *S.* Typhimurium LT2 were harboured in at least some of the *S.* Enteritidis isolates tested. Findings that isolates belonging to the phage types 4 and 21 seemed to harbour only few of the prophage genes from *S.* Typhi CT18 and *S.* Typhimurium LT2 according to the microarray data while a much higher proportion of these genes seemed to be present in the isolates belonging to the phage types 8, 9b, 11, 13a could possibly be explained in the context

of the overall prophage content in the *S.* Enteritidis isolates. Those isolates harbouring more of the prophage genes from *S.* Typhi CT18 and *S.* Typhimurium LT2 seemed to be lacking the ST64B phage in part or total in the putative prophage location ΦSE20. This might be caused by the presence of one phage conferring immunity towards infection with the same type of virus to the bacterium. In a microarray-based study by the working group of Michael McClelland using a different array, two groups of *S.* Enteritidis isolates were determined based on the presence of a subset of phage regions (Porwollik *et al.*, 2005). They were grouped based on absence / presence patterns for regions similar to the phages ST64B, Fels-2, ST27 and ST35. These two groups were seen to almost perfectly overlap with the separation into phage type 4-like isolates (including phage types 1, 4, 4b, 6, 7 and 24) and phage type 8-like strains (including phage types 2, 8, 13a and 23) based on LPS core structure (Guard-Petter, 1999; Liebana *et al.*, 2002; Porwollik *et al.*, 2005). These LPS differences are thought to make the strains resistant or susceptible to a phage present in the current typing scheme (Porwollik *et al.*, 2005; Ward *et al.*, 1987).

A comparison of the findings by Porwollik and co-workers with the results from this study showed some consent but more detailed information obtained in this study stress also some differences. In addition to that isolates belonging to phage types not included into the study by Porwollik and co-workers were also analysed in this study. A comparison of the results has been compiled in Table 4-1. As there was only one gene from the *S.* Typhi CT18 phage ST27 present on the array used in this study, results for this phage were not included into Table 4-1.

Strain	PT	ΦSE20 (ST64B-like)	Fels-2	ST35	Group according to Porwollik *et al.*
125109	4	+	3/66	0/30	PT 4-like
Leipzig	4	+	5/66	0/30	PT 4-like
VAC E[1]	4	+	5/66	0/30	PT 4-like
04-03158	1	+	23/66	6/30	PT 4-like
05-01906	8	-	57/66	29/30	PT 8-like
03-03059	9b	-	58/66	27/30	na
04-03092	11	50 %; 3':+	58/66	28/30	na
03-03561	13a	67 %; PCR -	60/66	28/30	PT 8-like
02-00191	20	33 %; 5': +	7/66	0/30	na
02-06391	21	+	4/66	0/30	na
04-03909	21c	-	5/66	0/30	na

Table 4-1: Comparison of prophage content results from this study with results by Porwollik *et al.*, 2005.

[1]AviPro® SALMONELLA VAC E

The results for the phage type 4 and 8 isolates match very well with the results by Porwollik *et al.*, and the phage type 21 and 9b isolates show a similar pattern but such phage types were

not included into the study by Porwollik and co-workers. In their study, Porwollik and colleagues put 6 different isolates belonging to phage type 13a into the PT 8-like group and found the ST64B-like phage to be absent in these isolates. Microarray data from this study however, indicated parts of the ST64B-like phage being present in several scattered small blocks of few genes totalling up to about 67 % of those genes represented on the array. Therefore it is unclear whether a phage type 13a isolate with a different phage content was used in this study or if the array used by Porwollik and co-workers wasn't able to detect the ST64B genes in the phage type 13a isolates. It would be of interest to test their isolates with the array used for this study. The phage type 11 isolate from this study showed a similar pattern like the phage type 13a isolate; no such isolate was tested by Porwollik and co-workers. The phage type 1 isolate tested in this study carries some Fels-2-like genes while the isolate tested by Porwollik and co-workers seemed to lack Fels-2-like genes and was therefore categorized in the PT 4-like group. This might be explained by the same reasons as for the phage type 13 a isolate, and it has to be kept in mind that there were only 26 of the Fels-2-like genes tested with the array used by Porwollik and colleagues while there were 66 Fels-2-like genes present on the array used in this study allowing a more accurate analysis. All facets of phage presence and absence in parts or total seem possible, and the phage type 20 isolate which harbours only part of the ST64B-like genes while lacking the Fels-2-like genes seems like the phage type 1 isolate to be somewhere in between the PT 4-like and the PT 8-like group with respect to the prophage content. The phage type 21c isolate tested in this study finally pointed up that lack of the ST64B phage did not necessarily coincide with presence of the Fels-2 and ST35-like genes as this isolate seemed to lack all of them.

4.3.6 General discussion of microarray results

The microarray analyses that were part of this study generated a tremendous amount of data. From this data set those data relevant for the scope of this study had to be extracted and analysed. Having done this, the array data was able to confirm some of the results obtained by the methods used in the first parts of this study and the assumptions that arose from the interpretation of these data. The microarray data gave a deeper, much more detailed insight into the putative prophage repertoire of the different *S.* Enteritidis phage types and was therefore also able to clarify and complement the data from the genome analysis and the PCR-based screening, making it a very valuable tool for the analysis of the *S.* Enteritidis prophage content. It became obvious that the prophage content differs enormously between the isolates

belonging to different phage types, and the whole spectra of presence and absence of phages in part or in total is possible. The putative phage locations seemed to consist of an assortment of phage genes also present in other serovars that are recombined frequently. The information obtained in these microarray experiments is by definition unidirectional. Only those putative genes present on the sequenced strains and represented on the array could be detected, but not those specific to other isolates of the same serovar unless they are present in a similar way in other serotypes represented on the array.

The three isolates belonging to the phage type 4 tested in this study showed only minor differences in the microarray data confirming homogeneity in prophage content within the same phage type that was already indicated by the PCR-based screening. There were no obvious differences between the vaccine strain AviPro® SALMONELLA VAC E and its parent strain Leipzig visible in the microarray data in relation to the prophage content and this was in accordance with the already known difference responsible for the attenuation of the vaccine strain that are not associated with prophage locations.

The GACK method seemed to be the most stringent of the three statistical methods applied and it was therefore used preferably for the final judgement to determine the presence or absence of a gene from the microarray data.

4.4 Prophage release and induction experiments

4.4.1 Prophage induction experiments

To investigate the inducibility of the temperate prophages previously identified in the genome comparisons, UV irradiation and mitomycin C, as one physically and one chemically inducing agent traditionally used for the induction of lysogenic cultures were applied to duplicate samples of SE125109.

No induction of any of the prophages could be detected with the experimental model used in this study. The genome analysis had already indicated the putative prophages ΦSE10, ΦSE12/ΦSE12A and ΦSE14 to be phage remnants that are very unlikely to be inducible. For ΦSE20 however, the genome analysis suggested that this prophage is probably inducible. As mentioned before, its *S.* Typhimurium analogue ST64B was isolated together with phage ST64T, and ST64B was inducible by mitomycin C but could not be propagated on any of the strains tested and failed to produce plaques on many diverse *Salmonella* and *E. coli* strains

(Mmolawa *et al.*, 2002; 2003a). A +1 frameshift mutation in ST64B is believed to be responsible for its inability to produce infectious virions, and reactivation of the phage can be achieved by a reversion of the +1 frameshift mutation (Figueroa-Bossi and Bossi, 2004). As the +1 frameshift mutation is not present in ΦSE20, it should be active like the ST64B revertants which lack the +1 frameshift mutation and arise spontaneously in cultures (Figueroa-Bossi and Bossi, 2004). Nevertheless no induction of ΦSE20 was seen in the experiments of this study. It might be possible that in *S.* Typhimurium additional genes are required for the activation of the phage like it has been suggested that the complete phage ST64T which propagates autonomously in the above mentioned *S.* Typhimurium strain might *trans*-activate the defective genome of ST64B and compensates for some ST64B deficiencies (Mmolawa *et al.*, 2003b). The genes might be lacking in SE125109. But also the experimental model chosen might be the reason why no phage induction could be detected. The indicator strain STm 576 used in the phage induction experiments might not have been ideal for the detection of released *S.* Enteritidis phages after induction. An indicator strain belonging to the same serovar might have had a higher probability for being susceptible to infection by the released phages. As hydrogen peroxide treatment has been shown to induce Gifsy-1 more effectively than mitomycin C in *S.* Typhimurium, the use of other inducing agents than UV irradiation and mitomycin C, like for example hydrogen peroxide might have been an alternative to induce any of the prophages in SE125109 (Figueroa-Bossi and Bossi, 1999). As the experiments focussing on the spontaneous release of phages from the *S.* Enteritidis isolates tested which were done in parallel and are discussed below produced interesting results very quickly, the route of phage induction wasn't followed any further in this study. However, other inducing agents or a different indicator strain could have lead to the detection of phage induction in SE125109.

4.4.2 Spontaneous prophage release experiments

As expected, no plaques were detected in any of the tester strains, when only medium was applied as a control. This confirms that the formation of the plaques detected is induced by the supernatants applied to the tester strains, and that the plaques themselves are not formed spontaneously. For all strains no plaques were seen when supernatants produced by the same strain were used. This is in accordance with the immunity against infection with the same bacteriophage when this phage is already present as a prophage in the respective strain. For the three phage type 4 isolates tested, the same pattern for plaque formation caused by

supernatants derived from this strains was seen with the whole set of tester strains. On the other hand, when the three phage type 4 isolates were used as tester strains, the whole set of supernatants caused the same patterns of plaque formation for these three strains. This is in accordance with findings from the previous analyses indicating a high level of homogeneity with respect to the prophage contents within the same phage type.

Several different patterns were distinguishable for the strains belonging to different phage types. While no plaque formation was seen when the phage type 13a and 20 isolates where used as the tester strains, the isolates belonging to the phage types 8 and 21c showed plaque formation with many of the supernatants tested. The other isolates behave somewhat in between and produce plaques with a few of the supernatants only. If the results are analysed from the perspective of the supernatants, the supernatants of the isolates belonging to the phage types 11 and 13a behaved in a very special way, as they were able to induce plaque formation in almost all tester strains. The supernatants from the phage type 8, 9b and 20 isolates on the contrary were not able to induce plaque formation in any of the tester strains used. It is not possible to directly correlate the results from the spontaneous phage release experiments with the results from the analysis of the prophage content. The patterns found do not necessarily correlate. While for example the supernatants derived from the phage types 8 and 20 showed a similar pattern in the plaque formation they induced, they differed in the analysis of the prophage content with ΦSE20 being probably present in the phage type 8 and absent in the phage type 20 isolate. On the other hand, the same prophage pattern was seen for the phage type 4 and 21 isolates, but these did not show the same pattern in the spontaneous phage release experiments both as the tester strain, as well as when they were used for the production of the supernatants. All possible combinations and differences can of course not be discussed here. The combinations mentioned above are examples only to illustrate that from the data generated in this study the prophage content and the lysis patterns can not be put into relation to each other. This is probably due to the fact that especially the non phage type 4 isolates included into this study might contain other or additional prophages to those identified in the phage type 4 isolates which could not be detected in the PCR analysis as this was based on the prophage content of SE125109 and not with the microarray analysis because phages not represented on the array could of course as well not be detected. These other or additional prophages could then give immunity against infection with certain phages present in the respective supernatants tested to these strains or in the cases when the supernatants produced from these strains were used spontaneously release their specific phages to these supernatants which could then infect some of the tester strains. Nevertheless, the spontaneous

phage release experiments were able to highlight certain phage types like 8, 9b, 11, 13a and 20. These are exactly those phage types that already popped up as being the most diverse from the page type 4 isolates in the PCR based screening and the microarray analysis which together is a strong indication for them to harbour a different set of prophages. As discussed in the previous chapter, ΦSE20 is the prophage most likely to be intact in SE125109 so that it could be released. However, the results obtained in the spontaneous phage release experiments do not allow to make a statement if this phage has been released from the phage type 4 isolates and induced plaque formation in some of the tester strains, as ΦSE20 has been found to be absent from the isolates belonging to the phage types 8, 9b and 21c, but plaque formation could be detected with the supernatants from the phage type 4 isolates in the phage type 8 and 21c isolates only. Certainly, further analysis of the released phages, what would have been out of the scope of this study, for example by phage specific PCRs would be interesting to gain more knowledge of the prophage content in different *S.* Enteritidis phage types.

4.5 Conclusions and outlook

Putative genes encoding virulence factors were found within the prophage regions identified in SE125109 in this study. The important role of bacteriophages in the movement of virulence factors among bacteria and their association with such virulence factors is supported by these findings. While in many cases the virulence associated effector proteins were highly conserved, the genes required for phage proliferation had obviously degenerated. This may suggest an important role for these virulence factors, although the presence of phage-encoded virulence genes themselves is not enough to determine if they are important to the pathogenicity of *S.* Enteritidis. Knock-out experiments targeting these genes would be a promising approach to further investigate the role of these putative virulence genes. Nevertheless the redundant character of the *Salmonella* genome might cover some of the effects posed by the knock-out of certain genes. The prophage regions identified in SE125109 display a mosaic of genes from related bacteriophages and the virulence associated genes that are carried by these prophages are also carried and distributed by a wide range of other bacteriophages isolated from other *Salmonella* serovars. The differences in the prophage content of *S.* Enteritidis PT4 (PT4), *S.* Typhimurium LT2 (LT2), and *S.* Gallinarum 287/91 (SGAL) are displayed in Figure 4-1. *S.* Enteritidis PT4 (PT4) and *S.* Typhimurium LT2 (LT2)

diverged from a common ancestor and already contained the prophage region ΦSE12A (or ΦSG12A in *S.* Gallinarum 287/91 respectively) before this divergence. Afterwards the prophage region ΦSE12 (or ΦSG12 in *S.* Gallinarum 287/91 respectively) were taken up. The divergence of *S.* Enteritidis PT4 and *S.* Gallinarum 287/91 then resulted in bigger differences in prophage content: While ΦSE10, ΦSE14 and ΦSE20 are present in *S.* Enteritidis PT4, they are absent from *S.* Gallinarum 287/91. ΦSE10 and ΦSE12 present in *S.* Enteritidis PT4 are similar to Gifsy-2 present in *S.* Typhimurium LT2.

A set of virulence genes harboured in one prophage in certain *Salmonella* serovars might be found in two prophages in other serovars. In this study this was shown for *gtgE* and *sodCI*, which are both harboured in Gifsy-2 in *S.* Typhimurium, while in *S.* Enteritidis *gtgE* is part of ΦSE10 and *sodCI* is part of ΦSE12. These findings reinforce the important role bacteriophages play in the movement of virulence factors among bacteria providing each serovar with an individual set of virulence-genes as a main mechanism driving the evolution of *Salmonella* pathogenicity. The rising number of sequenced bacterial genomes allows further comparisons like those performed for *S.* Enteritidis in this study that will probably lead to a better understanding of the prophage content and exchange between different *Salmonella* serovars and might help in understanding the relationship between these serovars.

While the PCR results obtained in this study indicated the prophage content to be conserved between the S. Enteritidis isolates, with those isolates belonging to the phage types 9b, 11 and 20 showing the biggest variation, the microarray results revealed a prophage content enormously differing between the isolates belonging to different phage types. No differences in the prophage content with the same phage type were seen irrespective of the source of the isolates tested (animal species, sampled organ, geographical region). Further genome research including additional phage types not included in this study would certainly be illuminating.

A proteome analysis or a gene expression analysis using a RNA microarray could lead to a deeper understanding of the importance of putative effector proteins present in the prophage locations in relation to their distribution between the different phage types.

It would also be possible to analyse the virulence of the different phage types in a chicken or mouse model to relate this to the virulence gene content.

Finally, an analysis of the released phages by further development of the PCRs used in the screening experiments would be a useful tool complementing the methods used in this study which proofed to deliver valuable data on the *S.* Enteritidis prophage content.

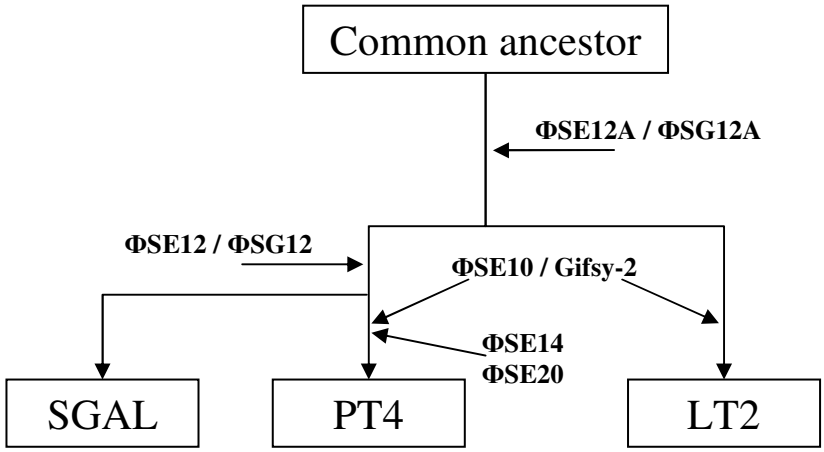


Figure 4-1: The line diagram illustrates the differences in prophage content of *S.* Enteritidis PT4 (PT4), *S.* Typhimurium LT2 (LT2), and *S.* Gallinarum 287/91 (SGAL). It is based on the assumption that following the divergence of *S.* Enteritidis PT4 and *S.* Typhimurium LT2 from a common ancestor *S.* Enteritidis PT4 and *S.* Gallinarum 287/91 have subsequently diverged. Branches are not intended to infer phylogenetic distance (modified from Thomson *et al.*, 2008).

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Abbreviations

° C	degree Celsius
3SD	3 standard deviations
A	adenosine
ACT	Artemis Comparison Tool
BfR	Bundesinstitut für Risikobewertung
BLAST	basic local alignment search tool
bp	base pair(s)
C	cytosine
CDC	Center for Disease Control
CDS	coding sequences
CE	competitive exclusion
CT	cholera toxin
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dd	distilled deionized
DDBJ	DNA Data Bank of Japan
dGTP	deoxyguanosine triphosphate
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DT	definitive type
DTT	Dithiothreitol
dTTP	deoxythymidine triphosphate
E	*Escherichia*
EDTA	ethylenediaminetetraacetic acid
EF	exclusion flora
EMBL	European Molecular Biology Laboratory
ERS	Economic Research Service
FLI	Friedrich-Loeffler-Institut
FU	Freie Universität
G	guanine

GDP	guanosine 5'-diphosphate
GFF	generic file format
gpr	genepix results file
gps	genepix settings file
GTP	guanosine 5'-triphosphate
IAH	Institute for Animal Health
IDT	Impfstoffwerk Dessau-Tornau
IUPAC	**International Union of Pure and Applied Chemistry**
IS	insertion sequence
JNK	Jun N-terminal kinase
LAH	Lohmann Animal Health
LAMP	loop mediated isothermal amplification
LPS	lypopolysaccharide
M	median
MLEE	multilocus enzyme electrophoresis
na	not available
Nal	nalidixic acid
NCBI	National Center for Biotechnology Information
nr	not relevant
NVSL	National Veterinary Services Laboratory
OMP	outer membrane protein
ORF	open reading frame
PCR	polymerase chain reaction
PDB	Protein Data Bank
PFGE	pulsed-field gel electrophoresis
PHW	Paul-Heinz Wesjohann
PT	phage type
Rif	rifampicin
RKI	Robert Koch-Institut
RNA	ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
RT	room temperature
S	*Salmonella*

SDS	sodium dodecyl sulphate
SLT	Shiga-like toxins
Sm	streptomycin
SNP	single nucleotide polymorphisms
SOD	superoxide dismutase
SPI	*Salmonella* pathogenicity island
SSC	Saline-Sodium-Citrate
STE	*Salmonella* translocated effector
T	thymine
T3SS	type III secretion system
TAE	tris-acetate EDTA buffer
Td^2	melting temperature
TER	terminus of replication
tif	tagged image file
TPB	tryptose phosphate broth
Tris	tris(hydroxymethyl)aminomethane
tRNA	transfer ribonucleic acid
U	units
UK	United Kingdom
US	United States
USDA	United States Department of Agriculture
UV	ultraviolet
WHO	World Health Organization

Abbreviations and symbols not mentioned are explained in the text. The International System of Units was used for physical units; chemicals were named according to IUPAC.

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Erklärung zur Dissertation

Hierdurch erkläre ich, dass ich meine Dissertation mit dem Titel *"Analysis and Characterization of the Prophage Content in Salmonella Enteritidis"* selbstständig verfasst, und die benutzten Hilfsmittel und Quellen sowie gegebenenfalls die zu Hilfeleistungen herangezogenen Institutionen vollständig angegeben habe.
Die Dissertation wurde nicht schon als Masterarbeit, Diplomarbeit oder andere Prüfungsarbeit verwendet.

Publications

Parts of the work that was part of this dissertation have been published:

Thomson, N.R., D.J. Clayton, D. Windhorst, G. Vernikos, S. Davidson, C. Churcher, M.A. Quail, M. Stevens, M.A. Jones, M. Watson, A. Barron, A. Layton, D. Pickard, R.A. Kingsley, A. Bignell, L. Clarck, B. Harris, D. Ormond, Z. Abdellah, K. Brooks, I. Chervach, T. Chillingworth, J. Woodward, H. Norberczak, A. Lord, C. Arrowsmith, K. Jagels, S. Moule, K. Mungall, M. Saunders, S. Whitehead, J.A. Chabalgoity, D. Maskell, T. Humphrey, M. Roberts, P.A. Barrow, G. Dougan, and J. Parkhill. 2008. Comparative genome analysis of *Salmonella* Enteritidis PT4 and *Salmonella* Gallinarum 287/91 provides insights into evolutionary and host adaptation pathways. *Genome Res.* **18:**1624–1637.

Windhorst, D., I. Schroeder, M. Watson, N.R. Thomson, and P.A. Barrow. Analysis and characterization of the prophage content in *Salmonella* Enteritidis. I3S International Symposium on *Salmonella* and *Salmonellosis*, Saint Malo, France 10^{th}-12^{th} May, 2006.

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