Genetic and phenotypic characteristics of importance for clonal success and diversity in Salmonella

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Genetic and phenotypic characteristics of importance for clonal success and diversity in *Salmonella*

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Preface

This research project was carried out at the National Food Institute, Technical University of Denmark at two different research groups (Research Group of Epidemiology and Microbial Genomics and Research Group of Microbial Food Safety) in collaboration with eight co-authors. The project was conducted from December 2008 until February 2012. An external research stay of two weeks at the Department of Food and Environmental Safety, Animal Health and Veterinary Laboratories Agency, United Kingdom was included. This project was financed by a grant of the Danish Ministry of Food, Agriculture and Fisheries (Grant no.: 3304-FVFP-07-721-01).

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Lastly, I would like to thank my parents for their support and for always being proud of me no matter what happens.

Abbreviations

ACSSuT	Ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracyclin
approx.	Approximately
a_w	Water activity
CFU	Colony forming units
aCGH	Array-based comparative genomic hybridization
CIP	Ciprofloxacin
DC	Dendritic cell
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
EU	European Union
h	Hour
IFN	Interferon
IL	Interleukin
LPS	Lipopolysaccharide
MIC	Minimal inhibitory concentration
min	Minute
MLST	Multilocus sequence typing
MLVA	Multiple-Locus Variable number tandem repeat Analysis
MR	Multidrug resistant (resistant to four or more antibiotic agents)
n	Number
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NOS	Nitric oxide synthase
NTS	Non-typhoid *Salmonella*

PAMP	Pathogen associated molecular pattern
PMN	Polymorphnuclear neutrophils
PCR	Polymerase chain reaction
PFGE	Pulse field gel electrophoresis
R	Resistant (resistant to less than four antibiotic agents)
RH	Relative humidity
RNA	Ribonucleic acid
ROS	Reactive oxygen species
S	Sensitive (fully susceptible to the tested antibiotics)
S.	*Salmonella enterica ssp. enterica*
SCV	*Salmonella* containing vacuole
SGI	*Salmonella* genomic island
SPI	*Salmonella* pathogenicity island
spv	*Salmonella* plasmid virulence
T3SS	Type three secretion system
TLR	Toll-like receptor
Q	Quinolone resistant (at least low level resistance to fluoroquinolones)
QRDR	Quinolone resistance determining region
WT	Wildtype
%	Percent
^{o}C	Degrees (Celsius)

Summary

Salmonella are zoonotic pathogens that cause food-borne illness and that are widely disseminated in nature. An estimated number of 93.8 million human cases of gastroenteritis caused by *Salmonella* occur annually throughout the world. The epidemiology of *Salmonella* is characterized by a temporal dominance of certain clones. These epidemically successful clones are often resistant to antibiotics and associated with severe human illness. They pose a major threat to public health and lead to heavy economic losses. So far, little is known about the environmental and bacterial factors leading to the emergence of successful clones. However, resistance to multiple antimicrobial drugs and quinolones seems to contribute to the epidemic success of *Salmonella* as it is associated with an increased severity of illness and epidemicity. In order to predict and prevent future outbreaks and epidemics, research should focus on the evolutionary mechanisms of emerging success clones.

The ability to spread in different food production sectors and to cause human disease seems critical for a clone to become successful. The aim of this PhD study was to identify common phenotypic and genetic traits of success clones that may have an epidemic importance with respect to the survival in the food chain and to human infection. Non-typhoid *Salmonella* isolates from food, production animals and humans were carefully selected and characterized as successful or non successful based on epidemiological data. In addition, the isolates were categorized based on their antibiotic resistance pattern into quinolone resistant (Q; at least low level resistance to one fluoroquinolone), multidrug resistant (MR; resistant to four or more antibiotic agents), resistant (R; resistant to less than four antibiotic agents) and sensitive (S; fully susceptible to the tested antibiotics).

The ability to survive and grow after physical stress experienced in the food chain is likely to contribute to a successful spread of *Salmonella*. Thus, the response of *Salmonella* isolates in exponential and stationary growth state to freezing in minced meat and to drying at two combinations of temperature and humidity was investigated. The results of this study are presented in **Manuscript I**. Stationary phase cells survived freezing for up to one year without major decline and initiated growth with a short lag time of 1.7 hours on average. Exponential phase cells showed reduced survival and a tendency to exhibit shorter lag times. Dehydration at 82 % relative humidity (RH) and 10 ˚C was less harmful for *Salmonella* than

49 % RH and 25 °C. Dehydration, in contrast to freezing, was differently tolerated by the *Salmonella* isolates but neither the tolerance to freezing nor to dehydration appears to contribute to the epidemic success of *Salmonella*.

MR and Q *Salmonella* seem to have a virulence or fitness advantage apart from the effect posed by the antibiotic resistance phenotype. **Manuscript II** describes a study aiming to identify genes and genetic structures in Q and MR isolates that might confer fitness or virulence attributes by the use of array-based comparative genomic hybridizations and PCR screenings. The largest fraction of the genes dominating in Q and MR *Salmonella* were single genes and gene clusters belonging to prophages, among these the virulence genes *sopE* (STY4609) and *gipA* (STM2599) and the genes *recT* (SG1183), *sugR* (STM3753) and *trhH* (AF261825_S012). The latter three genes were knocked out and the mutants were used in functional analyses. The mutants were not significantly different from the wildtype but RecT knockout mutants seemed to be more susceptible to the early defense of macrophages and exhibited increased spontaneous mutation rates. The largest group of genes dominating in sensitive isolates were related to metabolism indicating that many of these genes are typically absent or diverged in Q and MR *Salmonella*. The acquisition and maintenance of bacteriophages conferring fitness and virulence attributes may be part of the evolution of successful MR and Q *Salmonella*.

The bacterial and host factors leading to an increased severity of illness caused by quinolone resistant *Salmonella* were investigated as they are largely unknown. Quinolone resistance is mostly caused by point mutations and can develop in *Salmonella* during infection. As described in **Manuscript III**, the spontaneous mutations rates and virulence potential of ciprofloxacin (CIP) resistant *Salmonella* in presence and absence of CIP were assessed using cell cultures. The rate at which CIP sensitive isolates can experience a reduction in their CIP susceptibility after macrophage infection and in presence and absence of CIP was investigated. Strain dependent differences in the mutation rate, the ability to invade intestinal epithelial cells and to replicate within macrophages were found, but there was no correlation between virulence, mutation rate and resistance pattern. The presence of clinically relevant CIP concentrations decreased but failed to eliminate the number of CIP resistant intraphagocytic *Salmonella*. During macrophage infection, *Salmonella* adapted to higher CIP concentrations (to at least 2 – 8 fold the initial MIC). When CIP was added to the extracellular medium, the frequency of intraphagocytic bacteria exhibiting reduced CIP susceptibilities

increased markedly. Thus virulent *Salmonella* strains that are likely to persist in macrophages for prolonged times might become resistant to quinolones and the administration of quinolones may increase the rate of resistant mutants.

In conclusion, the emergence of successful clones is likely to be the result of a combination of factors including the acquisition and maintenance of bacteriophages encoding genes that increase the fitness or virulence of their bacterial host. Marker genes related to bacteriophages might be useful in the future for the early detection of these clones. Macrophages might play a role in the evolution of quinolone resistance in invasive *Salmonella* strains. Both in absence of antimicrobial drugs and when quinolones are administered, intraphagocytic *Salmonella* may become quinolone resistant and simultaneously may acquire other advantageous mutations. This highlights the importance of an antibiotic therapy that is effective in eliminating intracellular pathogens.

Sammendrag

Salmonella er zoonotiske patogener, der kan forårsage fødevarebårne sygdomme, og som er meget udbredte i naturen. Det anslåes, at der årligt er 93,8 millioner humane tilfælde af gastroenteritis (mave-tarm-katar) på verdensplan. Salmonellaepidemiologi er karakteriseret ved en temporal (tidsmæssig) dominans af bestemte kloner. Disse epidemiologisk succesfulde kloner er ofte resistente over for antibiotika og er associeret med alvorlig sygdom hos mennesker. De udgør en stor trussel mod folkesundhed og kan føre til massive økonomiske tab. Indtil videre vides meget lidt om de miljømæssige og bakterielle faktorer, der fører til fremkomsten af succesfulde kloner. Kloner der er resistente overfor adskillige antimikrobielle stoffer eller er og resistente overfor quinoloner menes at bidragei særlig grad til Salmonellas epidemiologiske succes, idet disse kloner oftere er associeret med alvorlig sygdom og evne til at spredes. For at kunne forudsige og forebygge fremtidige udbrud og epidemier bør forskningen fokusere på de evolutionære mekanismer, der ligger bag fremkomsten af nye succeskloner.

Evnen til at sprede sig i forskellige fødevareproduktionssektorer og til at forårsage sygdom hos mennesker ser ud til at have afgørende betydning for at kloner kan blive succefulde. Formålet med dette Ph.d-studium var at idetificere fælles fænotypiske og genetiske karaktertræk hos succeskloner, der kunne være af epidemisk betydning i forhold til overlevelse i fødevareproduktionskæden og til infektion hos mennesker. Non-typhoidal *Salmonella* - isolater fra fødevarer, produktionsdyr og mennesker blev omhyggeligt udvalgt og karakteriseret som succesfulde eller ikke-succesfulde på baggrund af epidemiologiske data. Derudover blev isolaterne kategoriseret efter antibiotikaresistensmønster; quinoloneresistens (Q; inklusiv lav resistens mod en fluoroquinolone), multiresistens (MR; resistens mod fire eller flere antimikrobielle stoffer), reistens (R; resistens mod færre end fire antimikrobielle stoffer) og følsom (S; følsom overfor alle testede antimikrobielle stoffer).

Evnen til at overleve og vokse efter fysisk stress i fødevareproduktionskæden bidrager sandsynligvis til en succesfuld spredning af *Salmonella*. Derfor blev det undersøgt, hvordan *Salmonella*-isolater i eksponentiel og stationær vækst responderer på frysning i hakket kød og på udtørring ved flere kombinationer af temparatur og fugtighed. Resultaterne af dette forsøg er præsenteret i **Manuskript I**. Celler i stationærfase overlevede frysning i op til et år uden større reduktion i antallet af levedygtige cellerog de initierede vækst med en kort latenstid på

1,7 time i gennemsnit. Celler i eksponentiel fase viste reduceret overlevelse og en tendens til kortere latenstid . Dehydrering ved 82% relativ fugtighed (RH) og 10 ˚C var mindre skadelig for *Salmonella* end ved 49% RH og 25 ˚C. Dehydrering blevr i modsætning til frysning tolereret forskelligt af *Salmonella*-isolater, men hverken tolerance overfor frysning eller dehydrering ser ud til at bidrage til Salmonellas epidemiske succes.

MR og Q *Salmonella* menes at have en virulens- eller fitness-fordel ud over effekten af antibiotikaresistens-fænotypen. **Manuskript II** beskriver et studie, der har til formål at identificere gener og genetiske strukturer i Q og MR isolater, der kan give fitness- eller virulensegenskaber ved brug af array-baseret komparativ genomisk hybridisering og PCR screeninger. Den største fraktion af generne, der dominerede i Q og MR *Salmonella* var enkelte gener og genklynger tilhørende profager, heriblandt virulensgenerne *sopE* (STY4609) og *gipA* (STM2599) samt generne *recT* (SG1183), *sugR* (STM3753) og *trhH* (AF261825_S012). De tre sidstnævnte gener blev inaktiverede og mutanterne blev anvendt i funktions analyser. Mutanterne var ikke betydeligt forskellige fra vildtypen, men RecT mutanten så ud til at være mere følsom overfor det tidlige forsvar af makrofager og RecT så ud til også at spille en mindre rolle i hæmning af spontane mutationer. Den største gruppe af gener, der dominerede i sensitive isolater, var relateret til metabolisme, hvilket indikerer at mange af disse gener typisk er manglende eller divergeret i Q og MR *Salmonella*. Optagelsee og integrering af bacteriofager, der giver fitness- og virulens-egenskaber kan være en del af evolutionen af succesfulde MR og Q *Salmonella*.

De værtsfaktorer og bakterielle faktorer, der fører til mere alvorlig sygdom og som forårsages af Q *Salmonella*, er for en stor del ukendte og blev derfor undersøgt. Quinoloneresistens skyldes hovedsaglig punktmutationer, som kan udvikles i *Salmonella* under infektion. Som beskrevet i **Manuskript III** blev den spontane mutationsrate og virulenspotentialet af cipofloxacin-resistent *Salmonella* med og uden tilstedeværelsen af cipofloxacin (CIP) målt ved brug af cellekulturer. Det blev også undersøgt, om CIP-følsomme isolater kan opleve en reduktion i følsomhed til CIP under makrofag-infektion og efterfølgende udsættelse for CIP. Stammeafhængige forskelle i mutationsrate samt evnen til at invadere tarm-epitelceller og til at replikere i makrofager blev påvist, men der var ingen korrelation mellem virulens, mutationsrate og resistensmønster. Ved klinisk relevante CIP-koncentrationer faldt antalet af CIP-reistente intrafagocytotiske *Salmonella*, uden at de blev helt elimineret. Ved makrofaginfektion tilpassede *Salmonella* sig højere CIP-koncentrationer (mindst 2-8 gange

den oprindelige MIC). Når CIP blev tilsat til det ekstracellulære medium, steg frekvensen markant af intrafagocytisk bakterier, der udviste reduceret CIP-følsomhed. Virulente *Salmonella*-stammer, der med høj sandsynlighed eksisterer i makrofager i længere tidsrum, kan således blive resistente overfor quinoloner uden tilstedeværelse af quinoloner, men især når værten behandes med disse antibiotika.

Det blev konkluderet, at fremkomsten af succefulde kloner formentlig skyldes en kombination af faktorer, herunder optagelse og integrering af bateriofag-kodende gener, som øger deres bakterieværts fitness og virulens. Markørgener for bakterofager kan muligvis anvendes i fremtiden til tidlig dektion af disse kloner. Makrofager spiller muligvis en rolle i evolutionen af quinoloneresistens i invasive *Salmonella*-stammer. Idet intrafagocytiske *Salmonella* udvikler reduceret følsomhed overfor quinoloner. Dette øges yderligere ved tilstedeværelse af fluoroquinolon og muligvis kan intrafagocystiske *Salmonella* blive quinoloneresistente og samtidig tilegne sig andre fordelagtige mutationer. Dette understregervigtigheden af antibiotikabehandling, der er effektiv ved eliminering af intracelluære patogener.

Background

Salmonella can cause zoonotic diseases and are widely disseminated in nature (4). It is a pathogen of global importance as it is one of the major causes of food-borne illness worldwide (150). This thesis has focused on non-typhoid *Salmonella enterica ssp. enterica* (NTS) that normally cause a localized self-limiting gastroenteritis in humans which does not require antibiotic therapy. However, a systemic infection can occur depending on the host immune status, the infectious dose and the pathogenic potential of the individual *Salmonella* strain. In these cases, antimicrobial therapy is necessary (56).

The population structure of *Salmonella* is clonal and the epidemiology of NTS is characterized by the temporal dominance of certain successful clones (137). Their occurrence and spread is usually followed by a decline and by the replacement with another clone (123). These epidemically successful clones are often resistant to antibiotics and associated with severe human illness (245). The pentaresistant (ACSSuT) *S.* Typhimurium DT104 clone is a prominent example of a success clone because of its global dissemination in the 1990s. Research should primarily focus on these clones because they pose an important public health threat and lead to heavy economic losses.

So far, little is known about the environmental and bacterial factors that lead to the emergence of successful *Salmonella* clones. These bacteria might be equipped with an increased virulence potential and/or might be fitter in surviving in the environment. Transmission of *Salmonella* from animals or the environment to humans usually occurs through the food production chain in form of contaminated foods. Not only the bacterial factors, also the food source plays an important role in the occurrence of outbreaks as some foods are more often associated with human disease. Denmark has established a comprehensive *Salmonella* surveillance program and monitors the prevalence of *Salmonella* in food animals, food products and humans. Some *Salmonella* subtypes seem to appear more often in food animals and food products and others in humans. Factors that cause a decrease or increase of these subtypes during this route of transmission are not well known. An understanding of these factors might help to elucidate mechanisms leading to the emergence of epidemically successful clones.

Purpose

A success clone may be identified as a leading pathogen after it has been discovered repeatedly over time by the surveillance systems (110). Similar to early warning systems, it will be a future challenge to predict and prevent the emergence of success clones before their wide dissemination. Three steps are important in this respect: First to recognize a clonal outbreak or epidemic in a very early stage, second to assess the potential danger of the clone in terms of spread and pathogenicity and third to implement effective intervention strategies. Before this can be realized, we require a lot of knowledge about genetic and phenotypic characteristics of successful *Salmonella* clones and about factors that influence their evolution. For instance, the identification of critical points in the food chain or during infection that promote the evolution of success clones, could help to guide food safety procedures or to give treatment recommendations, respectively.

Research approach

Salmonella isolates from recent years and various sources (animal, food and human) were collected with a focus on isolates from pigs, pork and humans and with a special emphasis on the serovar *S*. Typhimurium. *S.* Typhimurium is one of most common serovars in humans and in the pork production chain and pork contributes significantly to the public health burden of *Salmonella*. Each isolate was characterized as successful or non-successful. In this thesis, either one of two criteria was used to define bacterial success:

1. q-factor: This factor illustrates the relative ability of *Salmonella* subtypes to survive the food production chain and to cause human disease. It has been calculated by Hald et al. (104) using a mathematical model based on data from the Danish *Salmonella* Surveillance program. It varies among different *Salmonella* serovars and seems to increase in antimicrobial resistant strains. This factor has been used in this thesis to rank *Salmonella* isolates according to their epidemic success.
2. Antimicrobial resistance pattern: Multidrug and quinolone resistant *Salmonella* are frequently isolated from human outbreaks or epidemics. Moreover, they are associated with an increased severity of illness in humans. Thus, the drug resistance pattern of

Salmonella can be used as an indicator for bacterial success. In this study, *Salmonella* isolates were therefore categorized based on their resistance pattern into quinolone resistant (Q; at least low level resistance to fluoroquinolones), multidrug resistant (MR; resistant to four or more antimicrobial agents), resistant (R; resistant to less than four antimicrobial agents) and sensitive (S; fully susceptible to the tested antimicrobial agents).

The categorized isolates were subsequently used in phenotypic and genetic screenings followed by more targeted small-scale analyses.

Objectives

The overall objective of this study was to determine common genetic and phenotypic features of *Salmonella* isolates that were characterized as successful and non-successful based on the above mentioned criteria. These features may be of importance for clonal success and may help in the future to assess the potential danger of certain clones and to implement targeted interventions programs. This was achieved by accomplishing different studies with focus on the following objectives:

1. To analyze the tolerance of successful versus non-successful *Salmonella* subtypes to typical stress conditions that they are likely to experience in the food chain.
2. To identify specific genes or genetic patterns that are characteristic for multidrug and quinolone resistant *Salmonella*.
3. To determine the virulence potential and mutation frequencies of quinolone resistant *Salmonella* as well as the rate of occurrence of reduced quinolone susceptibilities under different conditions.

1. Taxonomy and typing of *Salmonella*

The genus *Salmonella* belongs to the family of Enterobacteriaceae. *Salmonella* are gram-negative, facultative anaerobe bacteria that diverged from *E. coli* ca. 100 – 160 million years ago and acquired the ability to invade host cells (60,170). Based on DNA relatedness, *Salmonella* is today divided into the two species *S. enterica* and *S. bongori*. *S. enterica* comprises over 2500 known serovars and each year new serovars are discovered. *S. enterica*, that has diverged from *S. bongori* ca. 71 – 100 million years ago, is further divided into six subspecies, shown in Figure 1. The majority of the bacteria causing disease in humans and warm blooded animals fall into subspecies *enterica* (I).

The serovars are classified by the Kauffmann-White scheme (186) on the basis of surface agglutination reactions of their O-, H- and Vi-antigens (Vi-antigens are only found in *S.* Typhi, *S.* Paratyphi and *S.* Dublin). *S.* Typhimurium and *S.* Enteritidis may be subdivided into phagetypes based on their lysis pattern after infection with a defined panel of bacteriophages (173). This is still the primary method for subtyping of *Salmonella* and often used in combination with antimicrobial susceptibility testing. However, this typing method has some limitations. Phagetypes are not always very stable and have been shown to change for example upon introduction of plasmids (39,139). Moreover, pure cultures from some serovars have shown variable expressions of minor O-antigens (114). Thus, apparently unrelated *Salmonella* can have a close phylogenetic relationship. Most of the human infections are caused by only view phagetypes of the two serovars *S.* Enteritidis and *S.* Typhimurium. To distinguish variants with small differences in these phagetypes, a high discriminatory power is needed which is provided by many molecular typing methods. The most frequently used methods are pulse field gel electrophoresis (PFGE), multi locus sequence typing (MLST) and multi locus variable number tandem repeat analysis (MLVA). It is suggested to use a combination of conventional and appropriate molecular typing methods to identify sources of human illness and to monitor the spread of successful clones in national or international outbreaks and epidemics (82).

For simplicity, the *Salmonella* strains mentioned in this thesis are abbreviated by *S.* followed by the serovar name and eventually the phagetype designation.

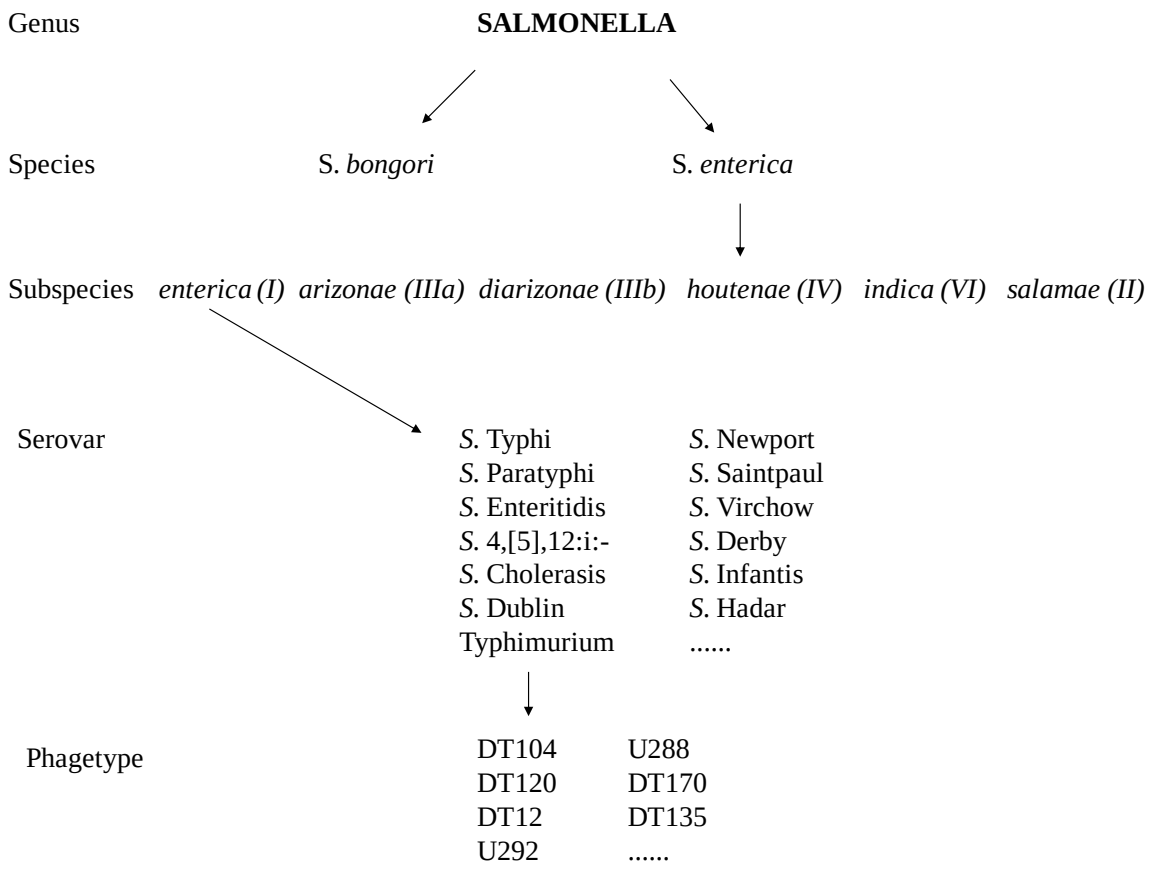


Figure 1: Taxonomic scheme of *Salmonella* serovars and phagetypes with relevance for this thesis (4).

2. Epidemiology of *Salmonella*

2.1. Incidence of human salmonellosis

An expected number of 93.8 million human cases of gastroenteritis caused by *Salmonella* occur annually throughout the world of which 80.3 million cases are estimated to be foodborne and of which 155000 infections result in death (150). Thus, human salmonellosis is a global public health problem and is of economic significance in many countries.

Important global epidemiological facts of human salmonellosis are listed below:

The age group that is most affected by salmonellosis are small children of 0 – 4 years (112.4 per 100,000 population in the EU in 2009) (74).

Most cases of enteric fever, caused by *S.* Typhi and *S.* Paratyphi, are associated with poor hygienic conditions and occur mainly in developing countries (177). In contrast, non-

typhoid *Salmonella* generally cause gastroenteritis and are transmitted through contaminated food. They are the leading cause of human salmonellosis in developed countries.

In the majority of countries, *S.* Enteritidis and *S.* Typhimurium are the two most prevalent serotypes in humans (115). The ten most prevalent serovars in the EU from 2008, when this project was launched, are listed in Table 1.

The world wide epidemiology of *Salmonella* is characterized by a constantly changing picture. Some *Salmonella* clones appear in a population, increase and persist for a certain time and are subsequently replaced by new clones (123).

In Denmark, human *Salmonella* infections followed a steadily decreasing trend during the past 14 years (17). In 1997, 95 salmonellosis cases per 100,000 inhabitants were reported whereas the incidence of human salmonellosis in 2010 was 28.7 cases per 100,000 inhabitants (17,217). This reduction was achieved through national control programs (254). Nevertheless, Denmark experienced a very high number of laboratory confirmed cases in 2008 (3656 cases) and 2009 (2129 cases) (105) due to several large outbreaks caused by *S.* Typhimurium. Hence, *S.* Typhimurium is the most common serovar isolated from human cases since 2008 (15-17).

Table 1: The ten most prevalent *Salmonella* serovars and *S.* Typhimurium phagetypes from confirmed human salmonellosis cases in Europe in 2009 according to the EFSA Journal, 2010 (73).

serovar	phagetype	N	%	N	%
S. Entertidis		70091	58		
S. Typhimurium		26423	21.9		
	U292			1021	19.1
	DT193			751	14.1
	DT104			731	13.7
	DT120			557	10.4
	RDNC			241	4.5
	U320			203	3.8
	NT			152	2.8
	U302			146	2.7
	DT135			141	2.6
	DT104b			134	2.5
	Other			1267	23.7
S. Infantis		1317	1.1		
S.Virchow		860	0.7		
S. Newport		787	0.7		
S. Agona		636	0.5		
S. Derby		624	0.5		
S. Stanley		529	0.4		
S. Bovismorbificans		501	0.4		
S. Kentuckey		497	0.4		
Other		18495	15.3		
Total		120760	100	5344	100

2.2. Sources of human salmonellosis

The routes for transmission of *Salmonella* to humans can be the environment, the contact with animals or the person-to-person contact but in industrialized countries, *Salmonella* are mostly transmitted through contaminated food (4). The main reservoir for *Salmonella* is the intestinal tract of a wide range of food animals and therefore salmonellosis can derive from various food sources, including plant and animal products. In Denmark, the human salmonellosis cases have peaked three times during the past 30 years. Each peak was attributed to a main food source: in the late 1980s boilers, in the mid 1990s pork and in the late 1990s eggs. Each time a new intervention program was successfully implemented (241).

In Denmark, the largest part of the human salmonellosis cases (46.3 - 47.4%) is however related to travelling (17). Human travel and worldwide trade are considered to be very

important for the dissemination of *Salmonella* and the introduction of new clones into importing countries (4,56,107). This highlights the need of national and international control programs and collaborations across countries in preventing the global spread of epidemically successful clones that emerged in one country.

3. Transmission of *Salmonella* in the food chain

Salmonella are mostly transmitted through contaminated food products of animal origin such as fresh meat and eggs (241). Transmission often occurs when the bacteria are introduced into the food preparation areas and when they are allowed to replicate in the food (74). *Salmonella* in the food can directly originate from the farm reservoirs. However, *Salmonella* can enter at practically any stage of the food chain through contamination from the environment, other foods, animals or humans. This chapter will exclusively focus on the pork production chain as this is the food sector most relevant for this thesis.

3.1. Risk factors in the pork production chain for *Salmonella* contaminated food

Pork contributes significantly to the public health burden of *Salmonella* (232). In Europe, up to 25 % of the human Salmonellosis cases are ascribed to the consumption of pork (68). The implementation of a nationwide control program in Denmark has resulted in a substantial decrease in the occurrence of human salmonellosis cases attributed to pork products (241). Nevertheless, pork is still an important source of human salmonellosis in Denmark. The estimated number of human cases attributed to Danish pork has increased from 2008 to 2010 from 8.8 % to 15.1 % (17). In 2010 Danish pork accounted for the most common food source for human *Salmonella* infections followed by imported pork which was estimated to contribute with 5.4% to the reported cases (17). EU-wide, the number of positive *Salmonella* samples in the pig slaughter houses ranged in 2009 from 0 – 13.7 %. At processing and cutting plants, *Salmonella* was found in up to 5.5 % of the pig meat samples and at retail it was detected in up to 3.5 % of the samples (74). *S.* Derby and *S.* Typhimurium are the most frequently isolated serovars from pig herds (breeding and production holdings) (Table 2). *S.* Derby was detected in 28.5 % and *S.* Typhimurium in 20.1 % of *Salmonella* positive production holdings in the EU (74). Among the *S.* Typhimurium phagetypes, DT120, DT193, DT12 and DT104 were most common in pigs and pork in Denmark in 2010 (17).

In pigs, the infection with *Salmonella* can be asymptomatic and therefore easily be spread from one animal in a herd to another via feces. A carrier pig can shed up to 10^6 *Salmonella* cells per gram of feces for prolonged times (168,171). As these animals are not recognized to carry *Salmonella*, no therapeutic treatment will be applied to eliminate the pathogen. However, other vectors than pig to pig transmission are observed on farm level. These sources include rodents, insects, birds, humans and contaminated feed. *Salmonella* are ubiquitous in the environment. They survive desiccation remarkably well and can persist in a farm once they have been introduced (55).

The infected pig constitutes the main reservoir for carcass contamination in slaughterhouses and a large part of the carcasses harbor *Salmonella* on the skin or in the rectum (232). However, the pig slaughter line is an open process with many possibilities for contamination. Around 30 % of all *Salmonella* on pig carcasses result from cross contamination during the slaughter line (232). An overview of a typical Danish slaughterline and the most important hazards of bacterial contamination are presented in Figure 2. An important source for contamination are the intestines as *Salmonella* can be found in the entire digestive tract of a pig (163). The time from killing until the pigs are cooled takes less than 1 h (30) during which the body temperature of the pig remains 37 °C, the optimal growth temperature for *Salmonella*. During the slaughterline, the pig associated bacterial numbers are reduced during scalding, singeing and chilling (232). However, the carcass can be recolonized by contaminations from tools and machines and internal organs of the pig. The equipment used for slaughtering includes big complex machines that are very difficult to clean and to sterilize and that provide niches for *Salmonella* to persist.

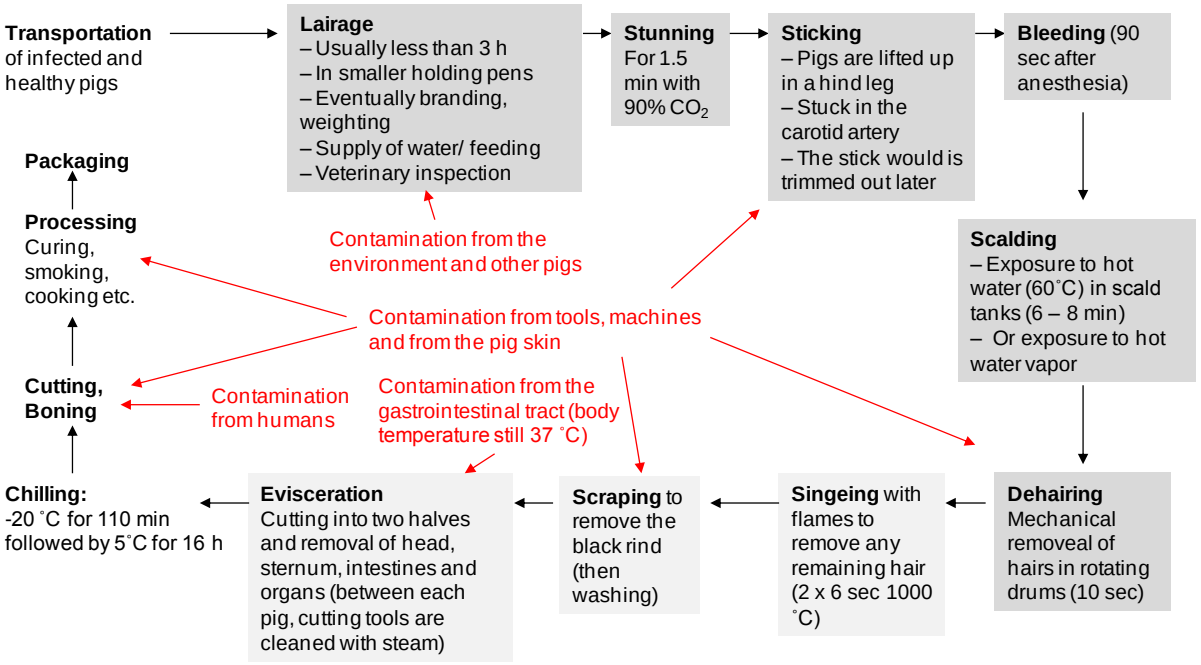


Figure 2: The slaughter process at a typical Danish pig slaughterhouse. Dark grey represents the black slaughterline and light grey the clean slaughterline. Hazards for contamination with *Salmonella* are displayed in the center of the diagram (in red). The temperature of the pig carcasses until chilling is 37 °C and the temperature of the slaughter area depends largely on the outside temperature and can vary from 10 - 25 °C. The temperature of the processing area after chilling is approx. 10 °C. (www.danishcrown.com, (30), personal communications)

The endpoint of the food chain is the kitchen of the consumers, restaurants or canteens. The environmental conditions that *Salmonella* has to face are very similar to those in the slaughter line. The main risk factors for domestic outbreaks have been associated to undercooking, improper storage and cross-contamination (123). The latter factor describes the situations where the handling of contaminated food in the kitchen, such as raw meat, results in the spread of the bacteria to the immediate environment followed by the contamination of ready to eat foods. Another risk factor might be that consumers freeze their food for long-term storage after which they thaw the food at room temperature for prolonged times before use.

Overall, *Salmonella* transmission in the pork production is a complex multifactorial problem due to the ubiquitous nature of the pathogen. Full eradication of *Salmonella* might therefore be impossible but the monitoring and control at critical points in the pork production chain seems essential to reduce the spread of *Salmonella* and guaranty pork safety.

Table 2: Distribution of the ten most common *Salmonella* serovars in pig meat and pig herds in the EU in 2008 according to the EFSA Journal, 2010 (73).

Pig meat			Pig herds		
Serovar	N	%	Serovar	N	%
S. Typhimurium	469	33.1	S. Typhimurium	1544	31.5
S. Derby	280	19.8	S. Derby	646	13.2
S. Agona	55	3.9	S. London	177	3.6
S. Infantis	49	3.5	S. 1,4,5,12:i:-	154	3.1
S. London	48	3.4	S. Livingstone	127	2.6
S. Bredeney	46	3.2	S. Cholerasuis	119	2.4
S. Rissen	37	2.6	S. Infantis	118	2.4
S. Enteritidis	21	1.5	S. Entertidis	112	2.3
S. Livingstone	20	1.4	S. Rissen	109	2.2
S. Brandenburg	9	0.6	S. Anatum	101	2.1
Other	383	27	Others	1690	34.5
Total	1417	100	Total	4897	100

4. Stress tolerance of *Salmonella*

4.1. Definition of bacterial stress

Microbial stress can be defined as sudden environmental changes that alter the behavior of the microorganism and reduce growth (100,198). However, usually stress is referred to environmental conditions that cause injury to the cell (242) which results in some loss of function (91). Sublethal injury damages but does not kill the microorganism (124) as it is able to respond to the stress and to repair the damage. Lethal injury eliminates the bacteria but in a bacterial population, a fraction of the bacteria cells may survive depending on the degree of injury and the time of exposure to the stress. Metabolic injury is related to damages of various components of the cells and is frequently distinguished from structural injury which refers more to damages of the cell wall or membranes. Both injuries are characterized by the failure of the bacteria to form colonies on selective media where uninjured cells would normally grow (242). Bacterial stresses can be generally classified into three categories: physical, chemical and nutritional (242).

4.2. *Salmonella* encounter various stress conditions in the food chain and during infection

While *Salmonella* passes the food chain and during the course of infection, it is exposed to numerous stress conditions (Table 3). As many foodborne pathogens, *Salmonella* can sense and respond effectively to divers stress signals inside and outside the host.

During food production, the food-associated microbes are exposed to food preservation techniques such as freezing, chilling, heating, smoking and curing. *Salmonella* might also survive on food processing surfaces where it experiences desiccation, nutrient limitation and where it gets in contact with disinfectants. Following, ingestion, an enteric pathogen, such as *Salmonella*, must cope with the low pH in the stomach, ranging from 2.5 – 4.5, largely depending on the alimentation (29). When *Salmonella* enters the gut, it is exposed to various stresses which are summarized in Table 3. Upon invasion, *Salmonella* is taken up by phagocytes where they encounters a new series of stressors. Unfortunately, *Salmonella* is known for tolerating a remarkable broad range of stress conditions and to also survive rapidly changing environments (29).

Table 3: Stress conditions that *Salmonella* may encounter in the food chain or during infection (242).

When?	Where?	What?
Food production	typically food associated	drying heating freezing thawing chilling high oxmotic pressure (curing, sugaring) antimicrobial compounds (e.g. spices, nitrite, decontamination agents) low or high pH competitive exclusion by protective cultures reduction of oxygen radiation (e.g. UV)
	typically associated with surfaces processing areas and equipments	limited nutrients and water disinfectants (e.g. chlorine, quarternary ammonium compounds, hydrogen peroxide) drying
Infection	stomach	low pH
	gut	bile acids antimicrobial peptides competitive exclusion by natural flora high osmotic pressure reduction of oxigen nutrient limitation
	phagocytes	low pH oxydative stress antimicrobial peptides limited nutrients

4.3. Cross-protection and virulence of stress adapted cells

Studies have shown that the exposure to sublethal stress can confer protection to the subsequent exposure to the same stress or other stress conditions (23). Dehydrated *Salmonella* cells can for example acquire high tolerance to heat, ethanol, high salt concentrations and UV irradiation (101). This is due to the production of stress proteins of the RpoS mediated general stress response system that maintain cell viability and thereby confer protection to different stresses. Thus, certain stress conditions have the potential to increase virulence because many of the host defense mechanisms show similarities to conditions that bacteria experience during food processing. For example acid or heat tolerant *S.* Enteritidis were observed to be more virulent in mice (122). Certain stress conditions that resemble host defense mechanisms

can also serve as signals for the expression of virulence genes, for example of the *spv* genes located on the *Salmonella* virulence plasmid (242). The general stress sigma factor RpoS is not only important for the regulation of stress proteins but is also involved in the regulation of the *spv* virulence genes (69). The stress conditions that enhance virulence in *Salmonella* include glucose starvation, low pH, elevated temperature and iron limitation (242). Preexposure to acids is known to induce resistance to gastric acid and thereby leads to a decrease in the infectious dose (251).

4.4. The role of the growth state during stress exposure

Lag times are good indicators for the severity of injury as they represent the time that the bacteria need to repair their damages and to prepare for growth. The lag time can vary markedly in length depending on the injury. Also on single cell level, considerable differences can occur (219) indicating a substantial heterogeneity of the degree of damage within a bacterial population. This variability might be explained by the growth states of the single cells at the time of stress exposure. Rapidly growing exponential cells are morphologically and physiologically distinct from cells in stationary phase. Cells in stationary phase are more robust whereas cells in exponential growth phase are more susceptible to stresses. For instance, *E. coli* that enters stationary phase synthesizes trehalose, an osmolyte that protects molecules and membranes from osmotic stress (189). Thus, an *E. coli* cell in stationary phase does not need to carry out as many physiological changes as exponential phase cells when they are exposed to stress. Exponential phase cells are presumably rare in the environment but in the food chain a significant amount of the single cells might grow exponentially when the temperature is adequate and nutrients available.

Under severe stress conditions, it can occur that bacteria are not able to grow on routine laboratory media but are still metabolically active. These bacteria are in a viable but non-culturable state and might remain pathogenic (242). In surveillance of food, this can lead to an underestimation of potentially harmful bacteria if molecular detection methods are not available. Furthermore, many stress conditions have shown to inhibit cell division leading to the formation of multinucleate filaments. In this case, the enumeration of CFU by direct plating would result in an underestimation of the cell concentration. These filaments can very rapidly divide and multiply when the bacteria enter favorable conditions and thereby increase the cell number in a short time (123).

4.5. The general stress response

Salmonella has complex regulatory systems to sense and respond to environmental conditions (123). Stress signals influence gene expression through a variety of mechanisms. Sigma factors play an important role in this process as they change the transcription specificity of the RNA polymerases. The general stress response in *Salmonella* that is induced under several different stress conditions (starvation, high osmolarity, non-optimal temperature or acidic pH) is regulated by RpoS, the alternative sigma factor (2). It activates up to 500 genes (116) indicating numerous alterations of cellular physiology and morphology. The general stress response allows to rapidly adapt to changing environmental conditions but also confers long-term protection to stress (208).

4.6. Impact of freezing and thawing on bacterial cells

On one hand, freezing injury is the consequence of dehydration of bacterial cells because of an increase in the extracellular solute concentration in the unfrozen fraction and the decrease of available water. On the other hand, freezing injury can be the result of a physical damage of the cells by intracellular or extracellular ice crystals (242). Freezing is generally recognized as an ineffective food preservation technique as many food components, such as peptides, proteins and glycerol act as cryoprotectants (149). Also fat has been suggested to contribute to the protection of bacterial cells by insulation (66). Moreover, the meet matrix might play an important role during freezing by binding free water and thereby reducing the ice formation (59). The cooling rate is an important factor during freezing as it highly influences the bacterial injury. Slow freezing results in the formation of large extracellular ice crystals whereas rapid freezing (between approx. 4 °C and 53 °C per min) leads to small extra- and intracellular ice crystal formation which is less detrimental for the bacteria (58,148,255). All bacteria cells need to maintain their cell turgor by an intracellular osmotic pressure greater than of the surrounding medium. During slow freezing, the extracellular medium freezes faster than the cell content, the osmotic pressure of the surrounding medium increases and the cytoplasmatic water flows out of the cell (148). This results in shrinkage of the cells and protein denaturation (89). The thawing rate has also an effect on the bacterial cell. Slow thawing allows small intracellular ice crystals to grow and coalesce before melting, which causes more damages than a fast thawing rate where the ice simply melts (180). Hence, quick freezing and thawing that are usually chosen to maintain product quality lead to less inactivation of microbes in the food. *Salmonella* is known for its tolerance to freezing stress.

It has been demonstrated to be quite stable in various foods frozen and stored between -18 and -22 °C (19). Repeated freezing and thawing and lowering the pH during freezing are however more detrimental for *Salmonella* (19).

4.7. Impact of drying on bacterial cells

The availability of water in foods is often expressed as the water activity (a_w). An a_w of 1 corresponds to pure water and an a_w of 0 means that no water is present (58). The optimal growth range for microorganisms is narrow (between an a_w of 0.995 and 0.980) (148). The minimal a_w value for *Salmonella* required for growth is approx. 0.95 (148). Decreasing the water activity is one of the most widely used food preservation methods. It reduces the amount of the available water to the bacteria and leads to a loss of water and a decrease in the cytoplasmatic volume similar to the effect observed during freezing described above (22). This will lead to an increase in viscosity, an increase in the intracellular concentration of salt and macromolecules, protein denaturation, DNA denaturation as well as to oxidative stress (213). *Salmonella* is known for being able to persist for long times in the environment leading to a risk of cross-contamination (88). It has to be distinguished between the increase of the solute concentration in the extracellular medium and desiccation (air-drying), the decrease of the extracellular water for example by evaporation. Under the first mentioned conditions, the bacteria can regain access to water by osmoregulation. To allow the water to cross the cellular membrane, the bacteria raise their intracellular solute concentration using compatible solutes, molecules that do not harm the cytoplasmatic enzymes and allow the functionality of the cell even at high concentrations (103). These solutes are either produced within the cell or are actively transported into the cell if present in the extracellular medium. A range of amino acids and related compounds serve as compatible solutes (148). In *Salmonella* glycine betaine, trehalose or proline are accumulated (148).

5. Pathogenesis of non-typhoid *Salmonella* in humans

5.1. A short introduction to salmonellosis

Salmonella serovars that cause disease in humans can be divided into typhoid and non-typhoid according to their disease pattern. Typhoid *Salmonella* including the human specific serovars *S.* Typhi and *S.* Paratyphi cause enteric fever, a systemic disease. The most common

disease outcome of non-typhoid *Salmonella* (NTS), such as *S.* Typhimurium and *S.* Enteritidis is a self-limiting gastroenteritis, a localized infection of the terminal ileum and colon that does not require antibiotic therapy. Depending on the host immune status, the bacterial strain and the infectious dose, a systemic infection can occur. In humans, this can manifest as septicemia or focal infection (4). A frequent model for studying salmonellosis is the oral infection of mice with *S.* Typhimurium which establishes a systemic infection in this animal model (Figure 3). This is different from the usual pathogeneses in humans and it is important to be aware of the limitations of this model. However, it has provided the opportunity to identify important *Salmonella* virulence factors and to study the course of a systemic *Salmonella* infection (202). This chapter will summarize the stages of salmonellosis in humans even though much of the current knowledge is based on animal models. In general, it is characteristic for NTS that they are able to colonize the gut, to cross the intestinal epithelial cells and to invade and survive in macrophages leading to inflammation of the intestines and to gastroenteritis. To establish a disease, *Salmonella* uses numerous virulence factors. This chapter will focus on some important and for this thesis relevant virulence factors.

5.2. The route of infection during *Salmonella* gastroenteritis

5.2.1. Infectious dose, passage through the stomach and colonization of the gut

In foodborne infections, the infectious dose of a pathogen depends on three factors: 1.) on host resistance, which is impaired in young, elderly and immunocompromised; 2.) on the food composition and matrix, with fat for example lowering the infectious dose (81), and 3.) on virulence and physiological state of the ingested bacterium (123). The infectious dose for salmonellosis is therefore variable. It is thought to be typically between 10^6 – 10^8 CFU for humans but can be significantly lower for example in outbreaks (123). After ingestion, *Salmonella* passes the stomach where it is exposed to an acidic pH (81). The surviving bacteria cells enter the intestines where *Salmonella* is able to colonize multiple sites of the small intestines, the colon and the cecum. The ileum is however the most frequently invaded site of *Salmonella* in humans (199).

5.2.2. Adhesion and invasion of intestinal epithelial cells

The epithelium of the intestines is covered and protected by a mucus layer. Thus, it has been suggested that *Salmonella* invades preferably the mucus-free M-cells situated in the Peyer's Patches and dendritic cells (DCs) that lie within the epithelium. The first cellular contact

presumably mediated by frimbriae (301) is followed by the invasion of the epithelial cells. This process is conferred by the type III secretion system (T3SS) 1, a protein complex that is associated with at least 20 structural and regulatory proteins (81). T3SS1 has a needle like structure and injects protein effectors into the epithelial cell. The translocated proteins cause cytoskeletal rearrangements in the host cell which leads to membrane ruffling and bacterial internalization through macropinocytosis (83,244). The internalized bacteria cells reside in a membrane bound vacuole (SCV) in which they cross the epithelia layer (200). Knodler et al. suggested that a subpopulation of *Salmonella* replicates within the cytosol of epithelial cells. These cells are thought to be extruded from the monolayer into the intestinal lumen (131). This host defense mechanism might serve as a reservoir of dissemination for *Salmonella*. The extruded cells can infect new host cells or can be excreted and eventually infect new hosts. Upon translocation to the lamina propria, bacterial flagellin functions as the major proinflammatory determinant (252). It is recognized by Toll-like receptor (TLR) 5 located on the basolateral side of epithelial cells and on other host cells (90). After translocation, *Salmonella* it thought to either invades macrophages or once again enter epithelial cells from the basolateral side (109).

5.2.3. Interaction of Salmonella with macrophages

Within the lamina propria, *Salmonella* is immediately taken up by mononuclear phagocytic cells (macrophages or DCs) associated with Peyer's Patches (200). Macrophages recognize pathogen associated molecular patterns (PAMPs) of *Salmonella*, such as flagellin, leading to an uptake of the bacteria (167). The contact of *Salmonella* with macrophages and other immune cells triggers the production of pro-inflammatory cytokines and chemokines (216). However, effector proteins of T3SS-1 early in infection have also been suggested to be directly implicated in eliciting the inflammatory response (211).

Inside the macrophage, *Salmonella* is located within the phagosome. During the first 2-3 h, before replication begins, *Salmonella* modifies the phagosomal maturation and creates as unique niche, the *Salmonella* containing vacuole (SCV) (99). It has been suggested that *Salmonella* arrests the degradative pathway of the phagosome by inhibiting its fusion with the lysosome (41). But contradictory studies exist that propose the survival of *Salmonella* despite the fusion of these two compartments (109,250).

Within the phagocytes, *Salmonella* faces a number of challenges, such as the procurement of nutrients, the defense against antimicrobial peptides, the protection against oxidative stress

and acidification. Upon phagocyte activation that occurs during uptake of *Salmonella*, the phagocyte NADPH oxidase reduces molecular oxygen to cytotoxic superoxide (234). This constitutes the initial bactericidal phase (called the oxidative burst) and it is followed by a prolonged bacteriostatic phase in the presence of nitric oxide which is synthesized by the nitric oxide synthases (NOS) (234). Microarray studies have shown that *Salmonella* regulates up to 919 genes differently when it senses the specific intravacuolar environment in macrophages (72). It is thought that genes needed for the early stages of infection, such as T3SS-1 and flagellin are repressed (109). The genes that are upregulated include a second *Salmonella* secretion system, the T3SS-2. It translocates effector protein across the vacuolar membrane into the cytsol. Among others, T3SS-2 is involved in maintaining and positioning of the SCV, in the defense against killing by the NOS and NADPH oxidase and in the delay of the apoptotic cell death (125,239).

During the course of infection, *Salmonella* can remain within the SCV of macrophages for several hours to several days (109). It may be spread systemically but *Salmonella* can also cause macrophage apoptosis allowing it to disseminate to new macrophages and to infect the epithelial cells from the basolateral side. Macrophage lysis results in the release of proinflammatory cytokines and inflammation (199). If this process is beneficial for *Salmonella* is still controversially discussed (230). It allows *Salmonella* to colonize the gut associated tissue but also helps the host in clearing the infection.

5.2.4. Neutrophil recruitment, antimicrobial products and diarrhea

Within 12 to 72 h after infection, the patient experiences diarrhea, nausea, vomiting and intestinal cramping. CXC chemokine production, in the lamina propria results in the attraction of polymorphnuclear neutrophils (PMN) to the side of infection. The resulting neutrophil migration through the intestinal epithelium develops into a massive PMN efflux associated with a loss of epithelial integrity and a flow of a protein rich fluid into the intestinal lumen (254). This is likely to clear the infection and to prevent a systemic spread of the bacteria. Diarrhea is also associated with a loss of nutrients in the intestinal lumen. This affects the commensal microbes as well as *Salmonella*. However, *Salmonella* seems perfectly adapted to this environment as it efficiently uses mucus carbohydrates as an energy source (201). It has therefore been suggested that, *Salmonella* has a fitness advantage over the intestinal microbiota under inflammation resulting in an increased number of *Salmonella* in the intestines (218). Thus, *Salmonella* seems to exploit this unique environment of inflammation

created by the defense mechanism of the host. In relation to this, it has been hypothesized that, a fraction of the *Salmonella* population invades the intestinal barrier on a "self-destructive mission" whereas the other fraction benefits from the inflammatory changes in the intestinal lumen (191).

Another important part of the innate immune response to *Salmonella* is the release of antimicrobial proteins and peptides from epithelial cells into the lumen of the inflamed intestines. In order to be able to colonize the intestines during infection, *Salmonella* possesses virulence mechanisms protecting it from the antimicrobial effectors. One example is the *iroBCDEN* gene cluster that confers resistance towards lipocalin-2, an antimicrobial that prevents bacterial iron acquisition (191).

5.3. Systemic infections established by non-typhoid *Salmonella*

In healthy adults, salmonellosis is usually limited to the intestines where it causes an early inflammatory response and diarrhea as described above. Systemic infections caused by NTS is therefore rare in industrialized countries but it occurs frequently in Africa due to malnutrition, malaria and HIV (98). Not much is known about the bacterial and host mechanisms leading to a systemic infection of NTS. Gordon et al. suggested an intracellular persistence of invasive NTS and a failure of the immune system to control these bacteria (98) for example the lack of establishing a strong PMN influx (65). The survival within macrophages seems to be an essential prerequisite for dissemination to mesenteric lymph nodes or other parts throughout the reticuloendothelial system. From there *Salmonella* can enter the blood stream and reach different organs around the body (65,109). Reaching high intracellular densities would lead to a spatial and nutritional limitation of the intracellular bacteria and to an outgrow of the macrophages, risking the detection by the immune system. DCs are also likely to mediate the systemic spread of *Salmonella*. In DCs, *Salmonella* does not appear to replicate but remains viable (228). Genes encoded by the T3SS-2 seem to suppress antigen presentation by DCs which limits the immune response (239). Once *Salmonella* has reached different organs, it might multiply by replicating inside the cells and by inducing apoptosis (81). However, there is evidence that *Salmonella* rather multiplies by expanding to new immunological unprimed foci without undergoing massive intracellular growth (228). Overall, the ability to remain relatively undetected by the immune system, as known for *S.* Typhi, might be a key mechanism in systemic infection caused by NTS (65,209).

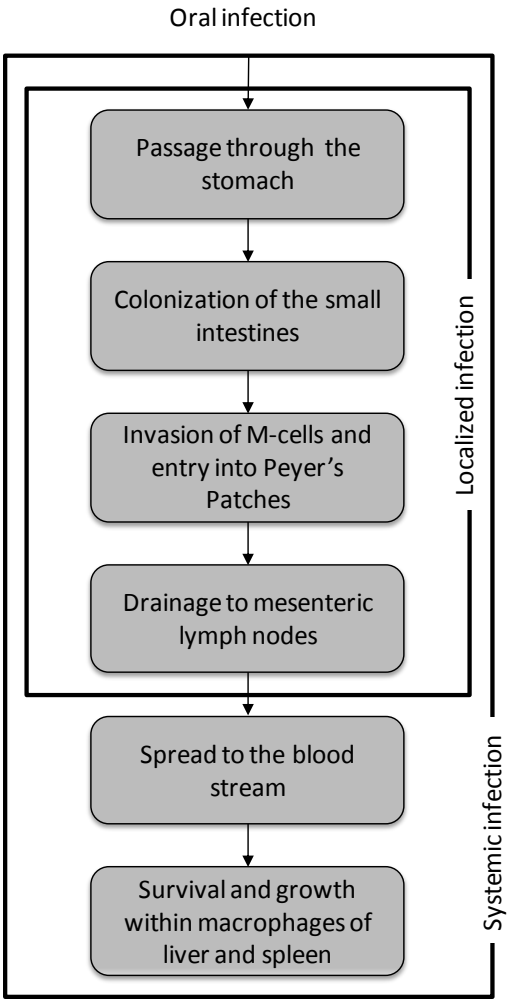


Figure 3: The course of an infection with *S.* Typhimurium in the mouse after oral administration, modified after Wray and Wray, 2000 (86).

5.4. Important virulence factors

A broad range of genes related to stress tolerance, nutrient uptake and metabolism is necessary for a *Salmonella* infection. Many of these genes are found in other bacterial species than *Salmonella*. In addition, *Salmonella* has acquired an arsenal of genes encoding classical virulence factors that are directly linked to a successful infection within the host. Some of these virulence factors are limited to a number of subtypes and contribute to the differences in pathogenicity and virulence of *Salmonella*.

5.4.1. Salmonella pathogenicity islands

Many virulence factors in *Salmonella* are encoded within large regions of the bacterial chromosome, within the *Salmonella* pathogenicity islands (SPIs). They are defined as regions that encode virulence genes and that are absent from the genome of *E. coli* K12 (77). They were probably taken up by horizontal gene transfer as they are composed of a distinct G+C content relative to the genome backbone and as they often carry remnants of transfer genes (1). Moreover, SPIs are often inserted adjacent to tRNA genes and insertion sequences, similar to mobile elements. The distribution and composition of many SPIs vary among *Salmonella* (28). In the following, SPI-1, SPI-2 and SPI-3 will be described more in detail.

SPI-1 encodes T3SS-1 that enables *Salmonella* to enter many types of host cells. Its major role is the invasion of the intestinal epithelium as strains that harbor mutations within this gene island are reduced in virulence when administered orally but not when given systemically (77). By transporting at least 13 effector proteins into the cytosol of the host cell, it promotes the uptake of *Salmonella* cells as described above (5.2.2). Many effectors are located outside of SPI-1, on other pathogenicity islands or bacteriophages (28). SPI-1 is ubiquitous among *Salmonella* but absent from the closely related *E. coli* lineage and was presumably responsible for the divergence of these two genera approx. 100 - 160 million years ago (60,170). The acquisition of SPI-1 allowed *Salmonella* to cross the intestinal epithelium and to access a new niche. In the following, *Salmonella* adapted to this new environment by acquiring new genes, such as genes of the SPI-2 (77).

SPI-2 is mosaic in structure and probably the result of independent horizontal transfer events (117). It encodes T3SS-2 that is required for survival and growth in macrophages as well as in epithelia cells and that is expressed 2-3 h post infection. It is induced intracellular, after formation of the SCV as a response to various signals, such as low osmolarity, nutrient depletion and acidification (81). It functions in translocating different effector proteins into the cytosol that interact with targets in the host cell (81,239). The region of the island encoding the T3SS-2 is present in all *Salmonella* except *S. bongori* (77). *S. bongori* carries another section of the island encoding a tetrathionate reductase required for anaerobic respiration (117). Similar as for T3SS-1, many T3SS-2 effectors are encoded outside of the SPI-2.

SPI-3 appears to have a mosaic genetic structure too. It is composed of three regions with variant G+C content and interspersed with remnants of insertion sequences (27). The

transcriptional units located on SPI-3 have only little known functional relation to each other (103). Some parts of SPI-3 are differently distributed in some *Salmonella* suggesting a multistep process in the evolution of this island (27). The distribution of the genes *rhuM*, involved in epithelial cell invasion, and *sugR*, of unknown function, show the greatest variability as they are deleted in some *Salmonella* (222). The region encoding for example the *mgtCB* operon required for growth in macrophages is in contrast conserved (77). Other SPI-3 genes have been found to harbor translational stop codons in some serovars, such as *S.* Typhi, and are considered to be pseudogenes (193).

5.4.2. The role of prophages in virulence

Bacteriophages invade bacteria cells and contribute to a great extend to the evolution of their hosts (136). After uptake of a temperate phage, the host bacterium can acquire new phage encoded phenotypic properties (136). P22 for example expresses the genes *gtrABC* that modify the O-antigen side chains by glycosylation, a process called O-antigen conversion (136). This alters host recognition of the bacterium and contributes to the bacterial evasion from the immune system (34). Many of the above mentioned T3SS effector proteins are encoded on bacteriophages located outside of the SPIs (1). For example *sopE* is part of the lysogenic bacteriophage SopEΦ and only present in a part of the *Salmonella* strains. It is a T3SS-1 effector protein and co-expressed and transferred together with all the other T3SS-1 effectors (37). This shows that SopE has been fully integrated into the host metabolism which is also characteristic for other prophage gene products. Similar to other phage genes, *sopE* has been duplicated and diversified during the evolution of *Salmonella*. Other known phage virulence factors of *Salmonella* are presented in Table 4. However, these genes might only represent a small fraction of all existing phage genes that enhance fitness or virulence of *Salmonella*.

Paradoxal, the phage could also contribute to fitness of the host bacterial population when it is released from individual bacterial cells. Bacteria infected with a certain phage cannot be reinfected with the same kind of phage (136). If some bacterial cells in a population lyse, the released phage can act as a weapon against competitive bacteria. It attacks competitors whereas the bacteria of the host population that still carry the phage are immune (179).

A considerable part of the *Salmonella* genome is composed of cryptic phages that do not have the ability to enter the lytic cycle. During evolution, these phages became slaves of their bacterial hosts. Even thought the detailed mechanisms are not clear, it has been reported for *E.*

coli that these phages contribute drastically to the fitness of the host bacterium. A deletion of these phages reduces for example the resistance to sublethal concentrations of antibiotics, to osmotic and oxidative stress as well as to acidic pH (238).

Table 4: The most important bacteriophages or phage remnants of non-typhoid *Salmonella*, grouped after their phylogenetic relatedness and their encoded virulence genes (37,136).

Phage group	Salmonella phages	Virulence genes
P27 like phages	ST64B	
P2 like phages	Fels-2	
	SopEϕ	*sopE*
	PSP3	
lambdoid phages	Gifsy-1	*gipA, gogAB*
	Gifsy-2	*gtgAE, sodC1, sseI*
	Gifsy-3	*sspH1, pagJ*
	Fels-1	*sodCIII, nanH*
P22 like phages	ε34	
	ES18	
	P22	
	ST104	
	ST64T	
T7 like phages	SP6*	
others	ε15	
	KS7	
	Felix O1	
phage remnants		*sopE2, sspH2*

*lytic phage

5.4.3. The spv genes

The *spv* (*Salmonella* plasmid virulence) locus is conserved and is located on large plasmids (50 – 100 kb). However, also chromosomally encoded *spv* genes have been detected (77). Strains from many serovars lack the *spv* genes but *S.* Typhimurium and *S.* Enteritidis are known harbor virulence plasmids carrying *spv* (81). The *spv* genes are thought to facilitate intracellular replication in macrophages and systemic spread (204). In human macrophages, they appear to induce cytotoxicity (146). The locus consists of five genes, namely *spvABCD* and the positive regulator gene *spvR* (102). Their expression is among others regulated by

RpoS, and notably the growth state. It has been observed that more blood isolates harbor *spv* genes than isolates from stool but *spv* is absent from *S.* Typhi, indicating that NTS bacteremia is fundamentally different from typhoid fever (76).

5.5. The use of cell cultures to study *Salmonella* virulence

Human and animal immortalized cell lines have often been used to study virulence in *Salmonella*. But some limitations have to be considered when using cell cultures. In vitro, *Salmonella* is replicating inside the SCV within epithelial cells resulting in a large amount of bacteria after 6 hours post infection (78). This is different from what is observed *in vivo* where the bacteria rapidly cross the epithelium. Macrophages contain *in vivo* approx. 1 – 10 bacteria cells, whereas the cultured macrophages are fast outgrown by a larger number of *Salmonella* cells (228). These examples reflect that many factors of the host defense are absent in cell cultures.

Furthermore, the experimental setup might greatly influence the results. When cell lines are used, a gentamicin protection assay is usually applied. This method takes advantage of the inability of gentamicin to penetrate eukaryotic cells (206). Extracellular bacteria can thus be eliminated whereas intracellular bacteria are spared. However, this experimental procedure is not standardized. Varying parameters in cell culture models are the multiplicity of infection, using opsonized versus non-opsonized bacteria, pre-treatment of macrophages with IFN-γ versus no pre-treatment and the growth state of the bacteria before infection (228). The origin of the cell culture, for example mouse or human can also complicate the interpretation of results (228).

6. Antibiotic resistance in *Salmonella*

Antibiotics inhibit essential physiological or metabolic functions in susceptible bacteria cells (144). There are numerous different antibiotics available that are, based on their mode of action, divided into antimicrobial classes. Antibiotics are widely used in human and veterinary medicine. Hence, bacteria are frequently exposed to the selective pressure of antibiotics leading to the development of resistance mechanisms. Antibiotic resistances can be roughly divided into three categories depending on the mechanism: 1.) the destruction or modification of the antibiotic, 2.) the decreased influx or increased efflux of the antibiotic and

3.) the alteration of the cellular target of the antibiotic (144). Bacteria can be naturally resistant to certain antibiotics but this thesis focuses on antibiotic resistances that are acquired through genetic changes of the bacterium. These changes include the acquisition of new genes by horizontal gene transfer or the mutation of existing genes.

The resistance of bacteria is usually determined in the laboratory by measuring the minimal inhibitory concentration (MIC) of a drug by broth/agar dilution methods or by recording inhibition zone diameters from diffusion assays (7). Breakpoints for the characterization of isolates as susceptible, resistant or intermediate are usually established based on population MIC distributions or on clinical efficacy data (7).

Since the 1990, *Salmonella* resistant to a variety of antibiotics, including agents that are used for treatment of humans have emerged (81). Adults with respective symptoms (such as blood in stool and high fever) are today usually treated with fluoroquinolones, such as ciprofloxacin, whereas for children β-lactam antibiotics may often be prescribed (personal communications). Usually most salmonellosis cases are not treated with antibiotics but during some outbreaks up to 40 % of the cases may receive antimicrobial therapy (personal communications). The increasing prevalence of particularly multidrug and quinolone resistant *Salmonella* limits the possibilities to treat severe salmonellosis cases and is a major public health problem.

6.1. Transmission of antibiotic resistance

The main mechanisms for transmission of resistance in *Salmonella* are horizontal gene transfer and clonal spread of resistant strains (42,160). Resistance genes can be transferred between different *Salmonella* serovars or can originate from other bacterial species (7). Plasmids and class one integrons are the main mobile elements responsible for the transfer of resistance genes in *Salmonella* (7). These elements often carry multiple antibiotic resistance genes. Not much is known about the role of bacteriophages in the transfer of resistance genes but there is evidence that phages contribute to the transmission of resistance determinants in *Salmonella* (36,51).

The spread of resistant *Salmonella* and other resistant food borne pathogens is largely the result of the antimicrobial use in food animals but also the use of antimicrobials in human medicine is a cause for transmission of resistances (11,12,156,227). During the past centuries, livestock production has developed from small to extremely large herds. The enhanced numbers of the animals in the herds has increased the usage of antibiotics for both, therapeutic

and non-therapeutic reasons (171). This had implications for the emergence and spread of multidrug resistant pathogens and has raised public health concern. However, transmission of resistant *Salmonella* has also been suggested to occur independent on antibiotics present (228). Exposure to disinfectants and food preservatives might as well select for resistant *Salmonella*. The reason for this might be resistance mechanism that protect against a broad spectrum of toxic agents, such as increased expression of efflux pumps (7).

6.2. Multidrug resistance

In this study, multidrug resistance (MR) refers to strains that are resistant to four or more antibiotic agents as this definition has been used in previous publications (104,178). However, today NARMS and DANMAP define MR as resistant to at least three antimicrobial classes (18,45).

6.2.1. The Salmonella genomic island 1

Multidrug resistance in many *Salmonella* is often chromosomally encoded and due to the acquisition of the *Salmonella* genomic island 1 (SGI-1) and variants of it. In contrast to plasmid encoded antibiotic resistances, SGI-1 is stable also in the absence of the selective pressure (118). SGI-1 was first found in *S.* Typhimurium DT104 which probably acquired SGI-1 in the early 1980s. In the 1990s, this multidrug resistant *S.* Typhimurium DT104 clone was the most prevalent clone in many countries of the world and was reported to be more virulent in humans as an unusual high percentage of patients were hospitalized in the UK and in the US, this strain was more often isolated from blood than non-ACSSuT stains (165,226). It has been suggested that SGI-1 was responsible for the successful spread of this clone and more recent also of *S.* Paratyphi in Canada (118,166).

SGI-1 carries a set of resistance genes that are all located within the boundaries of a complex class 1 integron (142). In *S.* Typhimurium DT104, it confers resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline (ACSSuT). The genes that encode this resistance phenotype are *aadA2*, *floR*, bla_{pse-1}, *sul1* and *tet(G)* (31,33). In variants of SGI-1, some resistance genes have been gained, deleted or replaced (141). This indicates that SGI-1 is a highly dynamic genetic element that allows variation in otherwise genetically uniform bacterial clones (42) and that contributes to a rather quick evolution (43). Besides the resistance genes, other genes associated with DNA transfer and many genes with unknown function have been found on SGI-1.

SGI-1 is an integrative mobilizable element that can be transferred via conjugation with a helper plasmid (61). Isolates of the same serovar from different parts of the world harboring SGI-1 seem to belong to one clone, indicating that the transfer of the island is rather rare (106). An SGI-1 variant is also present in *Proteus mirabilis* and parts of the SGI-1 nucleotide sequence are found in *Shewanella* indicating a participation of these bacteria in the evolution of this gene island (210).

6.3. Quinolones resistance

6.3.1. A short introduction to quinolones and their use

For many years, chloramphenicol, ampicillin and trimethoprim/sulphamethoxazole were the drugs of choice for treatment of severe salmonellosis (176). Due to the increased occurrence of multiple resistant *Salmonella*, fluoroquinolones have frequently been an effective alternative when antimicrobial therapy was required. The first generation of quinolones begins with the introduction of nalidixic acid which is characterized by a limited activity against gram negative bacteria. In the following, structural changes have resulted in more potent antibiotics with broader activity spectra (248). Today, the broad spectrum fluoroquinolone ciprofloxacin is the most used quinolone (18). Unfortunately, treatment failures have been described in patients infected with strains exhibiting low level resistance (MIC 0.12 - 1 µg/ml; according to Eucast breakpoints) (214).

Quinolones have also been used in the veterinary medicine and it has been suggested that quinolone resistance can be transferred from food animals to humans (11). However, it can also emerge in susceptible strains within a patient during the course of treatment (157,181).

6.3.2. Mechanisms of action

Quinolones target the complex of DNA and type II topoisomerase (DNA gyrase or topoisomerase IV) during DNA replication (95). These type II topoisomerase enzymes are heterotetramers of two A subunits where the active site of the enzyme is localized and two B subunits: GyrA and GyrB in the DNA gyrase and ParC and ParE in the topoisomerase V. DNA gyrase induces negative coils into the DNA superhelix which is important for replication and topoisomerase V allows the two chromosomes to be separated at the end of the replication (3,237). The interaction of quinolones with these enzymes leads to a replication stop and induces the bacterial SOS response, an error prone DNA repair mechanism (248). Furthermore, it is thought to result in lethal DNA double strand breaks (126).

6.3.3. Mechanisms of resistance

6.3.3.1. Point mutations

The acquisition of point mutations in the target genes of DNA gyrase and topoisomerase results in a decreased affinity to the quinolone and is the main mechanism responsible for quinolone resistance in *Salmonella*. Mutations within the genes of these enzymes usually cluster within the quinolone resistance determining region (QRDR) (120). Most of the mutations appear in the QRDR of the subunit *gyrA* as it harbors the active site of the DNA gyrase (120). A single point mutation within this region can mediate full resistance to nalidixic acid and low level resitance to fluorquinolones (182). Mutations in *gyrB* also occur but mutations in *parE* and *parC* are rare.

6.3.3.2. Downregulation of outer membrane porins

The entry of quinolones into the bacterial cells occurs through outer membrane porins as these antibiotics are hydrophilic molecules. Alterations in the expression of these protein, such as OmpF can lead to low level resistance to fluorquinolones even though *gyrA* is not mutated (183).

6.3.3.3. Efflux pumps

A decrease in the intracellular accumulation of the antibiotic can be achieved by an increased efflux of the drug. This is mediated by antibiotic efflux pumps that mainly utilize the proton motive force to export the antibiotic actively out of the cell (185). Bacteria that express these efflux pumps usually show cross-resistance to a number of different antibiotics (185). In Enterobacteriaceae, the main efflux system for quinolones is encoded by *acrA, acrB* and *tolC*. This system is under the control of AcrR (a repressor), MarR (a positive regulator) and SoxS (a positive regulator; in presence of oxidative stress it is turned on by SoxR). Some strains can become resistant because they acquire point mutations in the genes encoding AcrR, MarR and SoxR resulting in an overexpression of the efflux pump (135,185,243). This efflux system seems to play a major role in fluoroquinolone resistance as the resistance level is highly reduced when it is inactivated even though multiple point mutations within the target genes of the type II topoisomerase enzymes are present (21).

6.3.3.4. Plasmid-mediated quinolone resistance

Even though it is still rare in Europe, plasmid mediated resistance to fluoroquinolones is increasingly found in *Salmonella* (178). The plasmid genes that confer resistance are *qnr*,

qepA, *oqxAB* and *aac(6')-Ib-cr*. The *qnr* genes are proteins that protect the DNA gyrase enzyme from the inhibition by quinolones. The genes *qepA* and *oqxAB* mediate efflux and *aac(6')-Ib-cr* is an altered aminoglycoside acetyltransferase an is capable of modifying certain fluoroquinolones, such as ciprofloxacin (220). Plasmid mediated quinolone resistance has only been reported to confer low level resistance. However it decreases the mutant prevention concentration and enhances the occurrence of chromosomal mutations (220).

7. The importance of gene transfer and mutation rates for virulence and resistance

Two main molecular mechanisms are responsible for the genetic differences among *Salmonella* strains: point mutations leading to the inactivation or modification of existing genes and lateral gene transfer leading to the acquisition of new genes mediated by bacteriophages, plasmids and transposons (84). This chapter describes briefly the genetic elements that drive diversity in *Salmonella* and mentions how mutation rates influence the evolution.

7.1. Prophages

Bactriophages exist in large quantities in the environment. An estimated amount of phage particles of 10^7 per g feces can be found within the mammalian gut comprising approximately 160 – 1200 viral genotypes (36,46). Approximately two thirds of the Gammaproteobacteria harbor intact bacteriophages or phage remnants (36). Presumably, all *Salmonella* carry a set of prophages which are thought to contribute significantly to the diversity of *Salmonella* (1). *Salmonella* phages can be divided into at least five groups which are presented in Table 4. Phage specificity is mediated by the ability of the phages to adhere to the host surface and by bacterial defense mechanisms, accomplished through nucleases and methylases (136). The phagetyping system that is used to subtype different *Salmonella* serovars is built on the diversity of phage specificities (Chapter 1). After attachment of the phage to the cell, the only component that enters the bacterium is the nucleic acid of the phage. Most *Salmonella* phages have linear double stranded DNA. The genomes of phages are typically composed of different units, including the tail and the head region and the regions for integration, excision and lysogeny (40).

Bacteriophages can undergo two lifecycles: a lytic and a lysogenic cycle (Figure 4). During the lytic cycle, the phages use the replication and translation machinery of the host bacterium to produce a big number of progeny. Multiplication is followed by the phage induced lysis of the bacteria cell (136). More than 100 new phage particles can be produced within 30 min after infection of a single host (36). During the lysogenic cycle the phages integrate into the bacterial chromosome by recombination. In the case of lambdoid phages, the DNA circularizes and recombines with the host DNA via site specific recombination mediated by a phage encoded integrase enzyme. In the following, the phage replicates together with the host bacterium. The expression of phage proteins leading to the excision of the phage and lysis of the host are repressed. These temperate bacteriophages can enter the lytic cycle which is elicited by environmental stress. Induction can for example occur when the host bacterium experiences DNA damage, such as during exposure to quinolones (36,154). While functional phages are integrated in the bacterial chromosome, they are named prophages (136). The genomes of all temperate prophages are mosaic in nature and variants of one kind of phage exist, indicating that recombination between phages occurs frequently.

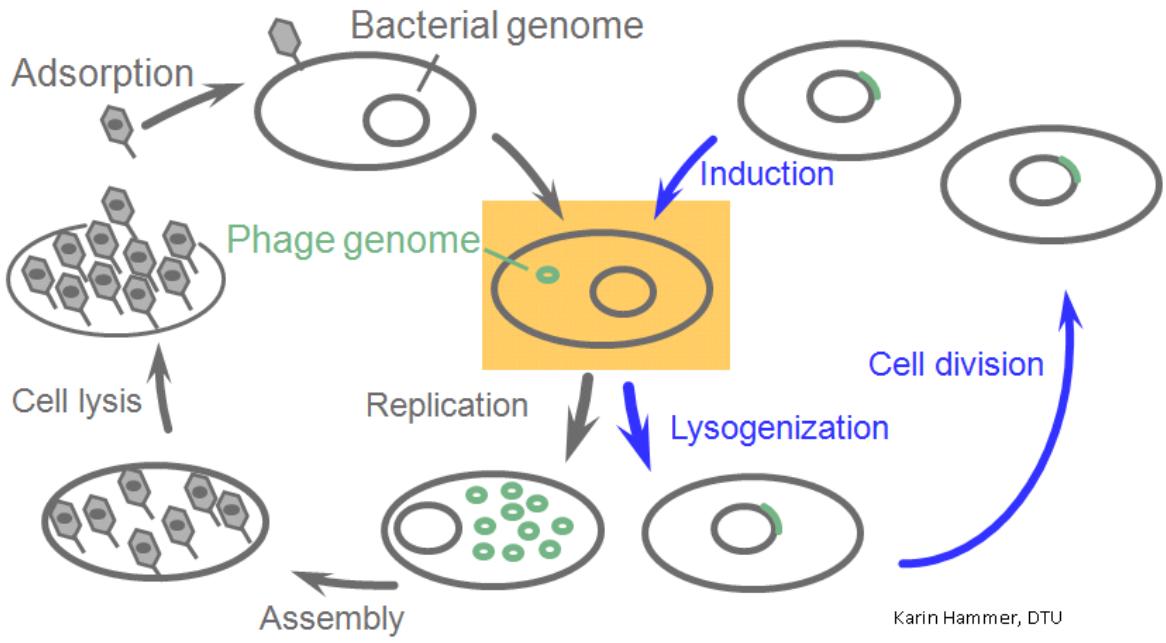


Figure 4: The lifecycles of bacteriophages (http://www.csm.bio.dtu.dk). The left side shows the lytic cycle and the right side the lysogenic cycle.

Phages can pack host DNA and transduce it to other bacteria (136). This can happen in two ways. General transducing phages can transfer virtually any gene of the host chromosome. The bacterial genome is degraded and parts of the host genome can accidentally be packed into the progeny heads of the phages. These particles are defective in their function as a virus. However, they can deliver the donor bacterial DNA into a recipient cell, where it is either degraded or recombined with the host DNA. The requirements for this type of transduction are that the packaging machinery of the phage is not too precise and that the host genome is not fully degraded. Both temperate and lytic phages can mediate generalized transduction (36). Specialized transducing phages can only transfer bacterial DNA adjacent to the phage integration site. This mechanism occurs only in temperate phages when the excision of the phage DNA upon induction is imprecise. The progeny phage might be functional or defective depending on the phage genes that have been replaced. The piece of host bacterial DNA can be subsequently transferred to a recipient and integrated into the chromosome along with the phage DNA (36). Both mechanisms can result in a change of the recipient's phenotype when the transduced DNA is expressed. A fully functional bacteriophage can also carry genes that are not required for phage replication and transfer. These genes also change the phenotype of the host bacterium, usually to its advantage and thus are also beneficial for the phage (37). This process is called lysogenic conversion (36). In principle, lysogenic conversion always occurs upon acquisition of a temperate bacteriophage because it generally confers immunity to the host bacterium to further infection by the same or similar bacteriophages (36). Lysogenic conversion is a key mechanism in the movement of virulence genes between bacterial strains. As described in Chapter 5 and 6, prophage elements (temperate phages and phage remnants) are important for *Salmonella* diversity particular with regard to the acquisition of virulence determinants.

7.2. Transposons

Transposons are gene elements that can change their relative position within the genome of a single bacterial cell. They can also jump on plasmids and thus be transferred to other cells. Transposons can be flanked by insertion sequences that encode the transposase enzymes (*insA* and *insB*). They can also be flanked by inverted repeats and express another kind of transposase gene (162). In *Salmonella*, antibiotic resistance genes are frequently located on transposons.

Transposons can carry integrons that accumulate and express gene cassettes. They encode the integrase Int1 which is similar to the phage integrases. In addition, they contain a promoter and an *attI* recombination site. Integrons capture and express gene cassettes that contain an *attC* site (79). They are themselves not mobile but in *Salmonella*, they have been found to lie on transposons, genomic islands or plasmids (7). Only class 1 and 2 integrons have been described in *Salmonella* (80). They are associated with multidrug resistance (79) because they can carry complex arrays of several resistance genes (162).

7.3. Plasmids

Plasmids are mobile elements that can replicate autonomously without integration into the host genome. They are transferred by conjugation to new hosts which can occur even across species boundaries (212). But not all bacteria have equal abilities to be efficient donor or recipient cells. Due to successive transfer events, plasmids have a mosaic genome structure.

Salmonella harbor different virulence and resistance plasmids. Most of the virulence factors of *Salmonella* are encoded chromosomally but certain serovars harbor serovar-specific virulence plasmids including Typhimurium, Enteritidis, Choleraesuis, Dublin and Gallinarum (195). They all share the conserved *spv* region mentioned in Chapter 5. The plasmids of *S.* Typhimurium (pSLT) and *S.* Enteritidis (pSEV) carry an additional virulence gene cluster, the *pef* (plasmid encoded fimbriae) operon contributing to adhesion to host intestinal cells (196). Virulence plasmids of *Salmonella* are large low copy number plasmids. Only the pSLT is self transmissible whereas the transfer region of the other virulence plasmids is absent or incomplete. Putative integrase and resolvase genes, insertion sequences and putative DNA repair genes can also be found on the *Salmonella* virulence plasmids (195). Resistance derivatives of these virulence plasmids have been identified in *S.* Enteritidis, *S.* Typhimurium and *S.* Cholerasuis (pSCV). And resistance plasmids carrying virulence determinant have been found in *S.* Enteritidis, *S.* Typhimurium and its monophasic variant *S.* 4,5,12:i:- (195).

7.4. Elevated mutation rates and mutator bacteria

The role of point mutations is well known for the development of antibiotic resistance mediated by alteration of target or transporter genes (48). Point mutations are less frequent in the evolution of virulence genes (153). However, it might be an important mechanism in the inactivation of antivirulence genes. During adaptation of a pathogen to its host, certain genes may become dispensable or even create a disadvantage. These genes are termed antivirulence

genes (155). In *S.* Typhi for example, several genes are inactivated by point mutations as they are not required in the specific human environment to which the bacterium has adapted.

Bacteria with elevated mutation frequencies are usually termed mutator or hypermutator strains (if the mutation rate is at a very high level) (161). A mutator phenotype can be regulated (transient) or permanently expressed (48). It has been suggest that mutator bacteria that exhibit constitutively high mutation rates and that usually account for a small part of a bacterial population are an important driver of the evolution in pathogenic bacteria like *Salmonella* (253). Hypermutator populations can have an increased mutation rate of around 10000 fold and are usually defective in their methyl-directed mismatch repair system or proof-reading function (153). These strains have the potential to develop rapidly resistance and virulence attributes but are considered to be less fit than the wildtype strains in the absence of the selective pressure. It has been shown that *E. coli* expressing increased mutation rates have an advantage *in vivo* as long as adaption is achieved. Thereafter, their fitness decreases compared to the wildtype because the hypermutator phenotype also results in the accumulation of deleterious mutations (92). Mutators probably survive in natural population because they are continuously enriched when bacteria encounter unfavorable conditions (71).

Transient mutators express only temporarily increased mutation rates. Certain environmental conditions can for example induce enhanced mutation rates. These conditions include the exposure to reactive oxygen species (ROS), to quinolone antibiotics or ionizing radiation and can be encountered in the food chain and/or during infection. They have the potential to induce DNA strand breaks, deamination, depurination and alteration of bases leading to a transversion event of the base pair (51). Quinolones elicit a replication arrest resulting in the exposure of single stranded DNA and DNA double strand breaks. Bacteria have developed different strategies to repair DNA. One of them is the SOS response which creates a transient mutator phenotype. During the SOS response, an error-prone DNA polymerase with a lower specificity that introduces mutations is used for replication of DNA (231).

Resistance to rifampicin is the result of at least 69 different point mutations in the *rpoB* gene and is frequently used to measure the mutation rates of bacteria (87). With this method, the number of mutants in a population can be estimated (not the number of single mutational events) (152). The spontaneous mutation rates in bacteria are generally around $10^{-10} – 10^{-9}$ per base pair per cell per generation (48). However, some genes are more subjected to mutations than others, depending for example on the size of the gene and number of duplications.

During growth under selective pressure, the mutation rates can be much higher and the genetic adaptation can thus be rapid (190). Also when bacteria do not grow or when they grow very slowly, for example during the course of infection, they can experience elevated mutation frequencies (152). This is because many stress conditions such as starvation have an enhancing effect on mutation frequencies (52). These findings suggest that mutation rates determined under standard laboratory conditions differ from those that occur in the natural environment.

8. Genetic and phenotypic diversity in *Salmonella*

8.1. The clonal nature of *Salmonella*

A clone is usually defined based on its similarity to different isolates determined with a certain typing method but only a few molecular methods are discriminatory enough. For instance MLVA typing is used to distinguish between different Salmonella clones (personal communications). The utility of this concept is the identification of strains that have a common ancestor and thus similar biological properties (26) and it is used to trace origins of infection, for example during outbreaks. The population structure of *Salmonella* is clonal with a comparatively low recombination rate between subspecies and a very high recombination rate within subspecies (137). New clonal *Salmonella* lineages can thus emerge by the replacement of one clone by another fitter clone or by the acquisition of phenotype-converting profitable genes (153). Despite their close genetic relatedness, the phenotype and epidemiology can vary substantially among *Salmonella* subtypes. In the following, this diversity will be described more in detail.

8.2. Phenotypic diversity

8.2.1. Differences of Salmonella isolates in their resistance to antibiotics

Some *Salmonella* subtypes are rarely antibiotic resistant compared to other subtypes. *S.* Typhimurium DT12 appears to acquire resistance only very slowly if at all (18) whereas only 6 % of the *S.* Typhimurium DT104 isolates that were collected from persons with clinical disease within a time period from 2003 to 2006, were fully susceptible (169). Interestingly, in 1998 DT12 was the far most prevalent phagetype isolated from pig farms but it declined

markedly and became replaced by DT120, DT170 and DT104. In contrast to DT12 which remained almost fully susceptible to antibiotics despite the increased use of antibiotics in the pig industry, DT104, DT170 and DT120 were to a large part multidrug resistant (18,71). It is not quite clear if one should conclude that the observed emergence of resistant *Salmonella* is the result of a change in the distribution of clones (70) or if it is the result of the acquisition of resistance genes and if some *Salmonella* subtypes have a higher ability to take up new genes than others.

In the US, the most common *Salmonella* serovars with a multidrug resistance phenotype are Typhimurium and Newport (45). Other clinical important serovars that are observed to be frequently associated with multidrug resistance are Heidelberg, Dublin, Agona, Enteritidis and Virchow (7). In a Danish study, Skov et al. reported that the proportion of multidrug resistant *Salmonella* for some serovars was much higher in humans than compared to the proportion in meat (213). These serovars were Typhimurium, Vichow and Hadar. For *S.* Saintpaul and *S.* Newport the opposite was the case. In this study, multidrug resistance was generally less a problem in *S.* Entertidis. In Denmark, 78 % of the human isolates attributed to outbreaks in 2010 were resistant to multiple antibiotics whereas 43% of the domestic sporadic human cases were multidrug resistant (18). This is reflected by data from the United States where a high number of human Salmonellosis outbreaks is caused by multidrug resistant strains (7). In Denmark and many other countries of the world, the most common multidrug resistance pattern is ACSSuT. It is conferred by the acquisition of SGI-1 as described in Chapter 6 and most often associated with *S.* Typhimurium DT104 (7,18). Other serovars than *S.* Typhimurium were SGI-1 has been identified are Agona, Albany, Cerro, Derby, Dusseldorf, Infantis, Kentucky, Kiambu, Meleagredis, Newport and Tallahassee (6,32,63,64,67,142,143). So far no SGI-1 could be found in other phagetypes of *S.* Typhimurium than DT104 (5,62,139).

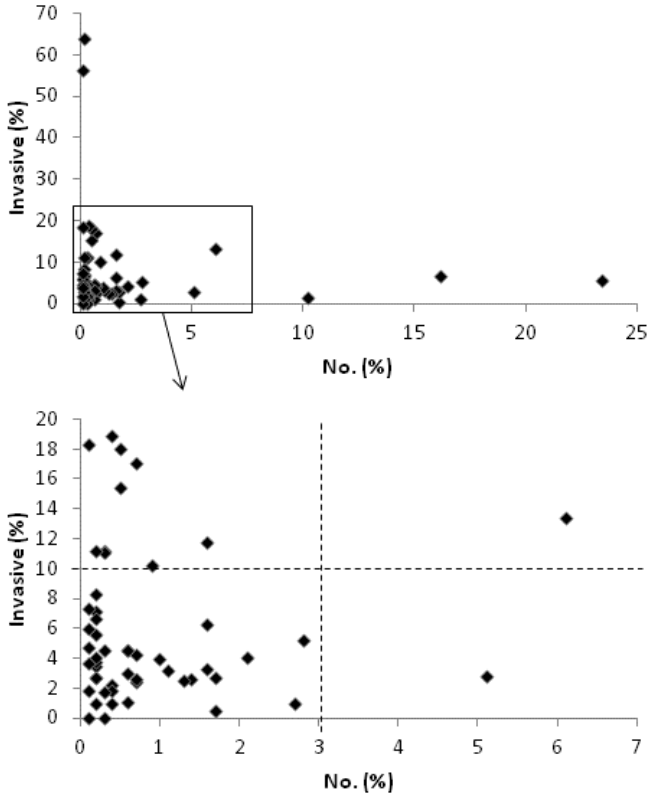
Skov et al. reported that nalidixic acid resistance was highest for *S.* Hadar, *S.* Newport, *S.* Kottbus and *S.* Virchow (213). In a large study from the United States, encompassing the years 1996 – 2007, it was shown that nalidixic acid resistance was the resistance pattern mostly associated with isolates from blood stream infection (54). The occurrence of high level fluoroquinolone resistant strains with an MIC above 2 µg/ml is still not very frequent among *Salmonella* (94). Higher MICs are more commonly observed in other pathogens, such as *E.*

coli (94). Overall, the antibiotic resistance pattern in *Salmonella* seems to be associated with the *Salmonella* serovar or phagetype and with the source of isolation.

8.2.2. Virulence and host preference

Salmonella cause disease in a wide range of animals, including warm blooded and cold-blooded animals, and humans. *Salmonella* serovars show a remarkable variety of host preferences. Some serovars are host specific, such as *S.* Typhi and *S.* Paratyphi for humans (53). These serovars are not able to cause disease in animals. Some serovars are host adapted, such as *S.* Dublin to cattle (215), and *S.* Cholerasuis to pigs which implicates that they are more often found to cause disease in these animals than in others. Some serovars are generalists and able to infect a broad host range, such as *S.* Typhimurium and *S.* Enteritidis (129).

Most human *Salmonella* infections are caused by one species, *Salmonella enterica* (127). Within this species, only a limited number of subtypes are responsible for the majority of salmonellosis cases. The disease outcome of salmonellosis can vary substantially ranging from mild gastroenteritis to severe extraintestinal infections with differences occurring even within strains of the same serovar (112,127,221). Even within the serovar *S.* Typhimurium, the disease outcome can vary tremendously. Some *Salmonella* serovars, such as Dublin and Cholerasuis are associated with severe invasive diseases (Figure 5). For other *Salmonella* subtypes, the disease outcome seems to depend on the antibiotic resistance pattern, with multidrug and quinolone resistant strains being more often associated with severe illness (Chapter 9) (38,111,113,121,140,151,229,233). By comparing surveillance data of non-typhoid *Salmonella* from food, food animals and humans, epidemiologists have calculated the ability of different serovars to cause disease and found remarkable differences (104,105,184,203,246). Variations in the number of human salmonellosis cases caused by different *Salmonella* subtypes also indicate different abilities to infect humans (105,246). These observations indicate that the disease burden of *Salmonella* is not only dependent on host and environmental factors but also on the pathogenic potential of the particular *Salmonella* strain. Figure 5 compares the prevalence of different *Salmonella* serovars in humans with the outcome of disease (127) as the pathogenic relevance depends mainly on these two properties: the degree of virulence and ability to be transmitted and thus to cause disease in a large amount of people (153).



Serotype	No. (%)	Invasive (%)
Typhimurium	23.4	5.7
Enteritidis	16.2	6.7
Newport	10.2	1.4
Heidelberg	6.1	13.4
Javiana	5.1	2.8
Oranienburg	1.6	11.8
Serotypes II/III/IV	0.9	10.2
Poona	0.7	17.1
Schwarzengrund	0.5	15.4
Panama	0.5	18
Sandiego	0.4	18.9
Brandenburg	0.3	11.2
Virchow	0.3	11.1
Dublin	0.2	64
Muenster	0.2	11.2
Urbana	0.1	18.3
Choleraesuis	0.1	56.4

Figure 5: The relative distribution of 58 *Salmonella* serovars from humans during 1996 – 2006 and their proportions showing an invasive disease outcome. The table presents only those serovars with a relative distribution in humans above 3 % or a relative proportion with an invasive disease outcome above 10 % (dashed lines); The data were taken from Jones et al., 2008 (127); Invasive means isolation of *Salmonella* from blood, cerebrospinal fluid, bone or joint fluid or a sterile site not further specified.

8.3. Genetic diversity

Salmonella is genetically closely related to *E. coli* but have an additional large number of virulence genes (108). The population structure of *Salmonella* is clonal and the chromosomal backbone therefore relatively conserved. However, the genome is interspersed with regions of variation including genomic islands and prophages (1). Microarray studies revealed that one-third of the *S. enterica* subspecies I pan-genome is variable (13). Prophage-like elements seem to constitute the major source of variation within *Salmonella* (24,256). Another genetic mechanism that leads to diversity among *Salmonella* is pseudogene formation. This is frequently observed in host specific serovars such as Typhi and Gallinarum. The largest *Salmonella* genome belongs to *S.* Typhi whereas the smallest belongs to *S.* Javania (103). *S.* Typhi is the serovar with the highest number of unique genes (genes that are absent in other

serovars of *Salmonella*). Among the non-typhoid *Salmonella*, *S.* Schwarzengrund, *S.* Tennesse and *S.* Newport carry the highest amount of unique genes whereas *S.* Typhimurium and *S.* Enteritidis contain the fewest number of unique genes (103).

The differently distributed gene islands in *Salmonella* are SGI-1 and SPI-3 described in Chapter 5 and 6. Apart from that, SPIs seem to be very well conserved in *Salmonella.* Nevertheless, genetic variations are often associated with pathogenicity. Surface structures, such as LPS, flagellin and fimbriae (77) underlie particular selective pressure as they are the main targets of the host immune defense. *Salmonella* serotyping after Kauffmann and White is based on the antigenic polymorphism of these structures (186).

8.4. Usage of DNA microarrays to study genetic diversity

Genomic microarrays usually contain DNA probes representing all identified open reading frames of one or more sequenced strains (205). Array-based comparative genomic hybridization (aCGH) is the comparison of these fully sequenced strains to unsequenced but related genomes. DNA microarrays have been used to study genetic diversity in *Salmonella*, frequently in relation to pathogenesis (1,13,24,174,187,188,192). The aCGH approaches are also used for typing and understanding of the evolution of *Salmonella* (313, 315, 320). DNA microarray based methods allow the comparison of a relatively large number of bacterial strains and to identify strain specific genes (1). With the rise of genome sequencing and the accumulation of whole chromosomal sequence data, microarray technology is constantly improving (1). In future, the coding sequence of virtually every gene might be known and this knowledge can be exploited in the design of microarrays (1). However, some limitations have to be considered when microarrays are used. False positive or negative results can be obtained with divergent genes. They might be displayed as absent when the sequence alteration prevents the hybridization to the probes on the microarray slide. They might eventually be shown as present when the gene alteration is not part of the probe fragment of the array (gene probes constitute gene parts and do not cover the whole nucleotide sequence of the gene). The amount of genes that can be detected is restricted to the probes present on the array. It is not possible to identify additional genes but only absent genes with respect to the strains that were used for constructing the array (1). Furthermore, it is important to consider that the detected genes are not necessarily expressed in a cell, which is the case for pseudogenes.

9. Evidence of increased fitness and virulence in multidrug and quinolone resistant *Salmonella*

Antibiotic resistance and virulence determinants share some common characteristics in terms of evolution and ecology (153). Both can be acquired by horizontal gene transfer and in both cases point mutations can play a role with respect to pseudogene formation and target gene alteration. Antibiotic resistance and virulence are both mechanisms that help to survive antibacterial defense systems. As antibiotics and host defense mechanisms, aim to prevent or to defend bacterial infections, there is evidence for a co-selection of antibiotic resistance and virulence determinants in the evolution of pathogenic bacteria (153).

9.1. Clinical and epidemiological data support the evidence of a linkage between virulence and resistance

The analysis of clinical data from hospitals has revealed that multidrug and quinolone resistant *Salmonella* are associated with an increased burden of illness in humans as patients infected with these bacteria have a higher risk for hospitalization, mortality and bacteremia as well as a longer duration of hospitalization than patients infected with fully susceptible *Salmonella* (111,140,151,229,233). SENTRY reported that quinolone resistant *Salmonella* were more frequently isolated from blood than from stool (42) suggesting that these isolates are more likely to be transported to systemic sites of the organism. Gorden observed an increase in the incidence of cases with bacteremia in children associated with the occurrence of multidrug resistant *Salmonella* in a hospital in Malawi over a period of seven years (98). The pentaresistant (ACSSuT) *S.* Typhimurium DT104 clone is a concrete example of a multidrug resistant clone with increased virulence. It was associated with increased severity of clinical illness as an unusual high percentage of patients were hospitalized in the UK and in the US, this strains was more often isolated from blood than non-ACSSuT stains (226). Epidemiological data from *Salmonella* surveillance programs could also show a linkage of virulence and resistance in *Salmonella*. In Denmark, the surveillance data show that antibiotic resistant *Salmonella* are more frequently isolated from human outbreaks than sensitive ones (18). In a study of Hald et al. (244), they used a mathematical model based on prevalence data of *Salmonella* from food, food animals and humans to estimate the relative ability of *Salmonella* serovars with different antimicrobial resistance pattern to survive the food

production chain and to cause human disease. Except for *S.* Saintpaul, this ability seemed to increase in antimicrobial resistant strains (244). Hence, multidrug and quinolone resistant *Salmonella* seem to be more successful in infecting humans and in causing a disease outcome with increased severity. In Section 9.1.3, particular mechanisms that may build a linkage between virulence and antibiotic resistance will be discussed.

9.1.2. The q-factor – The ability to survive the food chain and cause human infection

Based on prevalence data of *Salmonella* in food, food animals and humans from the Danish *Salmonella* Surveillance Program, Hald et al (104,105) developed a mathematical model to quantify the contribution of animal food sources to human salmonellosis cases. These calculations included a food source dependent factor accounting for the relative importance of a food source to act as a vehicle in foodborne salmonellosis. The model also included a subtype dependent factor, namely q-factor, accounting for the presumed differences of *Salmonella* strains in their overall ability to survive the food production chain and to cause disease in humans. The q-values were estimated for serovars with different antibiotic resistance pattern (Q, MR, R and S) but not for different phagetypes of *S.* Typhimurium as it was assumed that q for phagetypes with the same resistance level where equal. The q-factor for *S.* Enteritidis sensitive to antibiotics was set to 1 so that all q-values were estimated relatively to this one and to each other. As the overall ability to survive and to cause disease might be high in successful clone, the q-factor was used as a parameter to rank the isolates after their potential epidemic success. To separate successful from non-successful *Salmonella*, the cut off value q = 1 was chosen as it was the median of all q-factors provided. It is important to be aware that these factors are only indicators and that they are associated with a relatively big statistical uncertainty. Thus, Table 5 shows only if a given q-factor was above or below the cut-off value.

Table 5: The q-factors of selected *Salmonella* serovars of importance for this thesis.

		q-factor[2]	
Serovar	Resistance pattern[1]	Hald et al. , 2007 [3]	Data from Pires and Hald, 2009 (personal communication)
Typhimurium	Q	**> 1**	
	MR	**> 1**	
	R	*< 1*	
	S	*< 1*	
Virchow	Q	**> 1**	
	S	*< 1*	
Saintpaul	MR	*< 1*	
	S	**> 1**	
Newport	S	**> 1**	
Infantis			*< 1*
Derby			*< 1*

The combinations of serovars and resistance pattern estimated to have a high q-factor (>1) are indicated in bold and those with a low q-factor are indicated in italic (q<1); [1]Q: resistant to quinolones; MR: resistant to four or more antibiotic agents; R: resistant to less than four antibiotic agents; S: susceptible to the antimicrobials tested; [2]the q-factors range from 0.02 (*S*. Derby) to 3.4 (*S*. Typhimurium, Q; *S*. Saintpaul, S); [3]Reference: (104)

9.2. Mechanisms that connect resistance, fitness and virulence

Usually, the development of drug resistance and presumably also the acquisition of new virulence determinants are initially associated with a fitness disadvantage in the absence of the antibiotic (153,253). On one hand, this can be due to point mutations in the target genes that may alter the function of the gene and result in a fitness loss. On the other hand, resistance genes acquired by gene transfer might impose additional energy costs. However, compensatory second site mutations that restore or even increase fitness are observed to occur in *Salmonella* and other bacteria in the absence of the antibiotic (25,253). Since compensatory mutations can occur in several different genes they are more likely than the reversion of the resistance mutation (10). The compensatory evolution does however not take place in highly fluoroquinolone resistant *Salmonella*. They are observed to have a drastically decreased growth capability (95) and they are not able to compensate for their mutations (94). In contrast, intermediate fluoroquinolone resistant isolates have no or only little disadvantages compared to their wildtypes. Giraud could show that *in vivo* selected mutants show only intermediate resistance compared to *in vitro* selected mutants which showed high resistance to fluoroquinolones (94). Hence, the infrequent findings of highly fluorquinolone resistant

Salmonella might be because these bacteria are counterselected by competitive microorganisms in the absence of antibiotics.

Some stress conditions increase the rate of mutations (point mutations and horizontal gene transfer) (153). Point mutation rates are increased in the presence of quinolones and reactive oxygen species whereas horizontal gene transfer is enhanced *in vivo* and in the presence of antibiotics (153,159). These observations suggest that in presence of antibiotics and inside the host, the bacteria evolve more rapidly and a host treated with antibiotics may co-select for resistance and virulence determinants (153). But also in the environment, for instance in the food production chain, the bacteria might encounter antibiotics, in subinhibitory concentrations. They may also be exposed to other antibacterial agents such as disinfectants. Some of these substances have the potential to induce the rate of mutations. This can be detrimental for the bacteria but it can also lead to an increased adaptation of the bacterium to its environment. In the food chain, the bacteria may adapt to conditions that are very similar to host defense mechanisms (as mentioned in Chapter 4) increasing the pathogenic potential of the ingested strain (69,122,242,251).

Some resistance and virulence genes are located closely because they are encoded by the same replicon. Fluit et al. (79) observed that *Salmonella* isolates that harbored the virulence plasmid also carried resistance islands and/or integrons encoding resistance genes. In the course of evolution, these elements might have merged. And indeed, today virulence and resistance determinants are frequently found on the same plasmids in *Salmonella* (49,85,119,194,225). It has been reported that *Salmonella* virulence plasmids can capture genes conferring resistance to antibiotics. It has been shown that this is the result of a cointegration of virulence with resistance plasmids or of the acquisition of insertion sequences, transposons and integrons carrying resistance cassettes by virulence plasmids. Resistance plasmids that contain virulence genes, for example the *spv* region are also observed (195).

The correlation of resistance and virulence attributes in *Salmonella* could also be explained by defense mechanisms that are effective against both, antibiotics and the host immune system. And indeed, efflux pumps that are multidrug resistance determinants, such as *acrAB* of *S.* Typhimurium and *E. coli* have been reported to extrude bile salts conferring an advantage during the colonization of the gut (195,223). In addition, the multidrug resistance system SapA of *S.* Typhimurium has been reported to be involved in the active export of

antimicrobial peptides which constitute an important part of the host defense against *Salmonella* (Chapter 5) (175).

Finally, the exposure to antibiotics might induce virulence genes leading to an increased virulence of resistant strains when the antibiotic is present. This has been reported for *E. coli* in the presence of quinolones. These antibiotics induce the Shiga-toxin encoding bacteriophage leading to an increase in virulence (128).

Antibiotic resistance can also lead to a more severe disease outcome without being directly linked to virulence determinants. Treatment failure resulting from the choice of the antibiotic can for example result in a prolonged duration of the disease. However, salmonellosis is normally not immediately treated with antibiotics. Antibiotic treatment is only applied in the later stage of disease when complications have already arisen. This suggests that very virulent *Salmonella* strains are more likely to come in contact with an antibiotic and to develop resistance than those that cause only mild illness.

10. Success clones of *Salmonella* – Some examples of epidemiological importance

Characteristic for epidemics of *Salmonella* is that they are temporarily dominated by one clone. A period of increase is typically follows by a decline. The mechanisms of expansion and subsequent decline or replacement by another success clone are unknown (56). It can be the result of environmental and/or bacterial factors. Antibiotics are an important environmental factor that can select for clones that are resistant resulting in an overgrowth of theses clones and in a replacement of susceptible ones (153). Outbreaks or epidemics may also occur following the alteration of strains through mutations that enhance their fitness (including horizontal gene transfer). For instance, it has been suggested that the re-assortment of phage encoded fitness factors is largely responsible for the epidemic success of pathogenic clones (40). Overall, prevalent clones are often resistant to multiple antibiotics (245), may have additional virulence properties and an increased potential for transmission (195). But non-bacterial factors may also contribute to their success. Some of the recent *Salmonella* clones with epidemiological importance are presented below.

A well described example for a multidrug resistant successful *Salmonella* clone was *S.* Typhimurium DT104 with an ACSSuT (ampicillin, chloramphenicol, streptomycin, sulfonamide and tetracycline) resistant phenotype encoded by the SGI-1 (Chapter 6). The phage type DT104 is in fact quite heterogeneous representing different clones (137). This genetic island was probably taken up by one of these clones in the early 80s or a bit before and in the 90s, the multidrug resistant *S.* Typhimurium DT104 clone was of global importance because it became widely disseminated and was observed to be more virulent in humans (56,107,137,226). The global emergence of this DT104 clone was accompanied by a decline of other Typhimurium clones probably due to clonal competition as the total number of Typhimurium salmonellosis cases did not increase (107). Phagetypes that have been displaced were among others DT12 that were not resistant to antibiotics. The spread of this clone was in part the result of the increasing use of antibiotic but cannot fully be explained by that. The rapid dissemination of the multidrug resistant DT104 clone in the United Kingdom occurred after the pertinent antimicrobials were banned for use as growth promoters, indicating that the wide distribution of this clone is not primarily attributable to its resistance pattern (56). This suggests that other fitness or virulence attributes were associated with this clone.

Currently, the clonal dissemination of Typhimurium-like strains with the antigenic structure 1,4,5,12:i:- lacking the expression of phase 2 flagella is of concern (18,164). This serovar exhibits variable chromosomal mutations and deletions affecting the genes *hin*, *fljB* and *fljA* depending on the isolates (164). Interestingly, this serovar represents a considerable diversity of subtypes even within a given country (164). Also, the drug resistance pattern varies from pansusceptible to multidrug and quinolone resistant (164). The European isolates usually express a multidrug resistance phenotype whereas the American and Asian isolates appear to be rarely multidrug resistant (164). There is evidence in the literature that strains lacking phase 2 flagella are more virulent but the molecular mechanisms behind this increase in virulence are not clear (164). Overall, the clonal success of these *S.* Typhimurium isolates is not fully understood yet and remains to be investigated.

Systemic infections with non-typhoid *Salmonella* are rare in industrialized countries. There is an estimated annual incidence rate of 1.02 per 100,000 population (138). But in sub-Saharan Africa, cases of severe non-typhoid salmonellosis are now more numerous than cases of enteric fever caused by *S.* Typhi and *S.* Paratyphi. The recently discovered *S.* Typhimurium subtype ST313 has been reported to be uniquely associated with invasive non-typhoid

Salmonella infections in Africa. This clone is frequently multidrug resistant and is thought to be adapted to the human host (130). A distinct prophage repertoire has been found when the whole genome sequence of one member of this ST313 clone was analyzed. There was also evidence for genome degradation, including pseudogene formation and chromosomal deletion (130). Interestingly, these are also attributes of the human specific serovar *S.* Typhi.

11. Discussion

The epidemiology of *Salmonella* is characterized by a temporary dominance of certain non-typhoid *Salmonella* clones (123). It is of importance to determine genetic and phenotypic characteristics of these successful clones and to identify bacterial and environmental factors that influence their evolution in order to predict future epidemics and outbreaks in a very early stage of clonal dissemination. A comprehensive knowledge about these clones will also help to implement effective targeted intervention strategies. The overall objective of this study was to identify environmental as well as genetic and phenotypic bacterial factors that determine diversity in *Salmonella* and that are important for the emergence of successful clones. This was achieved by characterizing *Salmonella* isolates categorized as successful and non-successful using phenotypic and genetic screenings followed by more targeted small-scale analyses. This chapter of the thesis will discuss different aspects of the selection and categorization of the *Salmonella* isolates that constitute the basis of all experiments carried out (Section 14.1.). Thereafter (Section 14.2), the diversity of stress responses of *Salmonella* to conditions of the food chain and their importance for clonal success will be the subject of discussion (this comprises the work of Manuscript I and Manuscript II). In Section 14.3., the pathogenic potential of multidrug and quinolone resistant *Salmonella* will be discussed with focus on specific virulence genes and host pathogen interactions determined using eukaryotic cell culture models. This section includes the work presented in Manuscript II and III. The final Section (14.4.) of this chapter will discuss possible evolutionary mechanisms that may be involved in the emergence of successful multidrug and quinolone resistant *Salmonella* including the role of bacteriophages, spontaneous mutation rates and macrophages. This section deals with the results from Manuscript II and III.

11.1. The ranking of relevant *Salmonella* isolates based on their epidemic success

The bacterial isolates built the basis of the experimental work and their selection and categorization was thus an essential part of this thesis. The selection of isolates was based on prevalence data from the Danish *Salmonella* Surveillance Program encompassing the years 2007 - 2008 (14,15). The isolates originated from different sources with focus on the pork chain because pork is an important source of human salmonellosis in Denmark (17). An important criterion for the isolate selection was their relative distribution in pigs, pig carcasses

after slaughter and humans providing information about their decrease or increase during the transmission in the food chain. This information served as an indication of their ability to cause disease or to survive in the food or food preparation areas. The emphasis of this study was laid on *S.* Typhimurium as it is an important serovar in the pork chain and in humans and as different *S.* Typhimurium phagetypes vary substantially in their resistance to antibiotics and their epidemicity (18,71).

Using the comprehensive data of the Danish *Salmonella* Surveillance program, a source attribution model was implemented by Hald et al. (17) that estimated the contribution of different animal-food sources to human salmonellosis. This model included a subtype dependent factor q which accounted for the relative ability of *Salmonella* serovars with different antibiotic resistance pattern to survive the food production chain and to cause human disease (Chapter 9.1.2) (104). This factor has been used in this thesis to rank *Salmonella* isolates after their potential epidemic success. However, this categorization had some limitations. The q-factors did not distinguish between *S.* Typhimurium phagetypes and were not available for all possible serovar/antibiotic resistance combinations. Moreover, the values for q were associated with a relatively big statistical uncertainty. Interestingly, the q-factors increased in antibiotic resistant *Salmonella*, except for *S.* Saintpaul. Quinolone resistance was associated with the highest q-factors.

In accordance with the q-factors, antibiotic resistance in *Salmonella*, particularly multidrug and quinolone resistance, is associated with an increased human health burden with respect to both, an increased severity and incidence rate of illness (42,98,104,111,140,151,229,233). Hence, the antibiotic resistance pattern seems to be an indicator for the epidemic success and was used to group the isolates into quinolone resistant (Q; at least low level resistance to fluoroquinolones), multidrug resistant (MR; resistant to four or more antibiotic agents), resistant (R; resistant to less than four antibiotic agents) and sensitive (S; fully susceptible to the tested antibiotics). These definitions have been used in previous publications and where also used for the calculation of the q-factors of antibiotic resistant *Salmonella* (104,178). Today NARMS and DANMAP define multidrug resistance more correctly as resistant to at least three antimicrobial classes (18,45). An antimicrobial class includes those antibiotics that share the same mechanism of action. The classification of the isolates in this study was based on single antibiotic agents which might be a limitation of the study but in this manner the categorizations based on the resistance pattern and on the q-factors were comparable. When

q-factors of antibiotic resistant *Salmonella* are calculated in the future the categorization based on antimicrobial classes may be considered.

Overall, thanks to the availability of comprehensive epidemiological data, it was possible to rank a large number of field isolates after their potential epidemic importance.

11.2. *Salmonella* in the food chain – Varying tolerance to dehydration and limited diversity with respect to the freezing

11.2.1. Long-term freezing and thawing in minced meat is tolerated by all *Salmonella* without considerable cellular injury

A high prevalence of *Salmonella* in the foods and a successful spread in different food sectors may result in an increased number of human cases and in outbreaks. To achieve this, the bacteria must be able to survive within or on the food or in food preparation areas where they are exposed to different stresses. As freezing is a widely used preservation technique of fresh meat, the tolerance of *Salmonella* isolates to freezing of minced meat for up to one year was analyzed (Manuscript I). The impact of freezing and thawing on all *Salmonella* strains tested during the whole experimental period was very low. Within the meat, the bacteria cells seemed to be remarkably well protected from injury as *Salmonella* were capable of growing without reduction on selective media (35,242) and as the lag times in meat juice after thawing were relatively short (approx. 1.7 h). Growth was measured at room temperature (25 °C) and demonstrated that meat thawed on the kitchen table poses a risk for human health as *Salmonella* can readily multiply in the meat juice. Also other meat types than minced meat are associated with high survival rates of *Salmonella* (59,66). This indicates that meat requires specific attention with respect to food safety procedures.

The physiological state of the bacteria was shown to have an impact on the freezing tolerance. Cells in exponential growth phase were more susceptible to freezing but surprisingly, had shorter lag times after thawing. In the food chain, a fraction of the bacteria might appear in an exponential growth state posing a special risk if the meat is not thawed adequately. It is generally recommendable to thaw the meat in the fridge before transferring it to room temperature as slow thawing is more detrimental for the bacteria cells. Additionally, meat may be exposed to chilling before freezing to prevent that single bacterial cell are frozen in an exponential growth state.

As all *Salmonella* strains survived the freezing treatment equally well, it can be concluded that freezing and thawing in minced meat does neither contribute to diversity nor to clonal success of *Salmonella*. However, other stress conditions in the food chain might be better tolerated by successful *Salmonella* clones than by minor clones. A recent study could identify strain specific differences in survival of *Salmonella* on beef cuts subjected to cold storage (132). Interestingly, isolates that survived longer were *S.* Typhimurium DT104 and *S.* Enteritidis PT4 and PT8. These phagetypes belong to the most prevalent *Salmonella* phagetypes in food and humans in Denmark (16,17) and represent potential success clones. In contrast, *S.* Dublin, *S.* Derby and *S.* Infantis which are epidemically not so important showed shorter survival times during cold storage. The response of *Salmonella* to chilling requires further attention in order to determine if this is a treatment that favors successful clones.

11.2.2. Dehydration affects *Salmonella* subtypes differently but does not contribute to their clonal success

Water deprivation is experienced by microorganisms in a wide range of foods and on tools, machines and surfaces in food processing areas. The tolerance of different *Salmonella* isolates to drying under two different combinations of temperature and humidity simulating the condition in a typical Danish pig slaughterhouse and in private or public kitchens was investigated (Manuscript I). Due to the experimental setup of the dehydration experiment (bacteria resuspended in 0.9 % NaCl where subjected to air-drying), the bacteria experienced simultaneously an increase in osmotic pressure, nutrient limitations and temperatures below the optimum (10 °C or 25 °C). Interestingly, *S.* Infantis was most robust under these conditions which might explain why the relative distribution of this serovar on pig carcasses after slaughter is often observed to be higher than in pigs (14,15). The slaughter process decreases the overall number of carcass associated *Salmonella* due to scalding, singeing and chilling (232). But recontamination of the carcasses from insufficiently cleaned equipments and other sources accounts for approx. 30 % of all *Salmonella* on pig carcasses (232). *S.* Infantis could be able to persist and to recontaminate the carcasses in the slaughter line. In contrast, *S.* Derby and *S.* Typhimurium DT12 were impaired in surviving dehydration. They are both frequently isolates from pigs and pork but comparatively seldom cause human infection (14,15). They are eventually not able to persist in food processing areas, such as in kitchens which may result in fewer cross contaminations of ready-to-eat food. To conclude,

the tolerance to drying might play a minor role for some *Salmonella* to cause infection but it is not characteristic for success clones.

In line with these observations are the results from the array-based comparative genomic hybridizations (aCGH) (Manuscript II). They show that only two genes related to stress tolerance and survival dominated in successful MR and Q *Salmonella*. These genes encode an acid shock protein (Asr) and a putative copper homeostasis protein (CutC). Thus, it would be interesting to investigate copper and acid tolerance of success clones. Nevertheless, the overall results point out that the tolerance to stress conditions does not seem to be a major characteristic of epidemically successful *Salmonella* clones.

11.3. The pathogenic potential of quinolone and multidrug resistant *Salmonella*

11.3.1. Genes located on mobile elements seem to be involved in virulence of quinolone and multidrug resistant *Salmonella*

The impact of MR and Q *Salmonella* on human health has been the focus of several epidemiological studies that compared prevalence and clinical data of *Salmonella* with different antibiotic resistance patterns (20,104,111,151,229,233). These studies suggested that besides their selective advantage in presence of antibiotics, Q and MR *Salmonella* are more virulent and/or fitter than susceptible ones. An increased virulence potential could indirectly be the result of an improved ability to survive in the food chain. However, as discussed in Section 13.2., this evidence could not be proven experimentally. Consequently, factors that might determine the pathogenic potential of Q and MR isolates were studied with regard to virulence genes (Manuscript II) and host pathogen interactions in cell culture models (Manuscript III). Two genes with an experimentally proven function in virulence, *gipA* (STM2599) and *sopE* (STY4609) (246), and three genes that were suspected to be involved in virulence, *sugR* (STM3753), *recT* (SG1183) and *trhH* (AF261825_S012) were found to dominate in Q and MR *Salmonella*. All five genes are encoded by mobile elements, more precisely, by prophages (*sopE, gipA and recT*) and genomic islands (*trhH and sugR*). The genes *sugR, recT* and *trhH* were deleted and analyzed for their function.

RecT seemed to have a weak contribution in surviving the early defense mechanisms of macrophages. As this gene shows sequence similarities to a phage encoded recombination and repair protein in *E. coli* involved in double stranded break repair (134), *recT* might contribute to the reduction of lethal DNA damages in macrophages. Other *Salmonella* serovars that carry

recT are *S.* Gallinarum, *S.* Dublin, *S.* Partyphi and *S.* Typhimurium 14028s. These serovars are all highly virulent in their particular hosts (75,145,197,207) which supports the hypothesis that *recT* is of importance for virulence. RecT also seemed to have a week effect in preventing spontaneous mutations. However, its activity seemed to be enhanced by ciprofloxacin (CIP) or by the CIP induced DNA damages. It might be surprising that many Q and MR strains harbor an additional gene that is involved in reducing mutations because resistances towards certain antibiotics, such as quinolones, are conferred by gene mutations. The benefit of *recT* might be that it allows the bacteria to survive conditions that induce mutations such as the macrophage defense and quinolone exposure. The surviving bacteria may then profit from beneficial mutations generated under these conditions and for instance acquire quinolone resistance.

As expected, genes encoded by the *Salmonella* genomic island 1 (SGI-1) that is the cause of multidrug resistance in *S.* Typhimurium DT104 and in some non-Typhimurium serovars (6,32,63,64,67,142,143), were strongly associated with the Q and MR isolates. One of these genes *trhH* is annotated as a putative pilus assembly protein and was assumed to play a role either in bacterial cell or in host cell contact (31,33). But *trhH* did neither influence the adhesion to epithelial cells nor the conjugation frequencies with the Δ*trhH*::km^R mutant as the recipient. However, this gene may play a role in other cellular contacts that have not been investigated. For instance, it might be involved in the adhesion to M-cells which seem to be important in the infection process of *Salmonella* (109).

The gene *sugR* that was also found to dominate in Q and MR strains appeared to have a very weak positive effect on intraphagocytic growth. The gene *sugR* exhibits sequence homology to the ATP binding subunit ClpX of a Clp protease and might thus contribute to growth in macrophages because it degrades or repairs missfolded proteins during for example acid stress that is part of the phagocytic defense (224,240,247). The reason why the effect observed was only weak and not significant might be that the absence of this enzyme can be in part replaced by other chaperones (226).

The use of microarrays for the identification of virulence genes in a certain group of strains has some limitations. Only the genes that the test strains share with the strains that have been used to design the array can be identified. The microarray utilized in this study was based on the genomes and plasmids of seven distinct *Salmonella* serovars including virulent and/or antibiotic resistant strains, for example *S.* Typhi CT18 and *S.* Typhimurium DT104 (NCTC

13348). It was thus expectable that a wide range of *Salmonella* virulence genes was present on the array. Furthermore, the formation of pseudogenes that contributes to *Salmonella* pathogenesis cannot be detected with the help of microarrays which is a disadvantage when virulence attributes are studied. Thus, it might be a good alternative to use methods such as whole genome sequencing or optical genetic mapping that have the potential to identify novel strain specific virulence genes or small sequence alterations.

11.3.2. Diversity in epithelial cell invasion and macrophage survival does not contribute to the success of multidrug and quinolone resistant *Salmonella*

So far, the bacterial and host factors that contribute to the increased virulence potential of Q and MR *Salmonella* are unknown. Infections with these strains are often associated with bacteremia (42,98,233) suggesting that they must be capable to invade and disseminate systemically and macrophages are very likely to be a vehicle in this respect (109). The ability of MR and Q *Salmonella* to adhere and invade intestinal epithelial cells and to survive and replicate in macrophages in absence and presence of CIP was investigated (Manuscript III). The number of bacteria capable of adhering to epithelial cells was similar for all isolates investigated indicating that adherence is not a criterion that determines diversity in non-typhoid *Salmonella*. In contrast, considerable differences of the *Salmonella* strains to invade the intestinal epithelium were detected. These differences appeared to be strain specific and occurred within the serovars *S.* Typhimurium, *S.* Saintpaul and *S.* Derby. These results support other studies that have observed that the disease outcome of non-typhoid *Salmonella* in humans is divers and not only host but also strain dependent (77,112,127,249). Nonetheless, neither Q nor MR strains showed increased invasion of epithelial cells. The ability to survive and replicate in macrophages was similar for all test strains except for one MR *S.* Infantis isolate that exhibited reduced intracellular counts and a decreased cytoxic effect on the macrophages. This is in line with other studies that have shown that the survival within macrophages is strain dependent (172,235). In the time period from 2 h to 4 h post infection, the intracellular level of Q isolates increased slightly more compared to the other isolates. However, at 24 h post infection, this difference was not observed. Interestingly, in a study of Volf et al. (235) *S.* Typhimurium showed increased counts in porcine macrophages at 4 h post infection compared to *S.* Derby and *S.* Infantis but at 24 h post infection, this difference disappeared. As *S.* Typhimurium was thought to be more virulent in pigs than *S.*

Derby and *S.* Infantis, this might suggests that the 4 h sample in a cell culture model of macrophage survival is most indicative for the actual *in vivo* conditions.

The impact of CIP on survival in macrophages was analyzed using a Q *S.* Typhimurium DT104 isolate. The intracellular counts were not affected by the addition of CIP (in a concentration of 1 x and 20 x MIC) until 4 h post infection indicating that CIP reaches only subinhibitory concentrations in the *Salmonella* containing vacuole (SCV). After 24 h, the intracellular bacteria were reduced but not eliminated which was presumably the reason of macrophage apoptosis followed by the release and killing of the bacteria in the extracellular medium. If virulence was affected in *Salmonella* by the addition of CIP could not be concluded from these observations. In *Staphylococcus aureus* and Shiga toxin–producing *E. coli*, CIP has been shown to induce phage encoded virulence genes (96). As severe systemic diseases may be due to a dissemination of *Salmonella* within macrophages without a rapid induction of apoptosis (Section 5.3) (65,109), it is very important that antibiotics are effective against *Salmonella* within the SCV. Ofloxacin has been shown to be more effective in killing intraphagocytic *Salmonella* as it eliminated the intracellular bacteria in a concentration corresponding to 10 x MIC after 8 h post infection. (47).

To conclude, the mouse macrophage and human epithelial cell culture models used in this study were applicable to study pathogenic diversity in *Salmonella* but they did not seem to be suitable to investigate the virulence potential of successful MR and Q *Salmonella*. Other factors than the ability to adhere and invade epithelial cells and survive in macrophages may lead to the observed increased severity of illness associated with quinolone and multidrug resistance. Alternatively, the cell culture models were not sensitive enough as they were lacking many aspects of the host immune system. Cell culture models that are more similar to *in vivo* conditions, such as mucus-secreting cell lines (97) and PMN transepithelial migration assays (158) are available and should eventually be prioritized for virulence studies. The dissemination of *Salmonella* within dendritic cells to systemic sites of the host may be of importance in the pathogenesis of severe salmonellosis (228,239) and thus the persistence of Q and MR bacteria within these cells would be an interesting research subject. There have been several attempts to investigate virulence of the pentaresistant *S.* Typhimrium DT104 clone using cell cultures and mouse models (8,44) but these studies also failed to show an increased invasiveness of this clone. Thus, factors that are specifically related to humans and

that cannot be shown using mouse macrophages or mice may account for the increased virulence in MR and Q *Salmonella*.

11.4. Evolution of quinolone and multidrug resistant *Salmonella* - The role of phage repertoire, mutation rate and macrophage infection

11.4.1. Mobile elements, in particular prophage genes, are key elements in multidrug and quinolone resistant *Salmonella*

The dynamics in the genetic evolution of successful MR and Q *Salmonella* clones are still not well understood. As described in Manuscript II, most of the genes that dominated in Q and MR isolates were related to mobile genetic elements of which the largest fraction accounted for single genes or gene clusters of prophages. It may seem surprising that MR and Q resistance is associated with phagegenes as bacteriophages are not frequently reported to carry and transfer antibiotic resistance genes (190). However, *in vitro* studies have shown that bacteriophages are capable of transferring resistance determinants, including SGI-1 (51,204). Hence, it might be that the role of bacteriophages in the dissemination of antibiotic resistance is underestimated (36). Alternatively, phages might be indirectly linked to antibiotic resistance by the following mechanisms: 1.) Resistant *Salmonella* may have an improved ability to take up temperate bacteriophages or 2.) they might be more robust towards environmental conditions that induce phage lysis and are thus capable of maintaining their phages. These environmental conditions include certain antimicrobials, such as quinolones (57,236). Thus, in presence of quinolones, quinolone resistant *Salmonella* are protected from phage lysis and might instead take up new phages that are released by quinolone susceptible bacteria that are nearby.

The question has been ask if the take up of a single mobile element is enough to turn a minor clone successful (245). Of all the phage genes identified in the Q and MR *Salmonella*, the function of one particular gene (*recT*) was investigated. This gene was observed to have a weak effect on the phenotype (Section 14.3.1) which is supported by findings that individual prophages only slightly modify the fitness of a bacterium and confer only small advantages (40). Thus, the acquisition of one single phage would presumably not turn a bacterium into a successful clone. Instead, the quantity of converting prophages within a cell might lead to a fitter or more virulent phenotype. However, it is thought that the acquisition of a single mobile element (SGI-1) was responsible for the successful spread of *S.* Typhimurium DT104.

Along with resistance genes, this island encodes several genes with still unknown function that could be involved in virulence or fitness. But also the environmental conditions, the increased use of antibiotics, contributed to the fitness and spread of this clone.

To conclude, the evolution of successful clones is complex and several factors have to come together to enable the emergence of a success clone. Future surveillance and prevention strategies may have to concentrate on both, successful mobile elements as well as on successful bacterial strains and they have to consider the contribution of given environmental conditions to the emergence of outbreaks and epidemics. For the early assessment of the epidemic potential of a clone, molecular typing methods may be complemented with screenings for success marker genes, for example a combinations of certain resistance, virulence and phage genes.

11.4.2. The macrophage pathogen interaction might play role in the evolution of quinolone resistance and virulence

Quinolone resistant *Salmonella* are reported to exhibit the highest mortality rates in humans when compared to *Salmonella* that are susceptible or that harbor other resistances (111). The factors that drive the simultaneous evolution of virulence and resistance are not well understood. As quinolone resistance is often mediated by target gene alteration, it was hypothesized that mutation rates play an important role in the evolution of Q *Salmonella*. The results shown in Manuscript III point out that the mutation frequencies under static non-selective conditions are not elevated in CIP resistant *Salmonella*. This is contradicting with other studies that found a correlation of quinolone resistance and slightly elevated mutation rates (9,147). It might be a limitation of the study that the underlying resistance mechanisms of the isolates were unknown. However resistance to quinolones is most often caused by point mutations (113) and thus it is likely that the CIP resistance of most if not all the isolates was due to point mutations. A strength of the study is that the test group of CIP resistant isolates and the control group of CIP susceptible isolates were equal in size and in many aspects comparable (serovar, phagetype and resistance to other antibiotics than CIP). Hence, Q *Salmonella* might rather have transient elevated mutation rates, under conditions when an increased genetic adaptability is favorable such as during infection and in presence of antibiotics (93).

Macrophages are likely to be a vehicle of *Salmonella* during prolonged persistence and systemic spread. The macrophage defense activates the bacterial SOS response which may lead to increased mutation rates of intracellular bacteria that are able to survive the defense. The results discussed in Manuscript II show that the frequency of most CIP sensitive strains to acquire a reduced CIP susceptibly increases after macrophage infection. This frequency was substantially enhanced when CIP (in a concentration of 20 x MIC) was added suggesting that CIP penetrates the SCV. But overall, it seemed that the intravacuolar CIP concentration at that time is only subinhibitory for *Salmonella*. It is possible that the reduction of the CIP susceptibility predisposes the bacteria to become resistant to higher concentration when survival in macrophages is prolonged. Severe cases of salmonellosis are more likely to be treated with antibiotics and thus, virulent *Salmonella* may be more likely to come in contact with antibiotics. In this respect, macrophages may pose a risk for the acquisition of quinolone resistance. As a decrease in the CIP susceptibility occurs also in absence of the antibiotic, macrophages might generate resistant mutants already before antibiotic therapy is started.

When treating salmonellosis, an antibiotic in a concentration that efficiently eliminates the bacteria in the SCV should be given. The administration of agents that inhibit the bacterial SOS response might be considered in addition to quinolones (50,133). Alternatively, antibiotics to which bacteria cannot become resistant by a single mutation event might be prioritized.

12. Conclusions and recommendations

1) The tolerance to drying and rehydration was observed to be serovar and phagetype dependent whereas *Salmonella* exhibited a general robustness to freezing and thawing in minced meat. These observations underline that meat requires specific attention with respect to food safety procedures. However, interventions with the aim of reducing the spread of epidemically successful clones should presumably target other processes than freezing and dehydration.
2) Prophage acquisition seems to be of importance in the successful evolution of multidrug and quinolone resistant *Salmonella* clones. Prophage genes may be used in the future in combination with other methods to assess the potential epidemic importance of a clone in

an early stage of dissemination. However, the global spread of clones might also in the future be difficult to predict and prevent as they are not restricted to one country. Such a clone might emerge in developing countries which do not have a routine surveillance system. Thus, it is important to develop simple methods for the detection of these clones (that are easy to use and not too costly) that can be standardized and used in all national and international surveillance programs. In the future, whole genome sequencing may provide a tool that allows a fast detection of success clones but before this can be realized, the costs of this method must decrease and the data analyses must become more user-friendly.

3) Finally, the macrophage pathogen interactions seem to be important in the evolution of successful quinolone resistant *Salmonella*. The results suggest that *Salmonella* that are able to persist for prolonged periods within macrophages have a higher chance of becoming resistant to fluoroquinolones, particularly when fluoroquinolones are administered. The increased occurrence of invasive non-typhoid *Salmonella* infections highlights the importance of an antibiotic therapy with agents that are effective against pathogens within the *Salmonella* containing vacuole and to which bacteria cannot become resistant by a single mutation event.

Overall, the tolerance to drying and rehydration and the ability to invade intestinal epithelial cells as well as to survive in macrophages are important factors in the diversity of non-typhoid *Salmonella* but do not seem to contribute to clonal success. Success might be the result of a combination of factors and might include favorable environmental condition and a clonal group of bacteria with a selective advantage under the given conditions. The selective advantage might be conferred by the acquisition of prophage genes, genomic islands and resistance genes.

13. Perspectives

Successful clones of *Salmonella* should be prioritized for whole genome sequencing (245) in order to be used in comparative genomic analyses. This will allow to identify genetic markers that could be used for an early detection of clones that have the potential to spread and to cause disease. Along with sequence analyses, phenotypes and gene expression profiles of

success clones might have to be studied in order to get a comprehensive understanding of the relations of resistance, virulence and epidemic success. This understanding will be useful for the development of targeted intervention strategies.

In addition to the investigation of pathogen specific factors, environmental conditions along the food chain or within the host that have an impact on clonal success should be identified. This may include environments were phage transfer is enhanced or where mutation rates are increased. This might help to find hotspots of gene acquisition that pose a risk for the emergence of successful clones.

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Manuscript I

Survival and growth of epidemically successful and non-successful *Salmonella enterica* clones after freezing and dehydration

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Abstract

The spread of epidemically successful non-typhoidal *Salmonella* clones has been suggested as the most important cause of salmonellosis in industrialized countries. Factors leading to the emergence of success clones are largely unknown but their ability to survive and grow after physical stress may contribute. Epidemiological studies developed a mathematical model allowing the estimation of a factor (q) accounting for the relative ability of *Salmonella* serovars with different antimicrobial resistances to survive the food chain and cause human disease. Using this q-factor, 26 *Salmonella* isolates were characterized as successful or non-successful. We studied their survival and growth after freezing for up to 336 days in minced meat using stationary and exponential phase cells. We also investigated survival and growth after dehydration at 10 ˚C and 82 % relative humidity (RH) as well as 25 °C and 49 % RH for 112 days. Stationary phase cells declined less than one log unit during one year of freezing and initiated growth with an average lag phase of 1.7 h. Exponential phase cells showed reduced survival, but tended to have shorter lag phases. High humidity/low temperature were less harmful for *Salmonella* than low humidity/high temperature whereas the tolerance was highest for Infantis and one Typhimurium U292 isolate and lowest for Derby and one Typhimurium DT170 isolate. This study shows that dehydration, in contrast to freezing, was differently tolerated by the *Salmonella* strains. But the tolerance to freezing and dehydration does not appear to contribute to the emergence of success clones of *Salmonella*.

Introduction

Salmonellosis is one of the most reported zoonosis in the EU (5) and causes foodborne diarrheal disease worldwide (2). Denmark, known to have successfully achieved a reduction in human salmonellosis through national control programs (41), had a very high number of laboratory confirmed cases in 2008 (3656 cases) and 2009 (2129 cases) (4) due to several large outbreaks caused by *Salmonella* Typhimurium. In order to prevent outbreaks, it is important to continuously develop and implement effective intervention strategies.

A rapid spread of a limited number of epidemically successful *Salmonella* clones in different sectors of food animal production has been suggested as the most important cause of disease in industrialized countries (37,41). In other words, *Salmonella* subtypes differ in their importance for human health. This has been reflected in epidemiological studies, which used a mathematical model allowing the estimation of a subtype dependent factor (q) (22,23). This factor illustrates the relative ability of *Salmonella* serovars with different antimicrobial resistance pattern to survive the food production chain and to cause human disease. It varies among different *Salmonella* serovars and seems to increase in antimicrobial resistant strains (22,23) and can thus be used to rank *Salmonella* serovars according to their epidemic success. Circumstances leading to the emergence of success clones are largely unknown. The success of certain *Salmonella* subtypes seems to be primarily due to an increased virulence potential (27). Epidemiologists have demonstrated that antimicrobial resistant bacteria can cause infections with increased fatality rates in humans (24,29,40). But an association between virulence and resistance (13) or virulence and major worldwide clones (33) could not be proven by *in vivo* or *in vitro* studies. Therefore, other factors than virulence, such as the ability to survive the food chain may contribute to the epidemic success of specific clones. However, little is known about variations in tolerance of different clones to environmental stress in the food chain.

Raw meat, especially pork, is a common source of human salmonellosis (4,41). Bacteria entering the fresh meat line are commonly exposed to various stress conditions such as freezing and dehydration. Freezing is a widely used preservation method for controlling bacterial growth in fresh meat. Dehydration may be experienced when bacteria attach to surfaces of processing equipment for prolonged periods, and a reduction of the water activity in food is also a long established preservation method. Drying as well as freezing prevent

microbial growth but do not necessarily eliminate microorganisms, which may allow the bacteria to regain their growth capacity and their ability to cause disease when the environmental conditions become favourable (15,38).

Studies on bacterial survival during environmental stress are often carried out with stationary phase cells, possibly because they are more stress tolerant (3,8,14), and this will lead to more conservative estimates on growth and survival. The growth phase of pathogens contaminating the process line in the food industry, is however uncertain as bacteria in faeces, in waste material of the slaughter line and in food products may appear in mixed growth phases depending on temperature and nutrients available.

The aim of this study was to investigate the responses of *Salmonella* serovars and phagetypes with different antimicrobial resistance patterns to physical stress likely to occur in the fresh meat chain and to evaluate if the findings correlate with the level of epidemic success according to the ranking by the q-factor. We analyzed the response to freezing in minced meat using exponential and stationary phase cells and to dehydration at two different combinations of temperature and humidity.

Materials and Methods

Bacterial strains and culture conditions. We included 26 isolates of *Salmonella enterica* spp. *enterica*, representing different sero- and phagetypes originating from sporadic or outbreak cases from humans as well as from animal and food sources (Table 1). Most of the isolates were different phagetypes of *S*. Typhimurium as it is the dominant serovar in pork in Denmark (2). The isolates were characterized by their q-factor as successful ($q \geq 1$) or non-successful ($q < 1$). These factors were obtained from Hald et al. (22,23) and from the research group Epidemiology and Risk Modelling, Danish National Food Institute (unpublished data). Based on data from the Danish *Salmonell*a Surveillance Program, they used a mathematical model to calculate a factor (q) accounting for the presumed differences of *Salmonella* strains in their overall ability to survive the food production chain and cause disease in humans. To separate successful from non-successful *Salmonella*, the cut off value $q = 1$ was chosen as it was the median of all q-factors provided.

Overnight stationary phase cultures were prepared by inoculating a colony from calf blood agar into 20 ml Brain Heart Infusion (BHI) broth in a shaking incubator (at 37 °C, for approx.

18 h). To obtain inocula, the cells were collected by centrifugation (6000 g, 5 min), washed and resuspended in 0.9 % NaCl (w/v).

Cell counts were determined by plating five 10 µl droplets from relevant 10-fold dilutions prepared in 0.9 % NaCl (w/v) on Xylose Lysine Desoxycholate (XLD) agar (Oxoid) or BHI agar followed by overnight incubation at 37 °C. Two technical replicates were performed and the experiments were repeated twice if not otherwise stated.

Survival and growth after long-term freezing in minced meat. The optical density (OD_{600}) of the inocula was adjusted to 0.5 corresponding to a cell concentration of 10^8 CFU/ml and further diluted to obtain a second inoculum of 10^5 CFU/ml.

Minced pork was purchased in a local supermarket within one day prior to the experiment and stored at 5 °C. The fat content of approx. 10 % was determined as previously described (12). The water content of 70 % was measured by a gravimetric method (6). To obtain an equal distribution of the bacteria in the meat, smaller meat portions (100 g) were first mixed with 4 ml of the prepared inocula containing 10^5 or 10^8 CFU/ml of *Salmonella*. Then, 300 g of meat were added and blended giving a final *Salmonella* concentration of 10^3 CFU/g and 10^6 CFU/g respectively. As a negative control, meat portions inoculated with sterile 0.9 % NaCl (v/w) were prepared. The inoculated meat was finally divided into portions of 25 g, tightly packed into sterile plastic bags and frozen at -18 °C for 336 days. This time period was chosen as maximum storages times of 4 to 12 months in a freezer are recommended for minced meat (US Department of Agriculture. Freezing and Food Safety. Available at http://www.fsis.usda.gov/factsheets/focus_on_freezing/index.asp).

Duplicates of the 25-g meat portions were used to determine the number of *Salmonella* before freezing (at day 0) to confirm the initial cell number and after freezing for 1, 4, 7, 14, 28, 57, 84, 169, 259 and 336 days. For this purpose, the meat portions were allowed to thaw for 3 h at 5 °C, then transferred to 225 ml 0.9 % NaCl (w/v) and stomached (Colworth Stomacher 400) for 30 s to release the bacteria into the water phase. Bacterial counts on XLD agar were done as described above. In order to confirm the absence of *Salmonella* in the uninoculated minced meat, two negative controls were analyzed at each sampling day.

After approximately 180 days of freezing, 2 replicates of the 25-g samples were thawed at 25 °C for 3 h and 2 ml of meat juice were collected. The meat juice was subsequently incubated at 25 °C for 8 h. To assess growth, 100 µl meat juice where used for direct colony counts on XLD agar after 0, 1, 2, 2.5, 3, 3.5, 4, 5, 6 and 8 h. The temperature of 25 °C and the

time period of 8 h were chosen to imitate the behaviour of the consumers where meat might be thawed at room temperature during a working day (8-11 h).

Survival and growth of stationary and exponential phase cells after short-term freezing in minced meat. In a second freezing experiment, overnight stationary phase cultures of *S.* Typhimurium DT12 and U292 were prepared as described above using Luria Bertani (LB) broth. They were adjusted to 10^7 CFU/ml and used as inocula for stationary phase cells. For the preparation of the inocula of exponential phase cells, the overnight cultures were diluted to 10^4 CFU/ml and incubated at 37 °C for additional 4.5 h until the cell concentration had reached 10^7 CFU/ml.

The meat inoculation was carried out as for the long-term study with some modifications. First, 350 g of minced beef (fat content approximately 15 %) were blended with 13.5 ml of the inocula containing cells in stationary or exponential growth phase. Then, further 1000 g of meat were mixed into it leading to a final *Salmonella* concentration of 10^5 CFU/g. The meat was divided into portions of 60 g and frozen at -20 °C.

Growth curves in meat juice and the *Salmonella* concentration of the meat portions were determined before freezing (day 0) and after 1, 2, 8, 20, 34 and 49 days. For this purpose, the meat portions (60 g) were thawed over night at 5 °C. Then, 10 g of meat were removed, transferred to 30 ml 0.9 % NaCl (v/w) and stomached for 2 min. Direct colony counts on XLD agar were made as described above.

The remaining 50 g of meat sample were transferred to 25 °C for 1 to 2 h to collect meat juice which was subsequently incubated at 25˚C for 27 h. In this experiment, 50 g of meat were chosen as smaller volumes were not sufficient to obtain enough meat juice. The *Salmonella* concentration was measured after 0, 2, 4, 6, 8, 10, 27 h by plating 100 µl of meat juice on XLD agar.

Survival and growth after dehydration under two temperature/humidity conditions. Two days prior to the experiment, an air-tight chamber (Nalgene Labware) was moved to 10 ˚C and adjusted to 82 % relative humidity (RH) by placing 300 ml of a saturated NaCl solution on the bottom of the chamber. By a similar procedure, a second chamber was placed at 25 °C and adjusted to 49 % RH using a K_2CO_3 (Merck) solution. The conditions of the first chamber are representative for a typical cutting and deboning area in a Danish pig slaughterhouse, where the temperature is around 10-12 °C and the humidity very high

(personal communications). The conditions in the second chamber are likely to happen during summer in private kitchens.

The inocula were prepared as described above, adjusted to a concentration of 10^7 CFU/ml and 50 µl were transferred in triplicate into microtiter wells resulting in 5 x 10^5 CFU/well. Sterile 0.9 % NaCl (v/w) served as negative controls. After short and gentle shaking, cell counts and growth of *Salmonella* were established (day 0), and the plates were transferred to the respective chambers. Cell counts and growth were determined at specific time points from day 1 to 112 (Table 2), by rehydrating the bacteria with BHI. This was done by adding 400 µl to wells that were completely dried out and 350 µl to wells that still contained liquid. Vigorous pipetting ensured resuspension of the cells. From two wells, 100 µl were used to perform direct colony counts on BHI agar. The remaining 300 µl in the wells were used to establish growth curves in a Bioscreen C reader (Thermo Labsystems) for up to 2 days at 25 °C without shaking (except for a short shaking before each OD_{600nm} measurement every 20 min).

Calculation of lag phases and maximum growth rates. The log transformed and averaged data of the growth curves were fitted to a model of Baranyi and Roberts (9) using the web based application DMFit available at http://www.combase.cc/toolbox.html.

Statistical analysis. Two replicate experiments were performed, except for the growth analysis in the Bioscreen, which was carried out in triplicate. In case of colony counts, mean values of each data point were based on 4 to 12 values dependent on the number of dilutions plated. Colony counts were log transformed to calculate standard deviations. Statistical significances of differences in lag phases, growth rates and log reductions were calculated with one-way analysis of variance (ANOVA) followed by Turkey's multiple comparison test using GraphPad Prism (GraphPad Software).

Results

Survival of *Salmonella* during freezing in minced meat. For both inoculation levels (10^6 and 10^3 CFU/g), viable counts of the different *Salmonella* strains in stationary phase declined less than 1 log unit in the meat during 336 days of freezing (Figure A3 in the Appendix). When comparing the survival of *S.* Typhimurium DT12 and U292 during freezing for 49

days, the level of viable stationary phase cells also remained unchanged during the whole freezing period, whereas significant reductions of exponential cells were observed: 2.04 to 1.60 log for DT12 after 20 and 49 days .and 1.34 log for U292 at 49 days ($P < 0.05$) (Figure 1). The uninoculated controls were all found negative for *Salmonella.*

Growth of *Salmonella* in meat juice after freezing in minced meat. After thawing the meat, we established growth curves in meat juice at 25 °C. Neither the lengths of lag phase nor the growth rates differed significantly between strains in stationary phase ($P > 0.05$) independent on the start concentrations (Figure 2 and 3). When comparing growth of *S.* Typhimurium DT12 and U292 in meat juice, the average lag phases of both strains in exponential phase (3.51 h for DT12 and 1.83 h for U292) tended to be shorter compared to the stationary phase cells (5.50 h for DT12 and 5.04 h for U292). This difference in lag phase was only found statistically significant for U292 at day 5 and for DT12 at day 20 ($P < 0.05$). The growth curves of day 49 could not be fitted to the model of Baranyi and Roberts (9) and lag phases could not be established. Therefore, complete growth curves from the beginning (day 2) and the end (day 49) of the experiment are shown in Figure 3. The maximum growth rates for DT12 and U292 (exponential and stationary) ranged from 0.26 to 0.64 CFU/ml/h and were not significantly different ($P > 0.05$).

Survival of *Salmonella* during dehydration at two temperature/humidity conditions. After 7 days of storage in the low humidity chamber (49 % RH [SD 5.252], 25 °C [SD 0.420]), the inocula of all *Salmonella* strains were completely dried out. Until day 7, the viable cell counts of all strains remained almost the same, thereafter, the concentration of the bacteria decreased (Figure 4). Between day 14 and 28, the concentration of all *S.* Derby isolates and *S.* Typhimurium DT170 strain no. 32 were below the detection limit of 2 log CFU/ml. After 56 days only *S.* Typhimurium U292 strain no. 68, and the *S.* Infantis strains no. 127 and 10 could be detected with 2.0, 2.7, and 2.8 log CFU/ml, respectively.

The liquid of the inocula in the high humidity chamber (82 % RH [SD 2.158], 10 °C [SD 0.131]) never evaporated completely. At day 1, the viable counts of nearly all isolates increased compared to the initial counts of day 0. A decrease in cell counts was observed after 28 days but at the end of the sampling period (112 days), all isolates were still detectable. After 112 days, the highest reductions of more than 3 log units were observed for *S.* Typhimurium DT104 (no. 2) and DT12 (no. 16), *S.* Derby (no. 13) and *S.* Infantis (no. 127). However, the high decrease of the latter isolate occurred only after 84 days of exposure to high humidity. Before that, this strain decreased about 0.7 log units and belonged to the

group of strains with the lowest log reduction. A low log reduction, defined as a 1.0 log units or less during 112 days of dehydration was also observed for *S.* Typhimurium U292 (no. 68) and *S.* Infantis (no. 10). The controls were negative for *Salmonella*, during the whole sampling period (112 days).

Growth of *Salmonella* after dehydration at two temperature/humidity conditions. Lag phases and growth rates of the different *Salmonella* isolates were investigated. Figure 5 shows the length of lag phase at day 0 and after 1, 21 and 56 days of exposure to the two temperature/humidity conditions. To illustrate how the individual lag phases were scattered, the average lag phases at day 21 and 56 (dashed lines) are also shown. Lag phases after 1 day of dehydration were not much different from the untreated samples of day 0. Thereafter, the lengths and variations of lag phase increased constantly for all isolates. This observation was particularly characteristic for the low humidity condition. After 56 days at low humidity, no growth of *S.* Typhimurium DT104 (no. 02), DT12 (no. 16 and 23), *S.* Newport (no. 44), *S.* Virchow (no. 42) and *S.* Derby (no. 13 and 26) was detected. Under high humidity conditions, all strains were capable of growing after 56 days of dehydration. Under both conditions, *S.* Derby (strains no. 13 and 15), *S.* Typhimurium DT12 (no. 16), DT104 (no. 02), DT170 (no. 32) and U288 (no. 07) had a longer lag phase than the average after 21 and 56 day of dehydration, whereas the lag phases of *S.* Typhimurium U292 (no. 68), DT135 (no. 05), DT104 (no. 01 and 17) and *S.* Infantis (no. 127) were clearly shorter. The maximum growth rates were similar for all isolates at all sampling days under both humidity conditions (ranging from 0.11 to 0.22 h^{-1}) except for *S.* Derby (no. 13) which had generally a significantly ($P < 0.05$) lower maximum growth rate than most of the other strains.

Discussion

Salmonella serovars and subtypes hereof differ in their food safety importance (22,23,35,36) and diversity in robustness to food handling procedures might contribute to this divergence. Raw meat is a common source of human salmonellosis (41) and in Denmark, pork products are among the main sources in this respect (4). We hypothesised that specific handling procedures of the fresh meat chain, such as freezing, thawing, and dehydration, may affect *Salmonella* subtypes differently and may contribute to the epidemic success among particular *Salmonella* clones. Based on epidemiological data, *Salmonella* sero- and phagetypes with

different antimicrobial resistance pattern were characterized as successful or non-successful in their overall ability to survive the food chain and to cause human disease (22,23). This approach is new and differs from other comparative studies on *Salmonella* (17,25,26,36). We also included outbreak strains in this study, for example from the large outbreaks that Denmark experienced in 2008 (19). Since *Salmonella* has a relatively high dose response (7), a particular ability to grow in the food chain may be an advantage in relation to causing outbreaks. We compared the survival and growth capability of the *Salmonella* isolates subjected 1.) to freezing and thawing after inoculation of both, stationary and exponential phase cells into minced meat and 2.) to two temperature/humidity combinations and we examined if the responses correlate with the strain classification into successful and non-successful.

With an experimental setup imitating natural conditions, we showed that all *Salmonella* strains in stationary phase, independent of their characterization into successful or non-successful, survived at least one year in frozen minced pork without a major decline. The reduction of exponential phase cells of *S.* Typhimurium U292 and DT12 of more than 1 log units during 49 days indicated that exponential phase cells were more affected by freezing as reported earlier (8). The result obtained with stationary phase cells is in accordance with other studies on minced meat although these studies were of a shorter duration (10,20,21). Ice crystal formation and the increase in solute concentration are the principal factors causing freezing injury (28) and they are influenced by the freezing rate and the type of food. Meat may have a protective effect on the cells by reducing the freezing injury. It has been suggested that this could be due to an insulating effect of fat already at 10 % (16) or to the meat matrix that binds free water and thereby reduces the ice formation (15). In our study we used minced meat of approx. 10 and 15 % of fat and our survival rates could thus be influenced by this. The protective effect does not seem to be unique to minced meat because high survival of *Salmonella* has also been reported in other meat types (15,16).

The length of the lag phase of a cell after stress exposure is an indicator of the time needed to return to conditions that allow replication (15,16,42) and is therefore a useful parameter to assess the level of sublethal injury experienced during food processing. In the long-term freezing study, we demonstrated that after approx. 6 months of freezing at -18 °C, stationary phase cells of *Salmonella* were capable of growing in meat juice at 25 °C with an average lag phase of 1.93 h for the low (10^3 CFU/g) and 1.49 h for the high (10^6 CFU/g) inoculum. In the short-term freezing study, the lag phases of stationary phase cells were longer (Figure 3). The

explanation for this is not clear but it could be due to the different thawing temperatures of 5 °C used in the short-term study and 25 °C used in the long-term study. Slow thawing allows small intracellular ice crystals to grow and coalesce before melting, which is more detrimental than a fast thawing rate where the ice simply melts (34). For *S.* Typhinurium U292 and DT12, inoculated in exponential phase, we observed that they generally tended to have shorter lag phases after thawing than compared to stationary phase cells (Figure 3). This may be because their metabolic state was arrested in growth mode by freezing. Such a temporary arrest may be special to freezing, as other stressors may affect exponential cells differently. In a study of Mellfont et al. from 2004, (31) exponential cells of *S.* Typhimurium were subjected to osmotic stress and showed longer lag phases than stationary cells. They assumed that cells in exponential phase become more injured and that it takes a longer time for them to resolve the damage. Overall, the effect of freezing and thawing did not vary significantly between the selected *Salmonella* strains and does therefore not seem to contribute to the clonal success of *Salmonella* with respect to survival and growth capacity (length of lag phase and growth rate).

In the meat chain *Salmonella* may be attached to surfaces of process equipment for prolonged periods e.g. if cleaning and disinfection are inadequate. Thus, the bacteria will potentially experience environments of varying temperatures and humidities which could lead to dehydration of the cells. We investigated survival and growth of our selected *Salmonella* strains after prolonged exposure to high humidity (82 % RH) and low temperature (10 °C) and compared it to low humidity (49 % RH) and high temperature (25 °C). We generally observed that *Salmonella* survived high humidity and low temperature longer than lower humidity and higher temperature. The cell number of most isolates was observed to increase by approx. 1.5 log units during the first two days of exposure to high humidity/low temperature (Fig 4 A, B). As growth of *Salmonella* below 10 °C has been reported (14,30), this increase may be due to growth of the bacteria with leftovers of the BHI culture medium. In contrast to freezing and thawing, prolonged dehydration did impose variations in survival and length of lag phase among the 25 *Salmonella* isolates, most notably during exposure to low humidity. Even within the serovar Typhimurium variations were observed.

Furthermore, survival and length of lag phase after rehydration were observed to correlate. Isolates of *S.* Infantis as well as the *S.* Typhimurium U292 isolate no. 68 survived longer during the dehydration period and they had also shorter lag phases after exposure to both, low and high humidity conditions reaffirming the tolerance to dehydration. The *S.* Derby isolates

and *S.* Typhimurium DT170 isolate no. 32 where more susceptible as they did not survive low humidity conditions as long as other strains and as they had a tendency for longer lag phases. Like in the freezing and thawing study, the maximum growth rates after rehydration were, except for *S.* Derby no. 13, very similar among the isolates and did not change during the experimental period of 3 months indicating that maximum growth rates are independent of injury and metabolic state of the bacteria (28).

Except for *S.* Typhimurium DT104 no. 02, the isolates that were most affected by dehydration with lag phases longer than the average (*S.* Derby, *S.* Typhimurium DT12, U288, DT170 no. 32), were previously characterized as unsuccessful. *S.* Derby and *S.* Typhimurium DT12 are very common subtypes in Danish pork production but they are much less prevalent in humans (2). One possible reason could be the reduced tolerance to dehydration when attached to surfaces in slaughterhouses or kitchens. The isolates with a general shorter lag phase than the average are represented by both, successful and unsuccessful strains. In case of *Salmonella* Derby and Infantis, the response to dehydration might be serovar specific but for the other isolates, differences occurred within serovars or phagetypes. *S.* Typhimurium U292 and DT135 were very uncommon phagetypes before the outbreak that occurred 2008 in Denmark (19). Both, especially U292 no. 68, tended to show a higher tolerance to desiccation but this connection, if any, remains to be elucidated. The clinical U292 isolate no. 06 from the Danish outbreak in 2008 was however only moderately tolerant.

In conclusion, tolerance to freezing did not appear to be correlated with clonal success, as *Salmonella*, independent of its q-factor, survived remarkably long in frozen meat and was capable of growing again without major impairment. Thus, keeping meat free of *Salmonella* by preventing contaminations before the freezing process seems important. The ability of *Salmonella* to survive dehydration, most notably under low humidity conditions, was in contrast strain dependent and was shown to be reduced in a group of unsuccessful strains. Overall, the success of specific clones may rather be the result of still unknown factors with dehydration potentially playing a minor role. Interventions to reduce epidemically successful clones should presumably target other processes than freezing and dehydration. To prove this, further investigations should be carried out.

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Tables and Figures

Table 1: *Salmonella* isolates included in this study

No.[Ref.]	Serotype	Phagetype	Date of isolation	Source	Resistance pattern	q-factor[c]	Experiment 1	Experiment 2	Experiment 3
01[1]	Typhimurium	DT104	june 98	human	nal, amp, chl, str, smx, tet, cip, spe, aug2	q≥1	+		+
02[2]	Typhimurium	DT104	17-08-05	human	amp, chl, smx, str, tet, spe, (ffn)[b]	q≥1	+		+
17	Typhimurium	DT104	01-02-08	pork	spe, str	q<1			+
16	Typhimurium	DT12	15-01-08	pork	s[a]	q<1			+
23	Typhimurium	DT12	16-04-08	pork	s	q<1			+
04[3]	Typhimurium	DT12	06-06-05	human	s	q<1	+	+	+
03[4]	Typhimurium	DT120	10-07-08	human	s	q<1	+		+
31	Typhimurium	DT120	22-10-08	human	amp, smx, spe, str, tet, tmp	q≥1			+
05[4]	Typhimurium	DT135	16-09-08	human	s	q<1	+		+
32	Typhimurium	DT170	05-06-08	human	s	q<1			+
45	Typhimurium	DT170	02-06-09	human	smx, str, tet	q<1			+
07[5]	Typhimurium	U288	14-10-08	human	(str)	q<1	+		+
06[4]	Typhimurium	U292	28-07-08	human	s	q<1	+	+	+
68	Typhimurium	U292	30-06-05	pig	s	q<1			+
13	Derby		30-04-08	human	s	q<1			+
15	Derby		14-04-05	pork	tet	q<1			+
26	Derby		13-04-05	pork	s	q<1			+
09	Derby		15-10-08	human	s	q<1	+		
127	Infantis		20-06-07	pig	s	q<1			+
10[6]	Infantis			poultry	s	q<1	+		+
39	Newport		06-11-08	human	s	q≥1			+
44	Newport		15-06-07	human	cip, nal	q≥1			+
27	Saintpaul		26-04-09	human	s	q≥1			+
41	Saintpaul		20-11-07	human	apr, chl, gen, smx, spe, tet, (ffn), (str)	q<1			+
38	Virchow		11-06-09	human	tmp, tet, smx, neo, nal, gen, cip, amp	q≥1			+
42	Virchow		25-06-08	human	s	q≥1			+

[a]s: sensitive; [b]antibiotics in brackets: intermediate resistance; [c]q: factor expressing the overall ability to survive the food chain and cause disease, from publications of Hald et al. (22,23) and from the research group Epidemiology and Risk Modelling, Danish National Food Institute (unpublished data); q < 1 successful and q ≥ 1unsuccessful subtypes; + indicates that the isolate has been used in one of the experiments (1: Survival and growth after long-term freezing; 2: Survival and growth of stationary and exponential phase cells after short-term freezing; 3: Survival and growth after dehydration); Antibiotics: cip, ciprofloxacin; str, streptomycin; tet, tetracyclin; nal, nalidixic acid; ffn, florfenicol; sul, sulfonamides; neo, neomycin; smx, sulfamethoxacole; amp, ampicillin; chl, chloramphenicol; spe, spectinomycin; tmp, trimethoprim; gen, gentamicin; aug2, amoxicillin + clavulanat (2:1); Ref. (References): [1]32, [2]18, [3]39, [4]19, [5]11, [6]1

Table 2: Sampling times during dehydration when colony counts and growth were measured

		Time of dehydration (days)									
	Conditions	0	1	2	7	14	21	28	56	84	112
Colony	25 ˚C, 49 % RH[a]	+	+	+	+	+	+	+	+	+	+
counts	10 ˚C, 82 % RH	+	+	+	+	+	+	+	+	+	+
Growth	25 ˚C, 49 % RH	+	+	-	+	+	+	-	+	-	-
curves	10 ˚C, 82 % RH	+	+	-	+	+	+	+	+	+	-

[a]RH: relative humidity; + indicates that samples have been taken; - indicates that no samples have been taken

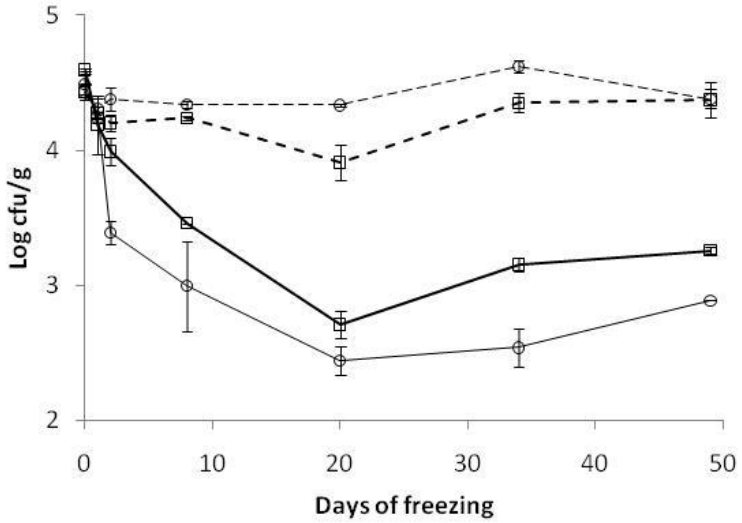

Figure 1 Effect of freezing (at -20 ºC) on colony counts of *Salmonella* Thyphimurium DT12 (circles) and U292 (squares) in minced meat in different growth phases; exponential cells (solid curves) and stationary cells (dashed curves). Error bars represent standard deviations between 2 replicates.

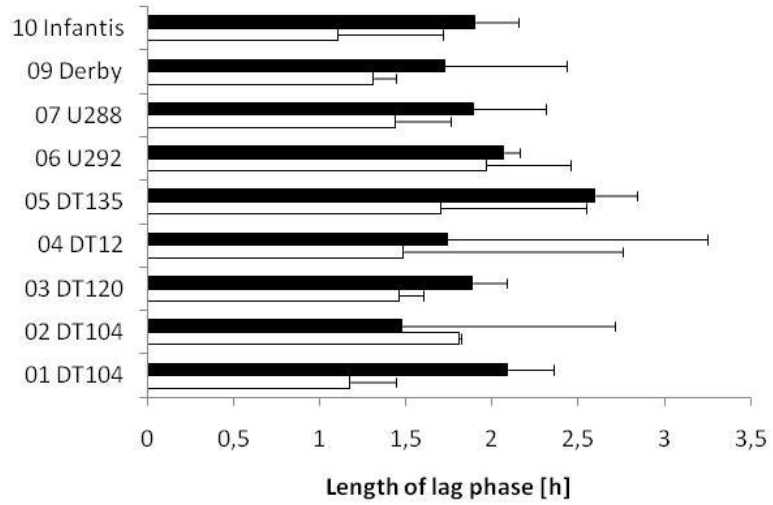

Figure 2: Lag phase duration [h] in meat juice at 25 °C after frozen storage in minced meat for approx. 180 days (black bars: start inoculum of 10^3 CFU/g; white bars: start inoculum of 10^6 CFU/g); Error bars represent standard deviations between 2 replicates.

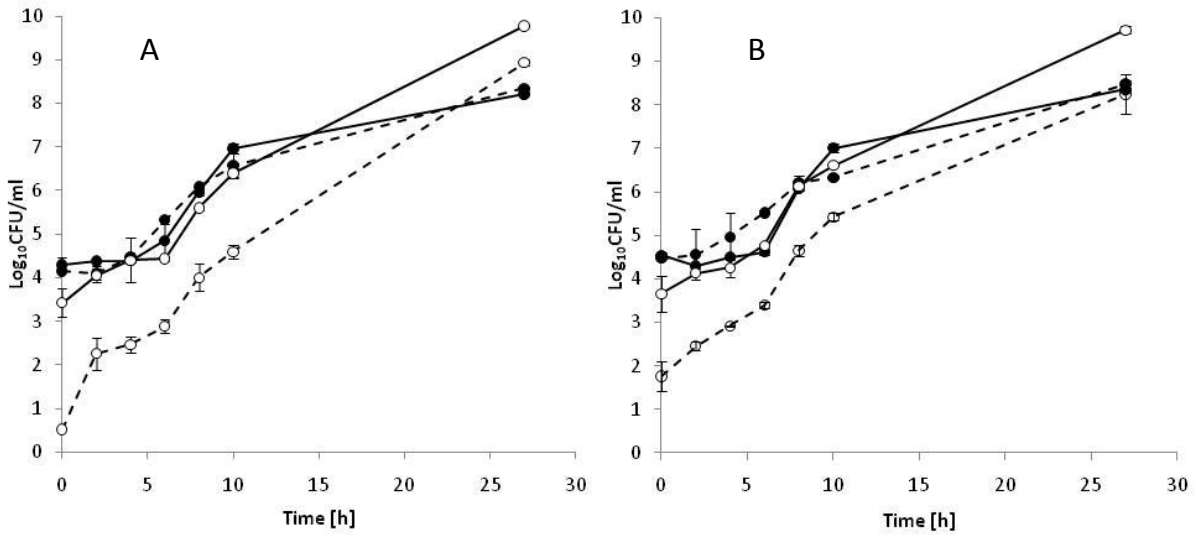

Figure 3: Growth (log CFU/ml) in meet juice at 25 ˚C after frozen storage in minced meat for 2 (closed circles) and 49 days (open circles); Typhimurium DT12 (A) and U292 (B) inoculated as exponential (dashed lines) and stationary phase cells (continuous lines); Error bars represent standard deviations between 2 replicates.

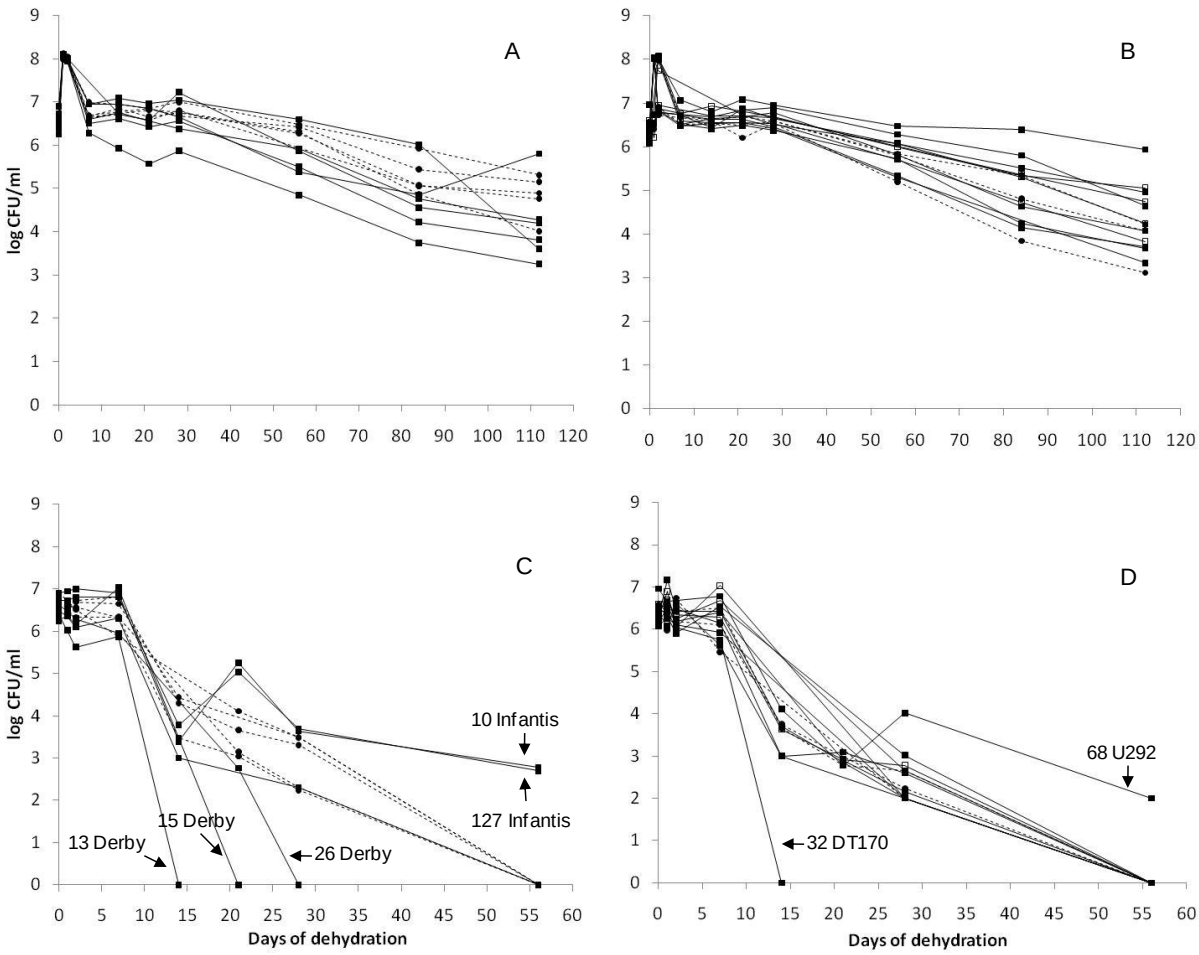

Figure 4: Effect of dehydration on colony counts of *Salmonella*; dashed lines with closed circles display successful *Salmonella* types; continuous lines with closed squares display unsuccessful *Salmonella* types ; continuous lines with open squares display outbreak strains of 2008; A, B: 10 ˚C, 82 % relative humidity; C, D: 25˚C, 49 % relative humidity; Standard deviations based on 2 replicates ranged from 0,06 – 0,38 log CFU/ml and tended to increase with the decline of colony counts (not shown).

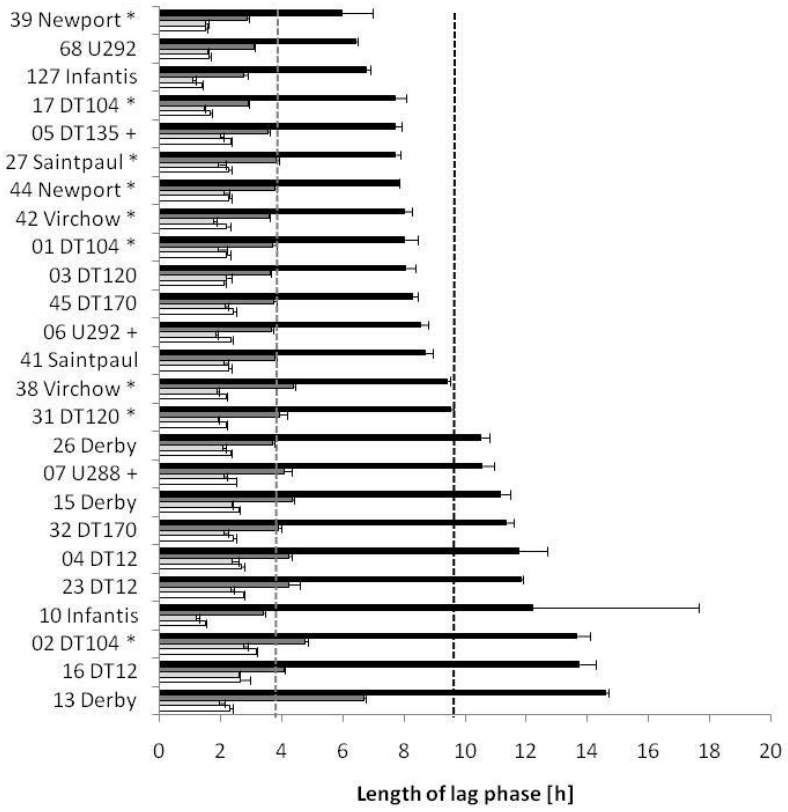

Figure 5: Lag phase duration [h] in BHI at 25 °C before (white bars) and after 1 (light grey bars), 21 (dark grey bars) and 56 (black bars) days of dehydration; the average lag phase duration of all the strains capable of growing after 21 (grey) and 56 (black) days is shown by a dashed line; *successful *Salmonella* types ; + strains from the 2008 outbreak; A: 10 °C, 82 % relative humidity; B: 25 °C, 49 % relative humidity; Error bars represent standard deviations between 3 replicates.

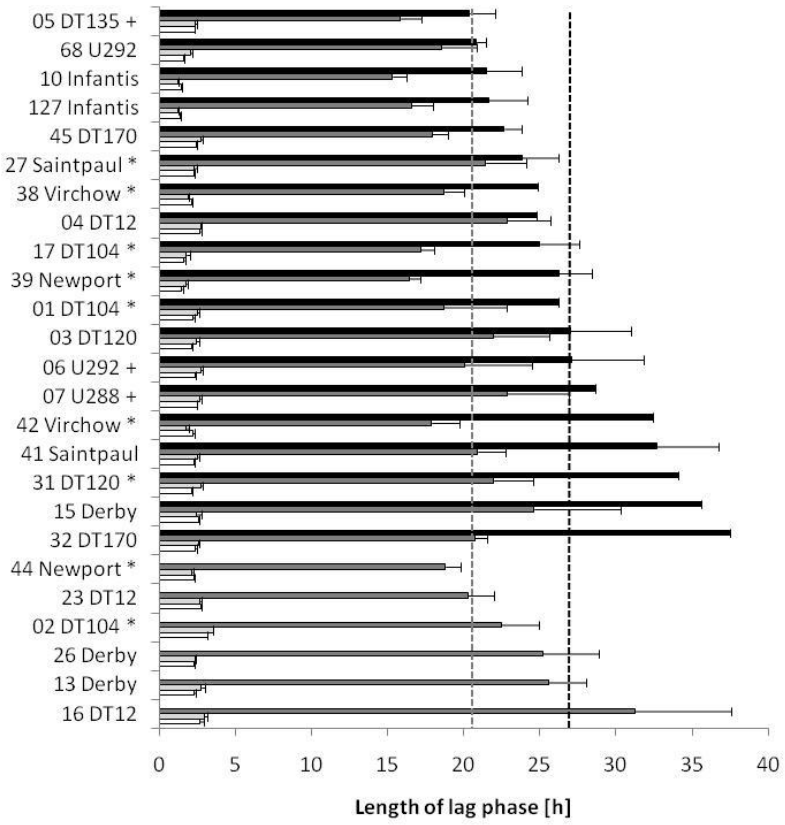

Figure 5 (continued)

Manuscript II

Identification of genetic features associated with multidrug- and quinolone-resistance in *Salmonella*

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Keywords: *Salmonella*, multidrug resistance, quinolone resistance, array-based comparative genomic hybridization, prophages, virulence factors

This manuscript is in preparation

Abstract

Multidrug (MR) and quinolone (Q) resistance in non-typhoidal *Salmonella* is associated with an increased burden of illness in humans implicating a virulence or fitness advantage in these bacteria apart from the antibiotic resistance. By means of comparative hybridizations using a pan-genomic microarray and PCR screenings of antibiotic resistant and susceptible *Salmonella* field isolates, we identified genes and genetic elements associated with MR and Q resistance. Three of these genes, *recT* (SG1183), *sugR* (STM3753) and *trhH* (AF261825_S012), were deleted for functional analyses. 74 genes dominated in MR and Q isolates compared to sensitive ones, of which 43 genes were related to mobile genetic elements. The largest fraction accounted for prophage genes, including five single genes and 23 genes attributed to three phage clusters (P22-like phages, Gifsy-1 and an unknown phage). The virulence genes *sopE* (STY4609) and *gipA* (STM2599) dominated weakly in Q and MR isolates. In contrast, genes involved in metabolism dominated in sensitive isolates. In none of the functional assays, the mutants were significantly different from the wildtype, but *recT* seemed to have a weak effect against the early defense of macrophages and to play a minor role in decreasing spontaneous mutation rates. The success of MR and Q resistant *Salmonella* could in part be explained by the acquisition of mobile elements, especially bacteriophages which are known to confer a fitness advantage and to be involved in virulence. Future research should examine if prophage genes can serve as epidemiological markers for emerging successful multidrug and quinolone resistant *Salmonella*.

Introduction

Salmonella enterica is a leading cause of zoonotic infections in the EU (77) and causes foodborne diarrheal disease worldwide (88). but *Salmonella* differ in their human health impact (38,45,47,88). As observed by the Danish *Salmonella* Surveillance program, antibiotic resistant *Salmonella* are more frequently isolated from human outbreaks than sensitive ones (11). It has also been reported that multidrug and quinolone resistant *Salmonella* clones are associated with an increased burden of illness in humans as patients infected with these bacteria have a higher risk of hospitalization, mortality and bacteremia as well as a longer duration of hospitalization than patients infected with fully susceptible *Salmonella* (40,52,54,81,83). Hence, multidrug and quinolone resistant *Salmonella* seem to be more successful in infecting humans and in causing a disease outcome with increased severity even in the absence of antibiotics. Moreover, antibiotic resistance might lead to treatment failures in antimicrobial therapy which is required in patient with severe cases of Salmonellosis (51).

A well described example for a multidrug resistant successful Salmonella clone was *S.* Typhimurium DT104 with an ACSSuT (ampicillin, chloramphenicol, streptomycin, sulfonamide and tetracycline) resistance phenotype encoded by the SGI-1 (*Salmonella* genomic island 1). This genetic island was probably taken up in the early 80s and in the 90s, the multidrug resistant *S.* Typhimurium DT104 clone was of global importance because of its wide dissemination in many countries. This clone was also reported to be more virulent as an unusual high percentage of patients were hospitalized in the UK and in the US, this strain was more often isolated from blood than non-ACSSuT stains (79). In general, *S.* Typhimurium belongs to the most important *Salmonella* serovars in many countries. In Denmark it is the dominant serovar in humans since 2008 (8,9,12). Another example of a success clone is a multidrug resistant clone of *S.* Newport that has emerged in the USA and that has been associated with tenfold more diseases than the sensitive *S.* Newport clones in the UK (71). However, *in vivo* or *in vitro* studies could not prove an association between virulence and resistance (4,25) or virulence and major worldwide clones (66).

There is evidence that resistance and virulence genes can be located close to each other, as they have been found on the same plasmids in *Salmonella* (26,36,41,70,78). Plasmid transfer

can thus be accompanied by a simultaneous gain of virulence and resistance traits. There might also be other factors than virulence, such as the ability to survive the food chain that may contribute to the emergence of salmonellosis cases caused by antimicrobial resistant clones (47). In a study by Hald et al. (38), they used a mathematical model based on data from the Danish *Salmonella* Surveillance program to estimate the relative ability of *Salmonella* serovars with different antimicrobial resistance pattern to survive the food production chain and to cause human disease. Except for *S.* Saintpaul, this ability seemed to increase in antimicrobial resistant strains (38).

The resistance to quinolones is most often conferred by point mutations that result in an aminoacid change of the DNA gyrase subunit GyrA. Transmission of quinolone resistance can be through clonal spread but it can also emerge in susceptible strains within a patient during treatment (6,49,55,68).

By means of array-based comparative genomic hybridization (aCGH) and PCR of antibiotic resistant and susceptible *Salmonella* isolates, we aimed to identify genetic elements or patterns that may be related to human infection and/or survival outside the host and that are characteristic for multidrug and quinolone resistant *Salmonella* clones. Overall, the objective was to gain knowledge about the genetic background of these clones and to identify novel fitness or virulence genes that potentially contribute to the success of multidrug and quinolone resistant *Salmonella* clones.

Material and Methods

Bacterial strains and growth conditions. This study included 81 field isolates (Table 1 and Figure 1) of *Salmonella enterica* spp. *enterica*, representing different sero- and phagetypes originating from sporadic or outbreak cases from humans. Isolates from pigs and pork were also included as pork is a common source of human salmonellosis in Denmark (see Figure 2 for a description of the whole experimental work flow) (8,88). The food and animal isolates were obtained from the Danish National Food Institute and the human isolates from the Statens Serum Institute. We used a field isolate of *Salmonella* Typhimurium DT104 (0703H64730, 109) resistant to streptomycin, sulfametoxazole and spectinomycin to generate isogenic mutants with the following gene deletions: Δ*trhH*::km^R, Δ*recT*::km^R and Δ*sugR*::km^R.

To investigate adhesion and invasion of epithelial cells, we used *S.* Typhimurium 4/74 and the isogenic mutant ΔinvH201::Tn*phoA* (86) as control strains. As donor strains for conjugation, we used *Alcaligenes faecalis* R214 (2) and *Salmonella* Agona 32 (1).

Overnight cultures were obtained by growing a colony from sheep or calf blood agar in 10 ml LB or BHI broth containing antibiotics where required for 16-20 h at 37 °C. For bacterial enumeration, colony counts were determined by plating 10 µl droplets on LB or BHI agar.

Plasmids and cell cultures. The plasmids pKD4 and pKD46 (28), used as tools for the inactivation of chromosomal genes, where provided by the Department of Veterinary Disease Biology (University of Copenhagen). The Int407 human epithelial cell line (ATCC CCL-6^{TM}) was obtained from the Danish National Veterinary Institute (Lindholm) and the RAW264.7 murine macrophage-like cell line (ATCC TIB-71^{TM}) were provided by the Danish National Food Institute. The cells were cultured at 37 °C in an atmosphere of 95% O_2 and 5% CO_2. Int407 cells were maintained in modified Eagle's minimal essential medium (MEM; ATCC) supplemented with 10% fetal bovine serum (FBS; ATCC) and 0.1 % penicillin/streptomycin (Gibco). RAW264.7 were cultured in Dulbecco's modified eagles medium (DMEM; Invitrogen) supplemented with 10 % FBS (Hyclone; Thermo Scientific) and 0.1 % HEPES buffer (1 M; Gibco), 0.1 % sodium pyruvate (100 mM; Gibco) and 0.1 % penicillin/streptomycin. One day prior to bacterial infection the cells were grown without antibiotics. During bacterial infection, the RAW264.7 cells were grown without FBS.

MIC determination. The minimum inhibitory concentrations (MIC) for fifteen antimicrobial agents were determined using commercially dehydrated antimicrobial agents in microtitre wells (SensititreTM; TREK Diagnostic Systems Ltd., UK) as part of the Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (10,87). Additionally, the resistance to rifampicin, tetracycline and trimethoprim was determined by use of the disc diffusion method (following CLSI guidelines).

***Salmonella* microarray.** The genomic DNA was extracted with the Easy-DNA kit (Invitrogen) according to the instructions and examined for purity and quantity by measuring the absorbance at 280, 260 and 230 nm with a nanodrop spectrophotometer. 29 *Salmonella* isolates (Figure 1) were selected to perform a comparative genomic hybridization (CGH) analysis using a pan-genomic *Salmonella* microarray comprising the genomes of *S. bonogori* 12419 (ATCC 43975), *S.* Typhi CT18, *S.* Typhimurium SL1344 (NCTC 13347), *S.* Typhimurium DT104 (NCTC 13348), *S.* Typhimurium LT2 (ATCC 700220), *S.* Gallinarum

287/91 (NCTC 13346) and S. Enteritidis PT4 (NCTC 13349). The design and the validation of the array as well as the experimental procedure have been described earlier by Pan et al. (65) and Wu et al (37).

A**nalysis of microarray data.** The array slides were scanned using the Genepix 4000B scanner (Axon Instruments, Inc.) and the images quantified with the Genepix Pro Software (Axon Instruments, Inc.). Normalization of the hybridization raw data was performed using the quantile normalization as implemented in the preprocessCore library for the R environment (16,17). The ratio of the cy5/cy3 signals was calculated and the geometric mean signal for each gene was used for further analysis. To distinguish between present and absent/divergent genes, a cut-off value of 6.6 was determined by plotting the log data of the signal intensity against the signal frequency. The data were validated by first verifying that the signals of empty and negative control probes were displayed as absent; second, by analyzing the presence of 15 housekeeping genes (*aroC, dnaN, hisD, purE, sucA, thrA, panB, icdA, mdh, aceK, glnA, tonB, recA, phoP, gabA*) in all *Salmonella* strains; and third, by examining the signal distribution of the lowest probes for each gene where the corresponding highest probe of the probe replicates was above the cut-off value. Based on all the genes present on the array, a dendrogram was established to ensure that the strains were grouped correctly after their phylogenetic correlation (Figure 1). The data were analyzed by comparing the present and absent/divergent genes within groups of isolates classified by their antibiotic resistance pattern and fully susceptible isolates.

PCR Screening. The DNA from boiling lysates of 52 additional *Salmonella* strains (Table 1) and from strains used in the microarray study was used in a PCR screening intended to verify the microarray results. PCR was also used to amplify the km^R (kanamycin resistance gene) cassette on pKD4 required for gene disruption (see below). Primers with 40 bp homologies to the ends of the target genes and 20 bp to the flanking region of the km^R cassette on pKD4 were used for this PCR. The gene disruption was confirmed by PCR amplification using primers from the ends or from the flanking sides of the target genes. Oligonucleotide primers (Table 2) were designed using Vector NTI Advance 10 software (Invitrogen). The primer specificity to the respective gene was confirmed using BLASTN (http://blast.ncbi.nlm.nih.gov/). The amplification was carried out in a T3000 thermocycler (Biometra). The PCR fragments were purified using the GFX purification kit (GE Healthcare) and one amplicon was sent for sequencing to Macrogen (Seoul, Korea) as a control.

Sequence analysis. Sequence analyses were performed using the software Vector NTI (Invitrogen), the CLC DNA workbench (CLC bio) and the web tools BLASTN and BLASTP as well as the MicrobesOnline operon predictions application. To verify the results of the CGH microarray, we performed an *in silico* screening of gene sequences in publically available sequenced and annotated *Salmonella* genomes.

Inactivation of chromosomal genes. 50 µl of electrocompetent cells of the *S.* Typhimurium strain no. 109, prepared as in reference 112, were transformed with 1 µl of the helper plasmid pKD46. Transformants were selected after incubation for 2 h at 30 °C on LB agar containing 100 µg/ml ampicillin. The deletion of the genes *recT* (SG1183), *sugR* (STM3753) and *trhH* (AF261825_S012) in strain no. 109 harboring pKD46 (109pKD46) was achieved by a method described by Datsenko and Wanner (28). Briefly, 5 µl of the purified km^R cassette were transformed into 100 µl of electrocompetent cells of strain no. 109pKD46. Transformants were selected after incubation for 2 h at 37 °C on LB agar containing 50 µg/ml kanamycin.

Growth curves. An overnight culture was diluted 1000 times in LB broth or DMEM supplemented with 0.1 % HEPES buffer and 0.1 % sodium pyruvate and grown without shaking at 37 °C in a Bioscreen C reader (Thermo Labsystems) for 24 h (measurement intervals: 10 min; OD: 600 nm). Growth was measured in triplicates.

Adhesion and invasion of epithelial cells. Bacterial adhesion and invasion of Int407 epithelial cells was determined by a gentamicin protection assay essentially as described by Watson et al. (86) and Galan et al. (35). The cells were seeded into 12 well culture plates at a concentration of 5 x 10^5 cells per well and incubated overnight. 1 h prior to inoculation, the monolayers were washed twice with MEM and incubated in 1 ml MEM. To obtain exponential cultures, overnight cultures were diluted to 10^7 CFU/ml and incubated for 3 h with shaking. The cultures were then washed and diluted to an OD_{600} of 0.1 with phosphate buffered saline (PBS). 100 µl were used to infect the Int407 cells using a moi of 20. After incubation for 1 h, the monolayers were washed twice with PBS and incubated with 1 ml MEM containing 100 µg/ml gentamicin. Before and 2 h after the addition of gentamicin, the cells were washed twice with PBS and lysed using 1 ml PBS per well containing 0,1% Triton X-100 (Sigma). For direct counts, appropriate dilutions of the lysates were plated on LB agar to determine the number of adherent (before gentamicin treatment) and intracellular (after gentamicin treatment) bacteria. Triplicate experiments were performed.

Macrophage survival. Analysis of survival and growth of *Salmonella* in macrophages was carried out essential as described by Humphreys et al. (44). RAW264.7 cells were seeded into 12 well tissue culture plates (Costar) at a concentration of 5 x 10^5 cells per well. After overnight incubation, the cells were washed once with PBS and incubated for 1 h in DMEM before bacterial inoculation. Bacteria of an overnight culture were washed and diluted to an OD_{600} of 0.1 with PBS containing 10% heat inactivated mouse serum and opsonized for 30 min. 100 µl of this cell suspension were used to infect the RAW264.7 cells using a moi of 20. The cells were centrifuged (1000 g, 5 min) to synchronize the infection and incubation for 1 h. Then, the monolayers were washed once with PBS and overlaid with 1 ml DMEM containing 100 µg/ml gentamicin. After an additional hour, the washing step was repeated and the cells were incubated with 1 ml DMEM containing 10 µg/ml gentamicin. 2, 4 and 24 hours post infection, the cells were washed as described above and lysed using 1 ml PBS per well containing 0,1% Triton X-100. For direct counts, appropriate dilutions of the lysates were plated on LB agar to determine the number of intracellular bacteria. Viability of macrophages was determined microscopically using 100 µl/ml Trypan blue (Invitrogen) to stain the dead cells. Triplicate experiments were performed but the viability of macrophages was only determined twice.

Spontaneous mutation frequency. A proportion of an overnight culture was transferred to fresh BHI medium to obtain a 1000 fold dilution. An equal amount of the overnight culture was plated on selective BHI agar plates containing 100 µg/ml rifampicin to confirm that no pre-existing mutants were present. The prepared culture was incubated at 37 °C with shaking until OD_{600} of 2 was reached. 10 ml were centrifuged (6000 g, 10 min), resuspended in 100 µl BHI and the entire volume was plated evenly on BHI agar containing 100 µg/ml rifampicin. In the same time, appropriate dilutions were plated on non-selective BHI agar to obtain total counts. The mutation frequency was calculated by dividing the number of spontaneous rif^R mutants by the total counts. Triplicate experiments were performed.

Conjugation frequency. The conjugation frequencies of the genes *tet*(A) and *dfr*A25 from the donors *A. faecalis*, and *S.* Agona conferring resistance to tetracycline (tet) and trimethoprim (tmp) were determined. Exponential cultures were prepared with 20 fold dilutions of an overnight culture followed by aerobic growth to an OD_{600} of 0.5. The cultures of the donor strains and spontaneous rif^R mutants of the recipients (wild type train no. 109 and the mutants 109Δ*trhH*::km^R, 109Δ*recT*::km^R and 109Δ*sugR*::km^R) were then equally mixed (1:1) and transferred to sterile filters (pore size 0.45 µm) on BHI agar plates. After incubation

for 1 h or 24 h, the filter was transferred to 10 ml 0.9 % NaCl (v/w). After resuspension of the cell material from the filter, appropriate dilutions were plated on donor-, recipient- and transconjugant-selective BHI agar plates containing 64 µg/ml tmp, 100 µg/ml rifampicin (rif) and/or 16 µg/ml tet. The experiment was at least carried out in duplicates.

Statistics. Colony counts were log transformed where appropriate and standard deviations (SD) or standard errors of the mean (SEM) were calculated. Statistical significances of differences were calculated with one-way analysis of variance (ANOVA) followed by Turkey's multiple comparison test or unpaired t test using GraphPad Prism (GraphPad Software).

Results

Validation of the microarray data. A *Salmonella* pangenomic microarray was used to compare the genomes of 29 *Salmonella* field isolates categorized according to their resistance pattern in order to identify genes that could contribute to a virulence or fitness advantage in successful multidrug and quinolone resistant clones. For 20 % of the probe replicates, the probes with the lowest signal intensity were below the cut-off value while the corresponding probe with the highest signal intensity was above the cut-off value. No positive signals were displayed from the negative controls. Two out of 15 housekeeping genes (*panB* [STY0200] and *gapA* [STY1825]) were absent in 86 % and 93 % of the isolates, respectively. The dendrogram, based on all genes present on the array, correlated the isolates according to their serovar and grouped them into two major clusters: the Typhimurium and non-Typhimurium isolates (Figure 1). The 16 Typhimurium and the 13 non-Typhimurium isolates had a core genome composed of 55 % and 56 % of the present genes, respectively. Within the non-Typhimurium cluster, isolates of *S.* Virchow showed the closest similarities to each other and isolates of *S.* Saintpaul were more distantly related.

Genomic comparison of *Salmonella* isolates categorized according to their resistance pattern. The comparative genomic hybridization analysis was carried out with the entire dataset of the present and absent/divergent genes of the 29 *Salmonella* isolates and separately with the datasets of either the Typhimurium (n = 16) or non-Typhimurium (n = 13) isolates. First, the isolates were classified according to their resistance pattern into four categories:

quinolone resistant (reduced susceptibility to at least one fluoroquinolone; Q), multidrug resistant (resistant to four or more antibiotic agents; MR), resistant (resistant to less than four antibiotic agents; R) and sensitive (susceptible to all antibiotics tested; S). When indicated, the Q resistant non-Typhimurium isolates were subdivided into isolates resistant to four or more antibiotic agents (QMR) and isolates resistant to less than four antibiotic agents (QR). A gene was defined as dominaant in isolates attributed to one of the mentioned resistance categories: 1) If it was present in 100 % of the isolates of that resistance category and absent in at least 70 % of the sensitive ones; 2) If it was absent in 100% of the sensitive isolates and present in minimum 60 % of the isolates of the respective resistance category; 3) If the total number of isolates carrying the gene was higher among all the resistant isolates than among the sensitive ones. No analysis was performed for MR non-Typhimurium as this category was only represented by one isolate but it was taken into consideration when genes dominating in two or more of the resistance categories were identified or when the entire dataset of Typhimurium and non-Typhimrium isolates was analyzed.

None of the *S.* Typhimurium DT104 isolates that were used for genomic hybridization were fully susceptible. Among the Typhimurium isolates, many of the genes that dominated in resistant strains turned out to be restricted to *S.* Typhimurium DT104. These genes were excluded from the results because it is impossible to distinguish between association with the phagetype DT104 or with the resistance pattern. We also identified genes that dominate in the sensitive *Salmonella* isolates as they provide information about genes that are typically absent or diverged in resistant isolates. These genes were determined in a similar manner and were defined as: 1) present in minimum 70 % of the sensitive isolates and absent in the Q and MR strains; and 2) present in 100 % of the sensitive strain and in not more than 30 % of the Q and MR strains.

Genes related to mobile genetic elements. In total, 74 genes were observed to dominate in MR and Q isolates (Table 3) and a big proportion of these genes also occurred in R isolates. Most of these genes (43 genes) were related to mobile genetic elements whereof the largest fraction (five single genes and 23 genes belonging to three gene clusters) accounted for prophage genes. Eight genes located within the region SDT0325 to SDT0359 were very common among resistant Typhimurium and almost always absent in the non-Typhimurium isolates. This gene cluster is homologous to P22-like phages such as ST104 and ST64T (104). The Gifsy-1 phage cluster (STM2590 - STM2617) belongs to the lambdoid *Salmonella* phages and is present in almost all Typhimurium isolates independent on their resistance

pattern but apart from that it dominates markedly in the Q resistant non-Typhimurium isolates. A small cluster of three genes (SL1943, SL1944 and SEN1935) that are homologous to genes of the P27-like phage ST64B, is present in almost all resistant Typhimurium isolates (DT104 and DT170) and generally absent in the non-Typhimurium isolates. Four genes within the region SDT1841 to SDT1857 seem to belong to a phage cluster as two of them encode phage proteins but none could be assigned to a known bacteriophage as no specific sequence database hits could be obtained using BLASTN and BLASTP.

As prophage genes seem to be important elements in MR and Q strains of *Salmonella* (33,34), we selected all 282 available genes on the microarray annotated as phage encoded and analyzed their signal distribution among the 29 isolates. Table 3 shows that resistant *Salmonella* among both, Typhimurium and Non-Typhimurim isolates, harbor most of these prophage genes. We furthermore investigated the distribution of known phage encoded virulence genes among the *Salmonella* isolates: *sopE* (137), *sodC* (138), *nanH* (139, 140), *gtgA* (139), *sseI* (141) and *sspH1* (142). The gene *sopE* (STY4609) was absent from Typhimurium but present in eight non-Typhimurium isolates: in all Q and MR isolates and three out of seven sensitive isolates. One Q isolate of *S.* Saintpaul (no. 34) hybridized to *sodC* (STY1682) and one susceptible Saintpaul (no. 27) harbored *nanH* (STY2483). The other three phage genes were absent from the microarray and were therefore screened by PCR for presence in all 83 *Salmonella* isolates included in this study. The genes *gtgA* and *sseI* were present in all the Typhimurium isolates and *sspH1* in 89 % of the Typhimurium isolates. These three phage genes were variable components among the non-Typhimurium isolates, but without any correlation to antibiotic resistance.

A cluster of 12 genes that dominated in the MR and Q isolates accounted for genes of the *Salmonella* Typhimurium genomic island 1 (SGI-1). Among the Typhimurium isolates, these genes were restricted to DT104, except for AF261825_S037 and SDT3847 which were also present in DT120 and DT170 respectively. Among the non-Typhimurium isolates, these genes were almost exclusively present in the Q (QR and QMR) strains of *S.* Saintpaul and *S.* Virchow.

Three single genes were found among the mobile elements dominating in the MR and Q isolates: HCM1.167, HCM1.217 and STY4657. The first was an integrase gene that is associated with integrons, which frequently lie on transposons or plasmids. The second gene

was a pseudogene which is partly (68%) identical to a transposase of IS26 and the last gene encodes the insertion element IS1 protein InsB.

The group of Q isolates was composed of QMR (resistant to at least 4 antibiotic agents) and QR (resistant to less than 4 antibiotic agents) isolates. Genes that are exclusively linked to quinolone resistance should be dominant in these two groups and absent in the MR and other isolates. Among the Typhimurium isolates, it does only apply to one gene: SEN1132, a probably phage encoded excisionase. Among the non-Typhimurium isolates, it is the case for many genes. Nevertheless, the Gifsy-1 phage cluster (STM2590 – STM2617) and SDT2013, a phage tail-like gene, seem to be most characteristic for non-Typhimurium Q strains as they were present in nearly all Q isolates.

To get an indication of genes that are absent or altered in resistant *Salmonella* strains, we identified 37 genes that dominated in susceptible *Salmonella* isolates (Table A1 in the Appendix). Eight (22 %) genes were related to mobile elements of which four genes belonged to a cluster originating form pSLT, the Typhimurium virulence plasmid. The remaining mobile elements were single genes: three phage encoded proteins and one plasmid encoded antirestriction protein.

Assuming that mobile elements implicate a greater genomic variability, we used the microarray results to estimate the genetic diversity of resistant *Salmonella* versus fully sensitive *Salmonella* isolates by subtracting the number of genes of the core genome from the number of genes of the pan genome. The percentage of the variable genes was 34 to 35 % in the resistant and sensitive isolates regardless if they belonged to the Typhimurium or the non-Typhimurium group.

Genes related to virulence. Among the Typhimurium isolates, 5 genes with an experimentally documented or putative function in virulence were found to dominate in the MR and Q isolates. One of them, *gipA* (STM2599), is listed together with the mobile genetic elements because it is encoded by the Gifsy-1 gene cluster mentioned above (Table 3). GipA was found to be involved in survival within Peyer's patches (74) and replication of *Salmonella* in macrophages (48). The gene *gtrA2* is a variant of *gtrA* (93% identical) from the P22-like phages (ST104, ST64T, SE1 and P22). It is involved in O-antigen conversion and has been suggested to contribute to long-term systemic infection in mice (50). No virulence associated genes could be identified to dominate among the susceptible *Salmonella* isolates (Table A1 in the Appendix).

Genes with other or unknown functions. 17 genes were related to other functions than virulence of which the largest part is involved in metabolism. However, two genes could be related to stress resistance: STY2115 (*cutC*), a copper homeostasis protein (144) and STY1582 (*asr*), an acid shock protein (72). Interestingly, orthologs of some of these genes are pseudogenes in *S.* Typhi. Genes of the *cbi* operon like *cbiJ* that are involved in cobalamin synthesis, are mutated in *S.* Typhi. Probably as a consequence of the *cbi* dysfunction, the adjacent *pdu* operon including *pduH*, involved in propandiol degradation, is not functional as well (56). The gene *ygbK* has also been reported to be a pseudogene in *S.* Typhi (supplementary note of McClelland et al. (56)). Among the 11 genes with unknown function, STY4728a is annotated as a pseudogene because it has a deletion of 913 bp within a putative *pspA* gene. Genes that are depleted of their function in certain Salmonella strains were also present among the above mentioned mobile elements and virulence genes: *sugR* (a pseudogene in *S.* Typhimurium SL1344 and *S.* Typhi) and HCM1.217.

Among the genes dominating in sensitive isolates, the fraction of metabolic genes accounted for the largest part (approx. 35 %) and is listed in Table 4 under genes with other function. One gene (*stiB*) involved in stress resistance could also be found to dominate in these isolates.

Verification of the microarray results by PCR, sequencing and *in silico* screening of genes. To evaluate the microarray results, the nucleotide sequences of the genes that were identified to dominate in MR and Q strains were screened *in silico* for their distribution in 41 sequenced and publically available *Salmonella* genomes. 72% of these genes were confirmed to be variable elements in *Salmonella* (Figure 3), including all the genes located on mobile genetic elements, except for AF261825_S037, three out of the five virulence genes (*sugR*, *gtrA2* and *gipA*) and 27 % of the genes with other or unknown function. Several genes belonging to mobile genetic elements (HCM1.217, SDT2013, STM2617 and some genes within the P22 cluster form SDT0325 to SDT0359), STY2208 and STY4728a turned out to have very heterogeneous sequences throughout the *Salmonella* genomes indicated in Figure 3 by shadings representing the different degrees of homology.

For a second evaluation, 22 genes from different gene categories (mobile, virulence, metabolic or unknown genes) dominating in Q and MR strains were chosen for a PCR screening using additional 52 *Salmonella* field isolates that were categorized after their resistance pattern in the same manner as the isolates used for the microarray analysis (Table 1). For 64 % of the genes, the microarray results could be reproduced by PCR. The distribution

of these confirmed genes in the 52 isolates of the PCR study and the 29 isolates of the microarray study are shown in Table 5. The microarray results could be confirmed for nearly all the genes located on mobile genetic elements and for three virulence gene (*sugR*, *gipA* and *sopE*), even though the dominance of the virulence genes in the MR and Q strains was not very apparent. All the tested genes with other or unknown function were ubiquitous among the 52 field isolates except for STY0439 (*malZ*), STY2249 (*pduH*) and STY4783 that were present in 98%, 92% and 96 % of the strains respectively. One control amplicon per gene was sequenced to confirm the primer specificity. The assembled sequences corresponded to the investigated genes except for SDT2013 with only 91 % identity. This reflects the results of the *in silico* screening that indicated that the sequence of SDT2013 is heterogeneous in *Salmonella*.

Phenotypical analysis of mutants. The genes *trhH* **(**AF261825_S012), *recT (*SG1183) and *sugR* (STM3753) were deleted in a *S.* Typhimurium DT104 field isolate (no. 109) and analyzed for their function by a series of phenotypical assays because we assumed that they play a role in virulence. The gene *trhH* **(**AF261825_S012) is located on SGI-1, belongs to the TrhH family of proteins and is annotated as a putative pilus assembly protein (19). We hypothesized that it has a function in mediating cell contact to host or bacteria cells. Using Microbes Online Operon Predictor (www.microbesonline.org), it seems to be the first gene of an operon including a second putative pilus assembly protein (AF261825_S011, *trhG*) and a hypothetical protein (AF261825_S010) (Figure A2 in the Appendix). RecT *(*SG1183) is a phage encoded recombination and repair protein involved in both, homologous and illegitimate recombination (73). It might contribute to the tolerance of conditions that induce DNA damage such as within macrophages. It is encoded within an operon including the gene for the exoribonuclease VIII (Figure A2 in the Appendix). The gene *sugR* (STM3753) is a variable pattern of the *Salmonella* pathogenicity island 3 (SPI3) (5,14) and 99% identical to the ATP binding subunit ClpX of a Clp protease of *S.* Weltevreden str. 2007-60-3289-1. This gene might function as a chaperone and allow tolerance to certain stress conditions such as low pH in macrophages (76). It is transcribed as the first genes of an operon including STM3754 encoding a hypothetical protein (Figure A2 in the Appendix). Based on *lacZ*-fusions, Blanc-Potard et al (14) also suggest that it is transcribed together with STM3755 (*rhuM*) even though the distance between the two genes is 580 bp. Mutants of the latter gene show decreased epithelial cell invasion (75).

No effect of the deleted genes on growth in LB and DMEM could be observed as the growth curves were equal for the wildtype and the mutant strains. To assess the impact of the genes on virulence, the epithelial cell adhesion and invasion capability and survival/growth in macrophages were analyzed using cell culture models. A decrease of 0.66 log CFU/well (SEM 0.12) for adhesion and 0.88 log CFU/well (SEM 0.04) for invasion of epithelial cells could be observed for the control mutant ΔinvH201::Tn*phoA* (86) compared to its wildtype strain *S.* Typhimurium 4/74. The three mutants generated in this study were not restricted in their ability to invade and adhere.

The direct counts of the inocula used to infect macrophages were slightly more variable probably due to the 30 min opsonization period directly before inoculation. Thus, to standardize the inocula, a correction factor was calculated by subtracting the actual counts from the inocula by 10^7 CFU/well (the ideal inoculum). The number of phagocytosed bacteria was determined by subtracting this correction factor from the direct counts of intracellular bacteria determined at 2 h post infection. At that time, the mutants Δ*recT*::km^R and Δ*sugR*::km^R exhibited slightly diminished but not significantly different ($P > 0.05$) intracellular counts of approx. 0.4 log CFU/well (Figure 4). The number of intracellular bacteria increased after 4 and 24 h post infection but no significant differences ($P > 0.05$) could be observed between mutants and wildtype although growth after 24 h post infection of Δ*sugR*::km^R and Δ*trhH*::km^R was approximately 0.51 and 0.37 log reduced (Figure 4). The cytotoxic effect of the *Salmonella* strains on the infected macrophages was determined by microscopical counts of viable and dead macrophages. The average number of viable macrophages ranged from 59% (SD: 6%) at 2 h post infection, 54 % (SD: 7%) at 4 h post infection to 31 % (± 4%) after 24 h post infection compared to the non-infected control were 75 % (SD: 3%) of the macrophages were viable during the whole time period. No significant differences ($P > 0.05$) in respect to cytotoxicity between the mutants and the wildtype were detected.

In order to estimate the importance of the disrupted genes for the ability of the bacterium to mutate, spontaneous mutation and conjugation frequencies were established. The spontaneous mutation frequency towards rifampicin was 2 times higher but not significantly different for Δ*recT*::km^R compared to the wildtype strain (Figure 5). The conjugation frequencies were equal for all recipients. They were on average $2.6*10^{-3}$ (SD: $1.46*10^{-3}$) for *S.* Agona and $5.14*10^{-4}$ (SD: $3.52*10^{-4}$) for *A. faecalis* as donor strains.

Discussion

Genetic characterization of successful antibiotic resistant *Salmonella* clones will add knowledge to the mechanisms involved in the concomitant acquisition of resistance and fitness or virulence determinants. In order to find genetic factors characteristic for these clones, we used a pan-genomic *Salmonella* microarray to compare the genomes of fully susceptible *Salmonella* strains to those with different resistance patterns. The array data were validated by PCR and the functions of three genes that dominated in MR and Q isolates of *Salmonella* were analyzed.

The importance of prophages and *recT* for Q and MR *Salmonella*. Using comparative genomic hybridization, we have shown that most of the genes that were predominant in Q and MR isolates were related to mobile genetic elements when compared to susceptible ones. Along with single genes from mobile elements, we could identify several gene clusters related to bacteriophages and Salmonella genomic island 1 (SGI-1). In contrast, metabolic genes were dominant in sensitive *Salmonella* isolates.

Phage DNA seems to be a key element in the genomes of Q and MR strains as prophage genes accounted for the largest group of genes dominating in these isolates. This might be surprising as resistance genes are more often reported to be mobilized via plasmids and transposons or integrons located on plasmids (62,64). Only three genes linked to transposons or integrons (*int*, *insB* and the putative transposase gene HCM1.217) where found to dominate in resistant isolates. Surprisingly, genes related to plasmids (pSLT) were more common among sensitive S. Typhimurium isolates. Bacteriophages can carry additional genes that are not required for phage proliferation (20) but provide and advantage for the bacterial host and thereby indirectly for the phage itself, especially those phages that integrate into the host genome and are dependent on the persistence and multiplication of the host. Many *Salmonella* prophages are reported to be involved in *Salmonella* virulence (reviewed in Brussow et al., (21). Among the phage gene clusters that dominated in Q and MR strains were Gifsy-1 and ST64B that play a role in Salmonella virulence (21,58). The phage encoded gene for the invasion-associated secreted protein SopE was present in 50% of the Q and MR strains towards 0% in R and 38% in sensitive strains. The putative phage encoded virulence protein GipA was present in 29 % of the Q strains towards 0 % in MR, 0% in R and 25 % in sensitive strains. The gene *gtrA* (STY2627a) was present in two MR Typhimurium isolates and exhibits

homology to the SPI-16 gene *gtrA* (STY0607) but it is not identical. Both genes seem to originate from *gtrA* located on phages such as P22 but seem to have undergone different sequence variations. The gene products mediate the transfer of glucose residues to O-antigen subunits. This modification of the O-antigen presumably results in an evasion of adaptive immunity (15). The blast results from the *in silico* screening of gene sequences also indicate the presence of several orthologous genes of *gtrA* as the sequences of this genes are heterogeneous among *Salmonella.* Other functional *Salmonella* phage encoded genes providing virulence or fitness advantages might exist but might not have been present on the microarray used. The findings of single phage genes and parts of prophages suggest the presence of phage remnants that are depleted for their function in phage particle formation and lysis. In *E. coli,* these cryptic phages have been reported to exhibit a positive effect on the host bacterium in adverse environmental conditions, like the presence of inhibitory concentrations of quinolones (85). Thus, bacteria that are more protected from being eliminated by an antibiotic might be more likely to become resistant to that antibiotic, which could explain the presence of phage remnants in antibiotic resistant bacteria. Moreover, viable prophages could also contribute to the virulence of their host cell. When phages are released, they might destroy competing bacteria whereas bacterial cells of the same population will be immune to a reinfection (67).

The repair of DNA damage is necessary for *Salmonella* virulence in macrophages as mutants in the genes *recA*, *recB* and *recC* are attenuated (23). The contribution of *recT* (STM2633) encoded by the phage Gifsy-1 to proliferation of *Salmonella* Typhimurium LT2 in macrophages has also been shown by Klumpp and Fuchs (48). This attenuation was documented at 7 h post infection and was relatively small. The *recT* gene (SG1183) that was found to dominate in Q and MR strains has no sequence homologies (neither nucleotide nor protein) to *recT* encoded by Gifsy-1 and Gifsy-2. Among others, it is present in *S.* Gallinarum, *S.* Dublin, *S.* Partyphi and *S.* Typhimurium 14028s and has 95 % sequence homology to the phage encoded *recT* gene in *E. coli*. In this study, it has been deleted in a *S.* Typhimurium DT104 field isolate for a functional analysis of the gene. No effect on adhesion and invasion of intestinal epithelial cells could be observed. In macrophages, the intracellular counts of the mutant 109 Δ*recT*::km^{R} at 2 h post infection were slightly reduced indicating a weak effect against the early defense mechanism of the macrophages, the oxidative burst (84). As expected, it also seems to play a minor role in reducing spontaneous mutations. It may seem contradictory that many Q and MR strains harbor an additional recombination and repair

protein that reduces mutations because resistances towards certain antibiotics, such as quinolones, are more frequently conferred by gene mutations than by horizontal gene transfer. But under circumstances where lethal mutations are induced, for example double strand brakes within macrophages, RecT might be involved in protecting *Salmonella* from being seriously damaged so that *Salmonella* can actually replicate and benefit from non-lethal mutations generated within macrophages. Quinolones belong to the group of antibiotics that are accumulated within phagocytes (39) suggesting that a host infected with susceptible *Salmonella* (maybe carrying *recT*) and treated with quinolones could serve as a source of quinolone resistant strains that are able to survive and proliferate in macrophages.

The roles of SGI-1 and *trhH* in *Salmonella*. Along with prophage elements, genes of the SGI-1 represented a big proportion of the genes dominating in Q and MR Typhimurium and non-Typhimurium isolates which is consistent with other studies suggesting the wide dissemination of variants of SGI-1 (3,18,30,53,57). In *S.* Typhimurium, SGI-1 includes a multidrug resistance regions encoding the genes *aadA2*, *floR*, $bla_{\text{pse-1}}$, *sul1* and *tet(G)* (102, 96) resulting in a pentaresistant phenotype, but according to the microarray hybridization results, genes from the island were also present in R isolates (resistant to less than four antibiotic agents). This is in agreement with the findings of variants of SGI-1 harboring less resistance gene (18,24). SGI-1 is listed here under mobile genetic elements as it can be mobilized via conjugative transfer with a helper plasmid (29). It has been suggested to be responsible for the successful spread of the *S.* Typhimurium DT104 clone with the ACSSuT resistance pattern and more recent also of *S.* Paratyphi in Canada (61). In addition to the resistance genes, this island and variants of it harbor genes for recombination, conjugation and regulation as well as genes with unknown function (60). The latter genes could play a role in virulence or fitness as *Salmonella* clones harboring the island are reported to be more widespread and virulent (61,79). But also gene products mediating conjugation, for example pili, might have a second function in virulence by improving the adhesion capability to host cells. We investigated if the SGI-1 gene *trhH* (AF261825_S012), presumably encoding a pilus assembly protein, is involved, in virulence, mutability and conjugation but no effect could be observed.

The roles of SPI-3 and *sugR* in *Salmonella*. The *Salmonella* pathogenicity islands (SPI) are also assumed to derive from mobile genetic elements (64). They are conserved among *Salmonella* (7,63,82) but SPI-3 is known to contain variable site (14). The gene *sugR*, that was found to dominate in Q and MR strains, is part of the left end of SPI-3 which is less conserved among *Salmonella*. Due to its homology to the subunit ClpX of a Clp protease, a

member of the stress protein family, and its location on a pathogenicity island, we assumed that the deletion of the gene could have an impact for virulence. No effect in adhesion and invasion of intestinal epithelial cells could be observed suggesting that no polar mutation affecting the expression of the downstream located gene *rhuM* was generated, as this gene was reported to be involved in epithelial cell invasion (75). After 24 h post infection, replication in macrophages seemed to be slightly diminished in *sugR* mutants. The cytotoxic effect on the macrophages was, however, not altered. These results are not in accordance with studies that have investigated the function of ClpXP. It has been reported that ClpXP is involved in down regulating macrophage apoptosis and in down regulating replication within epithelial cells (measured after 6 h post infection) (46). Reducing replication and apoptosis could be an important feature in establishing a systemic infection as macrophages are used by *Salmonella* as a vehicle for dissemination in the host organism (46). In addition to its regulatory function, ClpXP degrades missfolded proteins and might contribute to survival during the acidic conditions in the macrophages (377) which would explain why the mutant is slightly impaired in intraphogocytic multiplication. As expected, *sugR* had no influence on the conjugation or spontaneous mutation frequency of the host bacterium.

Genetic diversity in Q and MR *Salmonella*. As more mobile elements were found within Q and MR compared to susceptible isolates, we assume a greater genetic diversity among antibiotic resistant strains. By calculating the proportion of conserved genes (core genome) in resistant isolates compared to the proportion of non-conserved genes, we could not show that the overall genetic diversity for these isolates was higher than for sensitive ones. Saunders *et al.* (71) obtained different results by using an unweighted pair group method with arithmetic mean clustering, they could show that MR isolates of *S.* Newport showed a greater genomic variability than sensitive isolates of the same serovar.

Advantages and limitations of DNA microarrays for genomic comparison. *Salmonella* DNA microarrays have been successfully used to study diversity among *Salmonella* (42,43,65,69). They are powerful tools when the genomes of many strains are compared. However, some limitations have to be considered when microarrays are used. False positive or negative results can be obtained with divergent genes and the amount of genes that can be detected is restricted to the probes present on the array. Furthermore, it is important to consider that the detected genes are not necessarily expressed in a cell, which is the case for pseudogenes. We observed that several genes that dominate in Q and MR strains hybridized to probes originating from the genome of *S.* Typhi CT18 and belonging to genes that are no

longer expressed in *S.* Typhi. These genes are not necessary also pseudogenes in the test strains. We did not perform any further analysis on these genes and can therefore not draw any conclusions on their functionality in the test strains. Pseudogene formation is an important feature of host restricted *Salmonella* serovars, such as *S.* Typhi. It would be interesting to see if this also occurs in other serovars within more virulent clones. It proved to be important to validate the microarray data either by an *in silico* (using BLAST) or *in vitro* (using PCR) screening of genes. In our study, we used the hybridization results as a basis for the selection of genes for a functional analysis. The huge number of possible functional assays makes this approach laborious and time-consuming especially when no indications of the gene function are available but the genes that have been studied here exhibit sequence homologies to known genes providing information of their putative function. In order to find genetic factors characteristic for distinct clones within genetically similar *Salmonella*, it might be more effective to use methods that have the potential to identify completely new genes and small sequence alteration, such as whole genome sequencing. Nevertheless, to identify genetic patterns shared by distinct clones, comparative hybridization using a pan genomic microarray composed of genomes from different serovars covering the whole genus *Salmonella* is a useful method.

In conclusion, the success of multidrug and quinolone resistant *Salmonella* could in part be explained by the acquisition of mobile elements, especially bacteriophage DNA. Bacteriophages are known to confer a fitness advantage to their host bacterium and to be involved in virulence. To domesticate these phages by converting them into cryptic prophages might be a part in the evolution of successful resistant clones. The mechanisms behind the increased uptake and/or domestication of prophages are unclear. On one hand, multidrug and quinolone resistant *Salmonella* might be naturally more predisposed in taking up foreign DNA than sensitive ones. On the other hand, in presence of antibiotics, it might be facilitated for resistant *Salmonella* to reach and persist within host cells, like macrophages, were phage transfer and mutation frequency are enhanced. Studies have already successfully used prophage genes in order to discriminate between field isolates (27). Hence, a combination of resistance and prophage genes might serve as epidemiological markers and prospective tools for the early detection of emerging successful multidrug and quinolone resistant *Salmonella* clones. But further research has to be carried out in order to identify appropriate genes.

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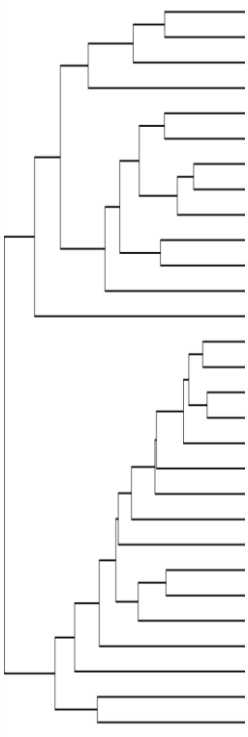
Tables and Figures



No.	Serovar	Phagetype	Source	Resistance pattern	Resistance group	Ref.
37	Derby		human		S	
15	Derby		pork	tet	R	1
26	Derby		pork		S	1
13	Derby		human		S	1
39	Newport		human		S	1
44	Newport		human	cip, nal	Q	1
42	Virchow		human		S	1
43	Virchow		human	cip, spe, str	Q	
38	Virchow		human	tmp, tet, smx, neo, nal, gen, cip, amp	Q (MR)	1
41	Saintpaul		human	apr, chl, gen, smx, spe, tet, (ffn), (str)	MR	1
27	Saintpaul		human		S	1
40	Newport		human		S	
34	Saintpaul		human	cip, nal	Q	
33	Typhimurium	DT12	human		S	
03		DT120	human		S	2, 1
32		DT170	human		S	1
16		DT12	pork		S	1
05		DT135	human		S	2, 1
23		DT12	pork		S	1
04		DT12	human		S	3, 1
29		DT12	human		S	
17		DT104	pork	spe, str, sul	R	1
31		DT120	human	amp, smx, spe, str, tet, tmp	MR	1
01		DT104	human	nal, amp, chl, str, sul, tet	Q (MR)	4, 1
06		U292	human		S	2, 1
45		DT170	human	smx, str, tet	R	1
07		U288	human	(str)	S	5, 1
02		DT104	human	amp, chl, smx, str, tet, spe (ffn)	MR	6, 1
28		DT104	human	amp, chl, cip, ffn, nal, smx, spe, str, tet, (aug2)	Q (MR)	

Figure 1: *Salmonella* field isolates used for comparative genomic hybridization, their phylogenetic relation based on all genes present on the microarrays and their resistance patterns; Antibiotics: cip, ciprofloxacin; str, streptomycin; tet, tetracyclin; nal, nalidixic acid; ffn, florfenicol; sul, sulfonamides; neo, neomycin; smx, sulfamethoxacole; amp, ampicillin; chl, chloramphenicol; spe, spectinomycin; tmp, trimethoprim; gen, gentamicin; aug2, amoxicillin + clavulanat (2:1); Resistance group: Q, reduced susceptibility to at least one fluoroquinolone; MR, resistant to four or more antibiotics; R, resistant to less than four antibiotics; S, senstitive to the antibiotics tested; Ref. (References): [1]Manuscript I, [2]32, [3]80, [4]59, [5]22, [6]31

Table 1: The number of Salmonella field isolates by serovar and phagetype used for PCR screening and their categorization based on their resistance pattern.

		Resistance group				
Serovar	Phagetype	Q	Q (MR)	MR	R	S
Derby					2	2
Infantis		1	1	2	1	3
Newport		1	1	1		1
Saintpaul			1		1	1
Typhimurium	DT104		3	1	1	2
	DT12					2
	DT120			2	2	2
	DT135					2
	DT170				2	2
	DT3					3
	U288			1	1	1
	U292					3
Virchow		1	1			1
	total	3	7	7	10	25

Q, resistant to less than four antibiotics and reduced susceptibility to at least one fluoroquinolone; Q(MR), resistant to more than four antibiotics and reduced susceptibility to at least one fluoroquinolone; MR, resistant to four or more antibiotics; R, resistant to less than four antibiotics; S, senstitive to antibiotics tested.

Table 2: The oligonucleotide primers used in this study

Gene name (synonym)	Purpose	Product size (bp)	Annealing temp. (ºC)	Sequence forward primer (5'-3')	Sequence reverse primer (5'-3')
STM3753 (*sugR*)		748	57	TTCGTTGCTTCAGACAGCT	GCTGGAGCTGTATTCCAGAT
AF261825_S012 (*trhH*)		927	57	GCTGCTTCTTTGTGACATCA	TCTTGTCAGTTAGCTCAGGG
HCM1.167 (*int*)		655	59	TTGCTGTTCTTCTACGGCAA	AATGGCCGAGCAGATCC
STY4657 (*insB*)		438	57	TTGGCCTCAACACGATTT	ACTTTGTCATGCAGCTCCA
STM2599		688	56	ATGCGTACTACGAGCGAA	TTCAGGGATTCGACGCAT
SDT0325		916	58	TCGATAGACTCCCAGCCA	ACAAACTGAAGGCAGAAGCA
SDT1852		333	59	TCCACATGCAGTCTGAATACCT	TAACGGCAAGGTATTTCACCTT
SEN1952		625	57	TTGCAGATAGCTCTCGAATTT	AGCACTTGGTGTACTGGAAGA
SDT2013		700	58	TAACTGAACATCTGCGACGA	TATCGGCCAGTATGCGTACT
SDT3832		362	56	AAGAGCTGCTGATAAGAATTGA	ATAAATTGGCGTCAACTCCT
SG1183 (*recT*)		423	57	TAACTGGTGCATCTTCGTTCT	TCAGCTTTATCAACCAGCCT
AF261825_S006		278	59	TGCTCGCAATGCTCTATCAT	TCCAGGACATTCCGTGGT
SEN1132		241	56	ATGAGCTTTGTGAGACTTGAAA	ACTTCTCCAGCAATGCACT
STY4609 (*sopE*)		490	58	AAGAACACTGAGTCTTCTGCA	TCGGCATAGCACACTCA
16S rDNA[1]		634	50	GACTACCNGGGTATCTAATCC	TGACGGGCGGTGTGTACA A
STY2231 (*cbiJ*)		616	57	TCCAGACAGGGCTGAACT	TTATGCCGACAACTGGGT
STY2249 (*pduH*)		162	57	AGCCTCTGGCACGAAGT	ATGTACGACCAGCGAGTGT
STY2208		456	58	TCGGTATTGAGTGGCTTGAT	AACGTGGAGCAAACAGTTCT
STY0594 (*fimH*)		606	59	CACCAGCGGCAATAATCA	ACGTTGGTACATTTGATAGCGA
STY2277 (*yeeF*)		431	56	GCCAGTACGGTGATAGTAGAGA	AACACTGTGATTGTGGTGCTA
STM2603		519	57	AGTGTTTGGGATAACGGGT	GAACGGGAAATACACCAT
STY4783		496	59	TTGTTGGCCTGCTCTCCT	ACCAGAGAGCCCAGACCA
STY0439 (*malZ*)		546	57	TCAGGACCGAGTGTATTACCA	AGCCAGTGACGAACGATACT
gtgA		659	58	TGGATTATCCCATCCTCATG	GTGGTGATGTGTGACCCAT
sspH1		698	56	AGGTCTTACCACCTTACCAGAC	CGTTATCTTCCAGTCCGAA
sseI		743	58	TGGTCAGCAGATTTGAACTG	GGCCATTCAGATTACTCATACCT
km^R_*recT*	For amplification of the kanamycin cassette on pKD4	1458	55/60[2]	TTATGCGGTCACGTTCTCTTCCACGCC AGCGCTTTCGACGTGTGTAGGCTGGAG CTGCTTC	ATGCCTAAACAGCCACCTATTGCAA AAGCCGACCTGCAAACATATGAATA TCCTCCTTAG
km^R_*sugR*		1458	55/60	ATGAGAATCGACAAACTTTCACTGCT TAACTTTCGTTGCTTGTGTAGGCTGGA TTACTCCTTTTTCCCTGATGCTTGGGC	TCACGCTGTGCAGTACATCATTACTT GGCTCGCCGTAGGTCATATGAATATC ATGAGAGTCTCTCCCCCTTACATTAG
km^R_*trhH*		1458	55/60	CGCAAGTAAAAGCTGTGTAGGCTGGA GCTGCTTC	AACACTATCTGCCTCATATGAATAT CCTCCTTAG
Δ*sugR*::km^R	For verification of the knock-out mutation	1245 (wt), 1612 (mt)	56	AACAGGTGAGGCTGGTGTA	CTGTTCCTTAACCGGAGGT
Δ*recT*::km^R		816 (wt), 1541 (mt)	59	GTTCTCTTCCACGCCAGC	CCTAAACAGCCACCTATTGCA
Δ*trhH*::km^R		1483 (wt), 1616 (mt)	57	CATCACCGACGGAATAAATAC	CACGAAACCTATTGGAGCA

[1]Reference: Bertelsen et al. (13); [2]5 PCR cycles with an annealing temperature of 55 ºC followed by 25 PCR cycles with an annealing temperature of 50 ºC

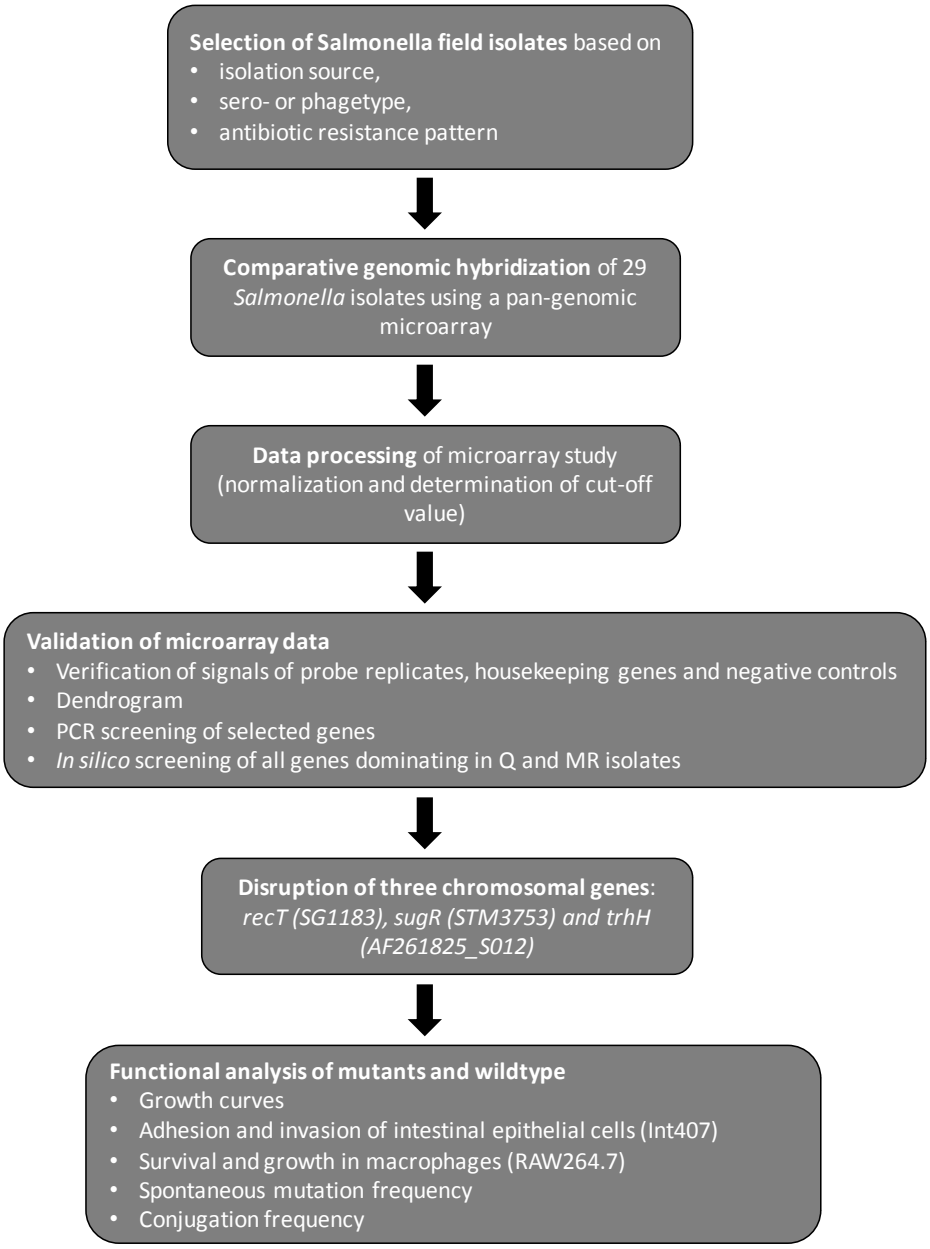


Figure 2: Flow chart of the experimental steps in this study.

Table 3 (next page): Genes dominating in quinolone and multidrug resistant *Salmonella* isolates determined by comparative genomic hybridization and PCR

Table 3 indicates the numbers of isolates where the respective genes were present determined by array-based genomic hybridization are shown; [1]The genes seem to belong to one phage cluster but none could be assigned to a known bacteriophage as no specific sequence database hits could be obtained using BLASTN and BLASTP; [2]The percentage indicates the relative proportion of the single genes within the given gene group compared to the total number of genes dominating in multidrug and quinolone resistant isolates; [3]a selection of genes was screened by PCR for presence in further 52 *Salmonella* isolates, a plus indicates if the results of this screening is in accordance with the microarray results; [4]Percentage of resistant isolates that contain the gene; [5]Percentage of sensitive strains that carry the gene; grey highlighted numbers indicate if a gene dominates in multidrug and quinolone resistant non-Typhimurium or Typhimurium isolates

Gene name	Function and other gene information	Genetic element	Typhimurium (n=16) Q (n=2)	MR (n=2)	R (n=2)	S (n=10)	Res (%)[4]	S (%)[5]	Non-Typhimurium (n=13) Q (n=4)	MR (n=1)	R (n=1)	S (n=7)	Res (%)	S (%)	Correlation of PCR to array results[3]
Mobile genetic elements					56%[2]						77%				
AF261825_S004	putative regulator protein	S. Typhimurium genomic island I (96, 103)	2	1	1		67	0	2				33	0	
AF261825_S006	putative regulator protein		2	1	1		67	0	2				33	0	+
AF261825_S012 (*trhH*)	putative pilus assembly protein		2	1	1		67	0	2				33	0	+
AF261825_S017			2	1	1	2	67	20	2				33	0	
AF261825_S026	putative ATPase		2	1	1	1	67	10	1				17	0	
AF261825_S037 (*groEL/intI1*)	GroEL/integrase fusion protein		2	2	1	1	83	10	2	1			50	0	
SDT3829	homologous to AF261825_S004		2	1	1	2	67	20	1			1	17	14	
SDT3832	homologous to AF261825_S008		2	1	1		67	0	2				33	0	+
SDT3844	homologous to AF261825_S023		2	1	1	1	67	10					0	0	
SDT3847	homologous to AF261825_S026		2	1	2		83	0					0	0	
SDT3848 (*tnpR*)	resolvase (homologous to AF261825_S027)		2	1	1	2	67	20					0	0	
SDT3850	aadA2 and partially int (homologous to AF261825_S028 and AF261825_S029)		2	1	1		67	0	1	1			33	0	
HCM1.167 (*int*)	probable integrase		2	2	1		83	0	2	1			50	0	+
HCM1.217	pseudogene, 68% identity to IS26 transposase			2			33	0					0	0	
SDT0325	probable integrase	homologous to P22-like phages (ST104 and ST64T)	2	2	2	3	100	30	1				17	0	+
SDT0330	Abc2 protein		2	1	2	2	83	20	1			2	17	29	
SDT0332C	bacteriophage protein 17		2	1	1	1	67	10					0	0	
SDT0341			2	1	2	1	83	10				1	0	14	
SDT0343	antiterminator protein		2	2	1	2	83	20					0	0	
SDT0356	probable DNA transfer protein		2	2	1	2	83	20				1	0	14	
SDT0358			2	1	2	2	83	20	1				17	0	
SDT0359	DNA transfer protein		2	1	1	2	67	20	1				17	0	
SDT1852[1]	putative DT104 specific prophage gene		2	1			50	0	2				33	0	+
SDT1857[1]	hypothetical phage protein gp12		2	1	1	1	67	10				1	0	14	
SDT2013	phage tail-like protein		2	1	2	9	83	90	4			1	67	14	+
SDT2672	putative phage-encoded DNA methylase		2	1	2	1	83	10				1	0	14	
SEN1132	excisionase (probably phage encoded)		2				33	0	1				17	0	+
SEN1952	phage protein					5	0	50	1	1	1		50	0	+
SG1183 (*recT*)	recombination and repair protein		2	1	1		67	0	2				33	0	+
SEN1935		homologous to ST64B phage	2	1	2	3	83	30				1	0	14	
SL1943			2	1	2	3	83	30				1	0	14	
SL1944			2	1	2	3	83	30				1	0	14	
STM2590	tail assembly protein I-like	Gifsy-1 phage (95)	2	2	2	9	100	90	4			2	67	29	
STM2592	phage tail component L-like protein		2	2	2	9	100	90	4			1	67	14	
STM2599 (*gipA*)	putative virulence protein			1	1	9	33	90	3				50	0	+
STM2601	minor capsid protein FII		2	2	2	9	100	90	4			1	67	14	
STM2603	phage head-like protein		2	2	2	9	100	90	4			1	67	14	variable
STM2604	phage head-like protein		2	2	2	9	100	90	4			1	67	14	
STM2605	head-tail preconnector-like protein		2	2	2	9	100	90	4			1	67	14	
STM2606	head-tail preconnector-like protein		2	2	2	9	100	90	4			1	67	14	
STM2608	terminase-like large protein		2	2	2	9	100	90	4			1	67	14	
STM2617	antiterminator-like protein			1	1	10	33	100	3				50	0	
STY4657 (*insB*)	insertion element IS1 protein InsB		1	2			50	0					0	0	+
Putative virulence genes					9%						0%				
STM3753 (*sugR*)	ATP binding protein, variable region of SPI-3 (98, 99)		1	2	1		67	0				1	0	14	+
STY0166 (*ppdD*)	major pilin subunit, inducer of IL-8 and TNF-alpha release in EHEC O157:H7 (97)		2	1	1	3	67	30	3			4	50	57	
STY0594 (*fimH*)	FimH protein precursor, contributes to adhesion and invasion (100)		1	2			50	0				2	0	29	ubiquitious
STY2627a (*gtrA2*)	bactoprenol-linked glucose transferase (O-antigen conversion, present on P22-like phages)			2			33	0			1	1	17	14	
Genes with other function					20%						15%				
SDT1840[1]	hypothetical lipopolysaccharide 1,2-N-acetylglucosaminetransferase		2	1	1	1	67	10	1			3	17	43	
STM0855	electron transfer protein beta subunit		1	2	1	1	67	10				1	0	14	
STY0043	possible sulfatase regulatory protein homologous to 2-keto-3-deoxygluconate			2		1	33	10	1			3	17	43	
STY0184 (*ygbK*)	permease		1	2	1	3	67	30	1			4	17	57	
STY1582 (*asr*)	acid shock protein				1	3	17	30	4		1	2	83	29	
STY0439 (*malZ*)	maltodextrin glucosidase			1	1	2	33	20	3	1	1		83	0	almost ubiquitious
STY0561 (*ybbB*)	tRNA 2-selenouridine synthase		1	2	2	3	83	30	1	1		6	33	86	
STY2115 (*cutC*)	putative copper homeostasis protein		2	1	1	2	67	20	2		1	5	50	71	
STY2231 (*cbiJ*)	precorrin-6x reductase (pseudogene)		1		1	1	33	10	4	1	1	2	100	29	ubiquitious
STY2249 (*pduH*)	involved in 1,2-propanediol degradation						0	0	3				50	0	almost ubiquitious
STY2277 (*yeeF*)	putative amino acid transporter protein			2			33	0				2	0	29	ubiquitious
STY2555 (*nuoE*)	NADH dehydrogenase I chain E					1	0	10	4			2	67	29	
STY2846 (*yfiM*)	homologous to alpha-ketoglutarate permease		2	2	2	3	100	30	3		1	7	67	100	
STY3210 (*gcvH*)	glycine cleavage system H protein			2		3	33	30	1				17	0	
STY3556 (*yhdH*)	possible oxidoreductase			2	1	3	50	30	2	1	1	3	67	43	
STY3732 (*rpoB*)	DNA-directed RNA polymerase, beta-subunit		2	2	2	3	100	30	3		1	7	67	100	
STY4364 (*rpsC*)	30S ribosomal subunit protein S3		1	2	1	3	67	30	1			5	17	71	
Genes with unknown function					15%						8%				
SDT1841[1]			2	1	2		83	0					0	0	
SEN1389			2	1	1	1	67	10	2				33	0	
STY0352			1	2	1	1	67	10	1			4	17	57	
STY0357 (*yafK*)					2	2	33	20	4		1	2	83	29	
STY0579			1	2		2	50	20	1			5	17	71	
STY1759				2	1	3	50	30	4		1	4	83	57	
STY2208			1		1	3	33	30	3				50	0	ubiquitious
STY4411				2	1	1	50	10				5	0	71	
STY4728a	pseudogene		1	2	1	3	67	30	2	1	1	6	67	86	
STY4783			2	1	1	3	67	30	3		1		67	0	almost ubiquitious

Table 4: The relative distribution of phage genes (in percent) in the *Salmonella* isolates compared to all phage genes present on the microarray (n=282)

No.	Sero- or phagetype	Resistance group	% Phage genes
	Typhimurium isolates		
2	DT104	MR	55
7	U288	S	54
28	DT104	Q(MR)	52
45	DT170	R	49
23	DT12	S	49
17	DT104	R	48
1	DT104	Q(MR)	48
4	DT12	S	47
29	DT12	S	45
32	DT170	S	45
33	DT12	S	44
5	DT135	S	43
16	DT12	S	42
6	U292	S	42
31	DT120	MR	33
3	DT120	S	29
	Non-Typhimurium isolates		
44	Newport	Q	48
43	Virchow	Q	46
26	Derby	S	37
38	Virchow	Q(MR)	37
34	Saintpaul	Q	35
40	Newport	S	34
42	Virchow	S	30
13	Derby	S	26
39	Newport	S	24
15	Derby	R	21
27	Saintpaul	S	21
41	Saintpaul	MR	20
37	Derby	S	17

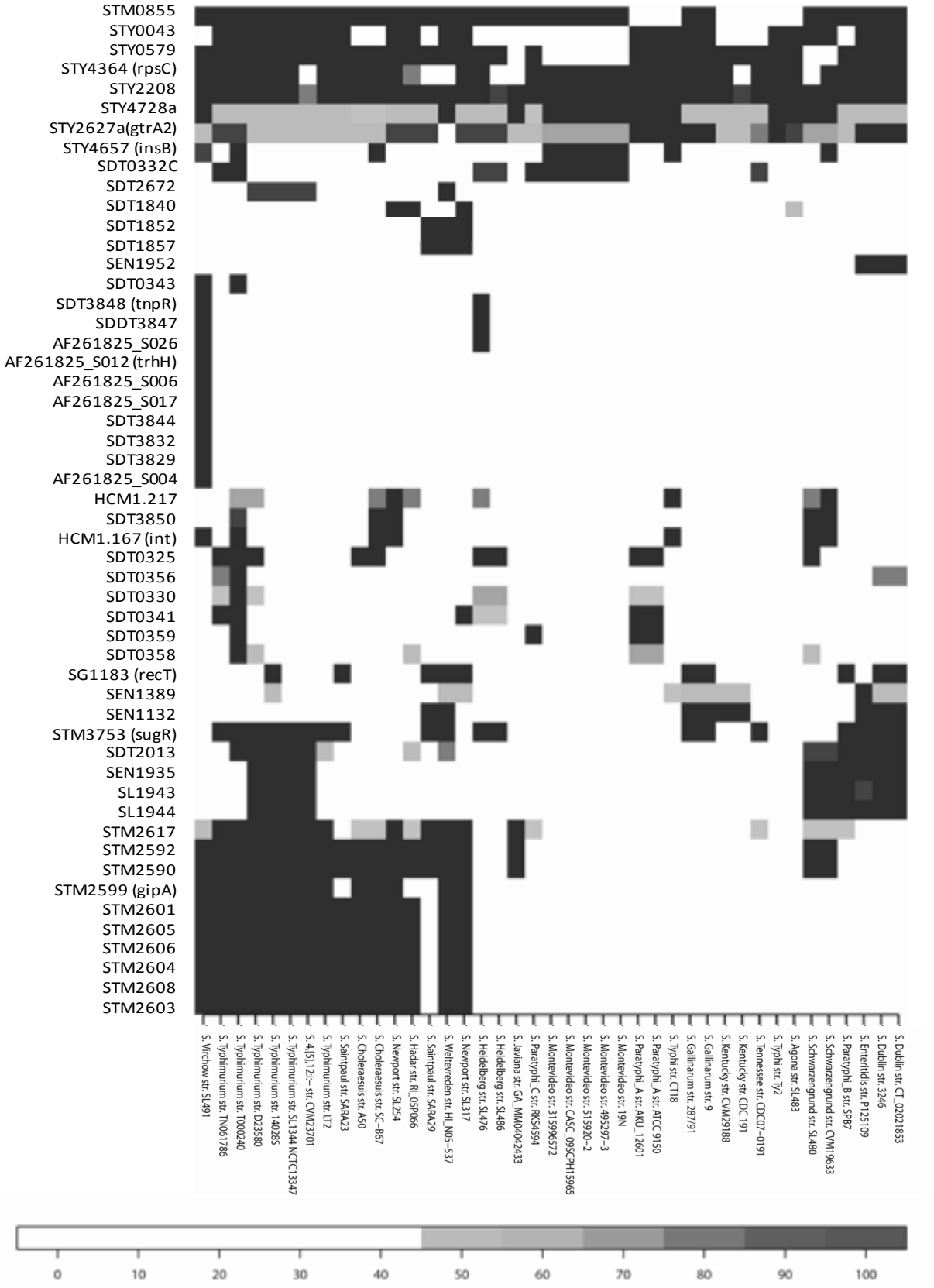


Figure 3: Sequence similarities of the genes that were dominant in quinolone and multidrug resistant field isolates (vertical axis) in sequenced *Salmonella* genomes (horizontal axis). Genes that were ubiquitous in all sequenced genomes are not shown. Sequence similarity is indicated by shading from dark (100% sequence identity) to light (0% sequence identity) in pairwise BLAST alignments.

Table 5: Percentages of selected genes in Salmonella field isolates (n=81) categorized after their resistance pattern determined by PCR and DNA microarray

	non-Typhimurium (n=35)				Typhimurium (n=46)			
Gene (synonym)	Q (n=12)	MR (n=4)	R (n=5)	S (n=14)	Q (n=4)	MR (n=7)	R (n=8)	S (n=27)
AF261825_S012 (*trhH*)	42*	25*	0	0	100*	43	25	4
SEN1132	8	25*	0	0	100*	29	13	7
STY4657 (*insB*)	8	25	0	7	25*	57*	25	4
HCM1.167 (*int*)	58*	50*	0	0	100*	71*	25	4
AF261825_S006	17*	0	0	0	100*	43*	25*	0
SG1183 (*recT*)	17	25	20	7	100*	43	25	7
SDT3832	17*	0	0	0	100*	43	13	4
SEN1952	8	50*	40*	0	0	29	25	26
SDT1852	8	0	20	7	100*	14	13	7
STM3753 (*sugR*)	25	50	20	14	75	100	88	63
STM2599 (*gipA*)	50*	0	0	7	0	57	75	89
SDT0325	8	0	0	0	100*	86	88	44
SDT2013	33	25	20	14	100	71	100	81
STY4609 (*sopE*)	75*	50	0	36	0	0	0	4

*Significant difference compared to sensitive isolates ($P < 0.05$)

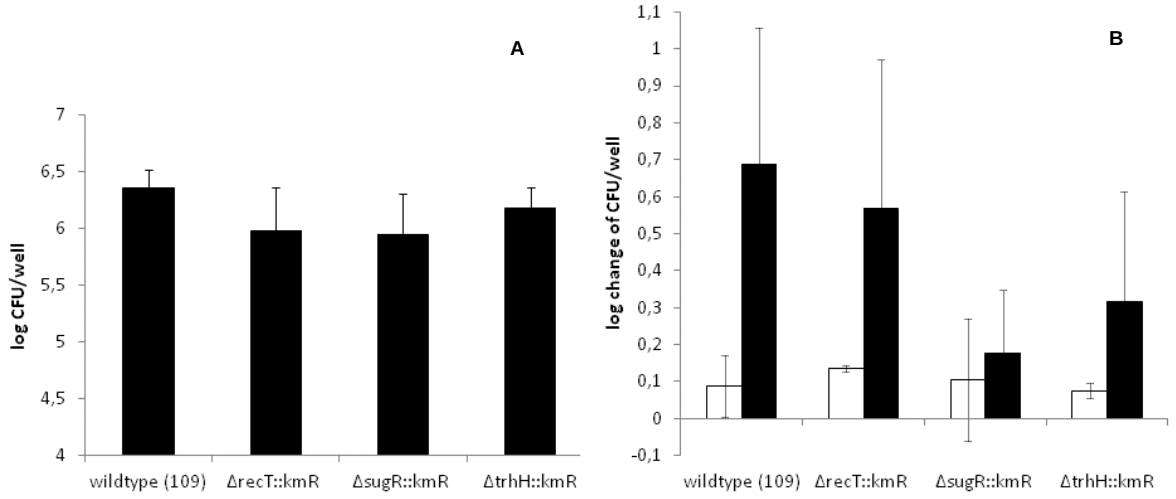


Figure 4: Phagocytosed (intracellular) bacteria at 2 h post infection (A) and subsequent increase of the intracellular counts (CFU/well) (B) at 4 h (white columns) and 24 h (black columns) post infection of the wildtype strains 109 (*S*. Typhimurium DT104) and three isogenic mutants in the macrophage cell line RAW264.7; Error bars represent standard errors of the mean (SEM) of three independent experiments

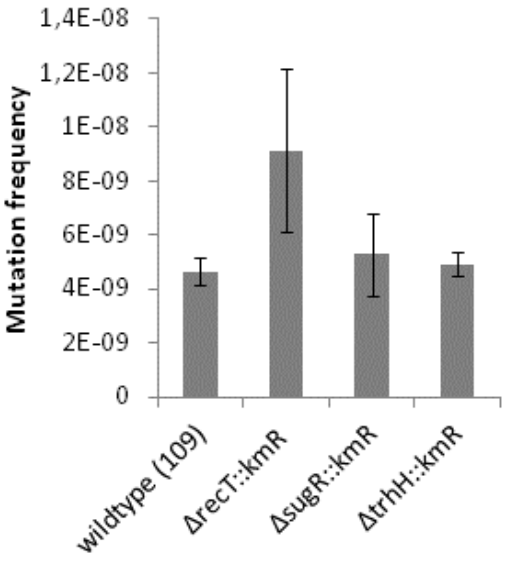


Figure 5: Spontaneous mutation frequencies of the wildtype strains 109 (*S*. Typhimurium DT104) and three isogenic mutants determined on 100 µg/ml rifampicin; Error bars represent standard errors of the mean (SEM) of three independent experiments

Manuscript III

The role of macrophages and mutation frequency in the evolution of virulent and quinolone resistant *Salmonella*

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Abstract

Quinolone resistant *Salmonella* are reported to cause more severe humans illness compared to sensitive ones but the underlying bacterial and host factors are largely unknown. Mechanisms that lead to the evolution of more virulent and quinolone resistant *Salmonella* are also not clear. Quinolone resistance is mostly caused by point mutations and can develop in *Salmonella* during infection. We assessed the capability of ciprofloxacin (CIP) resistant Salmonella to adhere and invade intestinal epithelial cells and to survive in macrophages in absence and presence of CIP using cell culture models (the epithelial cell line Int407 and the macrophage cell line RAW264.7). We determined spontaneous mutations frequencies of ciprofloxacin (CIP) resistant and susceptible *Salmonella* under non-selective conditions. Moreover, we analyzed the frequency at which CIP sensitive *Salmonella* isolates can acquire a reduced CIP susceptibility after macrophage infection in presence and absence of CIP. We found strain dependent differences in the mutation rate, the ability to invade intestinal epithelial cells and to replicate within macrophages but there was no correlation between virulence, mutation rate and resistance pattern. The presence of clinically relevant CIP concentrations decreased but did not eliminate the number of CIP resistant intraphagocytic *Salmonella*. We observed an increased frequency of cells that could acquire a reduced CIP susceptibility (2 – 8 x MIC) after macrophage infection (for 4 h). When CIP was added to the extracellular medium, this frequency increased markedly. Thus, more virulent *Salmonella* strains, that are likely to persist in macrophages for prolonged times might become resistant to quinolones, particularly when the host is treated with these antibiotics.

Introduction

Salmonellosis is a foodborne diarrheal disease that occurs worldwide and that is one of the most reported zoonotic infections in the EU (53,62). It is caused by a variety of *Salmonella enterica ssp. enterica* serovars but the incidence rate and the disease outcomes differ among *Salmonella* (34). It has been reported that the severity of disease for humans is increased by infection with quinolone resistant *Salmonella (13,28,29,32,55).* Salmonellosis usually leads to a self-limiting gastroenteritis but patients infected with quinolone resistant *Salmonella* have a higher risk for hospitalization, for mortality and for bacteremia and a longer duration of diarrhea compared to patients infected with susceptible *Salmonella*. Moreover, fluoroquinolones, frequently used to treat severe cases of salmonellosis, lose their efficacy in patients infected with *Salmonella* that exhibit low level resistance (MIC 0.12 - 1 µg/ml; according to Eucast breakpoints) to flouroquinolones which may worsen the disease outcome (52).

Fluoroquinolones are relatively new broad-spectrum antibiotics that began to receive attention in the 1980s and are now widely used to treat clinical and veterinary infections (16). Due to the increased occurrence of multiple resistant *Salmonella*, the usage of fluoroquinolones provided an effective alternative when antimicrobial therapy was required. In 2010, they belonged to the four most used therapeutic groups for humans in Denmark of which ciprofloxacin (CIP) was used most commonly (6). Flouroquinolones target the type II topoisomerase enzymes, DNA gyrase and topoisomerase IV, leading to a DNA replication arrest and double stranded DNA breaks (31,35). Quinolone resistance in *Salmonella* is most often conferred by point mutations that result in an aminoacid change of the DNA gyrase subunit GyrA. A single point mutation within this gene can mediate resistance to nalidixic acid and low level resistance to fluorquinolones (46). Mutations in the subunit GyrB of the DNA gyrase occur less frequently and mutations in parE and parC of the topoisomerase IV are rare and only found in addition to point mutations in gyrA. They might play a minor role in the acquisition of high level resistance (31). Furthermore, resistance often involves a decreased expression of outer membrane porins and/or an increased production of efflux proteins, mainly of the AcrAB-TolC efflux pump. Resistance can be acquired through point mutations in regulatory genes of this efflux system resulting in its overexpression (36,48,63). Plasmid mediated quinolone resistance also occurs and is increasingly reported (59). A combination of these resistance mechanisms may enhance the tolerance towards

fluorquinolones. It has been suggested that quinolone resistance can be transferred from food animals to humans (2) but it can also emerge from mutations in susceptible strains within a patient during the course of treatment (37,40,45). It has been shown that weak mutators (bacteria with higher constitutively expressed mutation rates) can drive the evolution of fluoroquinolone resistance (44) and indeed, quinolone resistant *Escherichia coli* and *Salmonella* are observed to exhibit slightly elevated spontaneous mutation rates under non-selective static laboratory conditions (38,39). Quinolones have been shown to accumulate in phagocytes by passive and active transport mechanisms. The intracellular upconcentration of CIP corresponds to approx. 4 - 10 times the extracellular concentration (9,10,25,57). The frequency at which susceptible *Salmonella* can become resistant or can reduce their MIC to fluoroquinolones in particular host environments, e.g. within phagocytic cells, where the bacteria are under strong selective pressure has not been investigated so far.

The aim of this study was to indirectly estimate the virulence potential of quinolone resistant *Salmonella* compared to susceptible ones using cell culture models of epithelial cells and macrophages. We studied macrophages infection of low-level CIP resistant *Salmonella* in presence of CIP to investigate if the antibiotic has an effect on survival and growth of the intracellular bacteria. Furthermore, we were interested in the impact of mutation rates and on the evolution of quinolone resistance. Thus, we investigated the spontaneous mutation frequency to rifampincin of quinolone resistant *Salmonella* under static non-selective condition and the CIP mutation frequency of CIP susceptible *Salmonella* after different pretreatments.

Materials and Methods

Bacterial strains and growth conditions. This study included 48 field isolates of *Salmonella enterica* spp. *enterica*, representing different sero- and phagetypes originating from sporadic or outbreak cases from humans, from production animals as well as from meat (Table 1). 21 isolates were quinolone resistant of which at least 17 isolates exhibited low-level ciprofloxacin (CIP) resistance (MIC 0.12 - 1 µg/ml; according to Eucast breakpoints). The remaining non-quinolone resistant isolates were characterized as multidrug resistant (MR, resistant to four or more antibiotic agents), resistant (R, resistant to less than four antibiotic agents) and fully susceptible to the tested antibiotics (S). As *S.* Typhimurium has been the dominant serovar in humans in Denmark since 2008 (3-5), we included 14 *S.* Typhimurium

isolates belonging to different phagetypes. The animal and food isolates were obtained from the Danish National Food Institute and the human isolates from the Statens Serum Institute. Table 1 gives an overview of the isolates and the experiments where they were used. In he study where we investigated quinolone adaptation within macrophages, we included the mutant Δ*recT*::km^R, a field isolate of *Salmonella* Typhimurium DT104 (no. 109) with a gene deletion in *recT* (SG1183) (unpublished manuscript) For the adhesion/invasion study, we used the control strains *S.* Typhimurium 4/74 and the isogenic mutant invH201::Tn*pho*A (61). To investigate spontaneous mutation frequencies, we included *Escherichia coli (E. coli)* ATCC 25922 and *E. coli* CSH 115, a hypermutator strain with a mutation in *mutS* (42), as control strains. Overnight cultures were obtained by growing a colony from sheep or calf blood agar in 10 ml LB or BHI broth for 16-20 h at 37 °C. For bacterial enumeration, colony counts were determined by plating 3 times 10 µl droplets on LB or BHI agar.

Cell cultures. The Int407 human epithelial cell line (ATCC CCL-6^{TM}) was obtained from the Danish National Veterinary Institute (Lindholm) and the RAW264.7 murine macrophage-like cell line (ATCC TIB-71^{TM}) were provided by the Danish National Food Institute. The cells were cultured at 37 °C in an atmosphere of 95% O_2 and 5% CO_2. Int407 cells were maintained in modified Eagle's minimal essential medium (MEM; ATCC) supplemented with 10% fetal bovine serum (FBS; ATCC) and 0.1 % penicillin/streptomycin (Gibco). RAW264.7 were cultured in Dulbecco's modified eagles medium (DMEM; Invitrogen) supplemented with 10 % FBS (Hyclone; Thermo Scientific) and 0.1 % HEPES buffer (1 M; Gibco), 0.1 % sodium pyruvate (100 mM; Gibco) and 0.1 % penicillin/streptomycin. One day prior to bacterial infection the cells were grown without antibiotics. During bacterial infection, the RAW264.7 cells were grown without FBS.

Antimicrobial susceptibility testing. The minimum inhibitory concentrations (MIC) for fifteen antimicrobial agents were determined using commercially dehydrated antimicrobial agents in microtitre wells (SensititreTM; TREK Diagnostic Systems Ltd., UK) as part of the Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (8). Additionally, the resistance to rifampicin (RIF) and CIP was determined using the agar gel dilution technique following CLSI guidelines (7).

Growth curves. For the macrophage survival study, we incubated the infected cell culture for 24 hours, a time sufficient for the bacteria to replicate. Growth curves in culture medium were established prior to the macrophage experiment to analyze for changes in thegrowth capability. An overnight culture was diluted 1000 times in LB broth or DMEM supplemented

with 0.1 % HEPES buffer, 0.1 % sodium pyruvate. The cultures were grown without shaking at 37 °C in a Bioscreen C reader (Thermo Labsystems) for 8 h (measurement intervals: 10 min; OD: 600 nm). Growth was measured in triplicates.

Adhesion and invasion of epithelial cells. Bacterial adhesion and invasion to Int407 cells was determined by a gentamicin protection assay essentially as described previously (107, 108). The cells were seeded into 12 well culture plates (Costar) at a concentration of 5 x 10^5 cells per well and incubated overnight. 1 h prior to inoculation, the monolayers were washed twice with MEM and incubated in 1 ml MEM. To obtain exponential cultures of the bacteria, overnight cultures were diluted to 10^7 CFU/ml and incubated for 3 h with aeration (in a shaking incubator with loose lids on the culture tubes). Then, the cultures were washed and diluted to an OD_{600} of 0.1 with phosphate buffered saline (PBS). 100 µl were used to infect the Int407 using a moi of 20. After incubation for 1 h, the monolayers were washed twice with PBS and incubated with 1 ml MEM containing 100 µg/ml gentamicin. Before and 2 h after the addition of gentamicin, the cells were washed once with PBS and lysed using 1 ml PBS per well containing 0,1% Triton X-100 (Sigma). For direct counts, appropriate dilutions of the lysates were plated on LB agar to determine the number of adherent (before gentamicin treatment) and intracellular (after gentamicin treatment) bacteria. Triplicate experiments were performed.

Survival within macrophages in presence and absence of ciprofloxacin. Survival and growth of *Salmonella* in macrophages was carried out essential as described by Humphreys et al. (33). RAW264.7 cells were seeded into 12 well tissue culture plates (Costar) at a concentration of 5 x 10^5 cells per well. After overnight incubation, the cells were washed once with PBS and incubated for 1 h in DMEM before bacterial inoculation. Bacteria of an overnight culture were washed and diluted to an OD_{600} of 0.1 with PBS containing 10% heat inactivated mouse serum and opsonized for 30 min at 37 °C. 100 µl of this cell suspension were used to infect the RAW264.7 cells using a moi of 20. The cells were centrifuged (1000 g, 5 min) to synchronize the infection and incubated for 1 h. Then, the monolayers were washed once with PBS and overlaid with 1 ml DMEM containing 100 µg/ml gentamicin. After an additional hour, the washing step was repeated and the cells were incubated with 1 ml DMEM containing 10 µg/ml gentamicin. 2, 4 and 24 hours post infection, the cells were washed as described above and lysed using 1 ml PBS per well containing 0,1% Triton X-100 (Sigma). To determine the number of intracellular bacteria, direct colony counts of the lysates were made as described above. When indicated, CIP was added to the cell culture medium at

1 h prior or 1 h post infection. Once CIP was added, the concentration (0.25 or 5 µg/ml) was continuously kept at the same level until the end of the experimental period. Viability of macrophages was determined microscopically using 100 µl/ml Trypan blue (Invitrogen) to stain the dead cells. Triplicate experiments were performed but viability of macrophages and survival in presence of CIP was determined twice.

Spontaneous mutation frequency. For this experiment, 38 *Salmonella* isolates were chosen. The 20 fluoroquinolone resistant isolates corresponded largely to the fluoroquinolone susceptible isolates with respect to the serovar, phagetype and the resistance pattern to other antimicrobials. A proportion of an overnight culture was transferred to fresh BHI medium to obtain a 1000 fold dilution. An equal amount of the overnight culture was plated on selective BHI agar containing 100 µg/ml rifampicin in order to confirm that no pre-existing mutants were present. The prepared culture was incubated at 37 °C with shaking until OD_{600} of 2 was reached. 10 ml were centrifuged (6000 g, 10 min), resuspended in 100 µl BHI and the entire volume was plated evenly on BHI agar containing 100 µg/ml rifampicin. In the same time, appropriate dilutions were plated on non-selective BHI agar to obtain total counts. The mutation frequency was calculated by dividing the number of spontaneous rif^R mutants by the total counts. Triplicate experiments were performed.

Mutation frequency after macrophage infection and treatment with ciprofloxacin. Infection and incubation of macrophages with quinolone sensitive *Salmonella* was carried out as described above. In parallel, macrophage-free culture medium (DMEM containing 0.1 % HEPES buffer and 0.1 % sodium pyruvate) was inoculated with 10^6 CFU/ml of the bacterial inocula which corresponds to the number of intracellular bacteria after phagocytosis (Figure 1). At 1 h post infection, CIP in a concentration corresponding to 2 or 20 times the MIC of the respective strain was added to the macrophage containing culture medium. At the same time, CIP in a concentration of 0.5 times the MIC was added to the macrophage-free culture medium. A control without antibiotics was included in both assays. At 4 h post infection, the cells were washed once with PBS and lysed using 1 ml PBS containing 0.1% Triton X-100 per well. The lysates and the samples containing the macrophage-free culture medium were diluted 1:10 with LB and incubated at 37 °C for 16 h to an approximate OD_{600} of 1. Then, 100 µl of the culture were plated evenly on LB agar containing CIP corresponding to 2 (0.032 µg/ml for strain no. 109 and 101), 4 (0.032 µg/ml for strain no. 2 and SH-9.2) or 8 (0.064 µg/ml for strain no. 2 and SH-9.2 or 0.128 µg/ml for strain no. 101 and 109) times the MIC of the respective strain. In the same time, appropriate dilutions were plated on non-selective LB

agar to obtain total counts. The mutation frequencies after the different pretreatments were calculated by dividing the number of colonies from the selective agar plates by the total counts. Triplicate experiments were performed.

Statistics. Colony counts were log transformed where appropriate and standard deviations and standard errors of the mean were calculated. Statistical significances of differences were calculated with one-way analysis of variance (ANOVA) followed by Turkey's multiple comparison test or unpaired t test using GraphPad Prism (GraphPad Software).

Results

***In vitro* adhesion and invasion of intestinal epithelial cells.** To indirectly investigate the virulence potential of quinolone resistant *Salmonella* isolates compared to strains that are sensitive or have another resistance pattern cell culture models were used. The number of bacteria capable of adhering to intestinal epithelial cells until 1 h post infection was very similar among all field isolates studied (Figure 2). On average, adhesion was achieved by 5.29 log CFU/well of quinolone resistant isolates, 5.24 log CFU/well of isolates with another resistance pattern and 5.29 CFU/well of sensitive isolates. The ability to invade was more diverse and varied even within isolates of the same serovar and phagetype. Sensitive *S.* Derby (no. 13), multidrug resistant *S.* Tyhimurium DT104 (no.2) and quinolone and multidrug resistant *S.* Saintpaul (no. 34 and 41) showed reduced invasiveness. The intracellular counts from the latter three isolates were significantly lower compared to all the other isolates whereas the counts of *S.* Derby no. 13 were only significantly lower than 5 of the other isolates, including *S.* Derby no. 26 ($P < 0.05$). Only one isolate of sensitive *S.* Staintpaul (no. 27) had higher intracellular counts than the reference strain *S.* Tyhimurium 4/74 but this was not significant ($P = 0.65$). No correlation between the capacity to adhere or invade and the resistance pattern could be observed. The validity of the epithelial cell culture model was verified by showing a decrease of 0.66 log CFU/well (SEM 0.12) for adhesion and 0.88 log CFU/well (SEM 0.04) for invasion of the control mutant ΔinvH201::Tn*phoA* (61) compared to its wildtype strain *S.* Typhimurium 4/74.

Survival and growth in macrophages in absence of quinolones. The direct counts of the inocula used to infect macrophages were slightly more variable probably due to the 30 min opsonization period directly before inoculation. Thus, to standardize the inocula, a correction

factor was calculated by subtracting the counts from the inocula by 10^7 CFU/well (the ideal inoculum). The number of phagocytosed bacteria was determined by subtracting this correction factor from the direct counts of intracellular bacteria determined at 2 h post infection. At that time, an average of 6.2 log CFU/well were taken up by the macrophages corresponding to approx. 1.1 bacteria per macrophage which was similar for all isolates. Figure 4 shows the percent change of the intracellular counts after 4 and 24 h post infection compared to 2 h post infection. The number of intracellular bacteria increased slightly over time of the experimental period, except for multidrug resistant *S.* Infantis (no. 97) that showed a decrease of 0.43 log CFU/well after 24 h compared to 2 h post infection. Except for this *S.* Infantis isolate and multidrug resistant *S.* Typhimurium DT104 (isolate no. 2), growth in culture medium (LB and DMEM) was equal for all bacteria. The area under the growth curves was 1.3 times smaller for these two isolates than the average. A trend that quinolone resistance has an impact on invasion and proliferation within macrophages could not be observed, except for a small tendency at 4 h post infection where the intracellular counts of the quinolone resistant isolates showed an average increase of 0.23 log CFU/well compared to 0.11 log CFU/well for the other bacteria. This was however not significant ($P < 0.30$). The cytotoxic effect of the bacteria on the macrophages was determined by microscopic counts of viable and dead macrophages (Figure A1 in the Appendix). The average number of viable macrophages ranged from 65% (SD: 10%) at 2 h post infection, 60 % (SD: 8%) at 4 h post infection to 38 % (SD: 17%) at 24 h post infection compared to the non-infected control were 75 % (SD: 3%) of the macrophages were viable during the whole time period. The percentage of viable macrophages varied among the isolates but did not seem to correlate well with the intracellular counts or with the resistance pattern of the bacteria.

Macrophage survival in presence of ciprofloxacin. To investigate, if the presence of quinolones has an effect on macrophage survival of *Salmonella* with low level resistance to fluoroquinolones, we analyzed the survival of *S.* Typhimurium DT104 (no. 28) within macrophages in presence of CIP at a concentration of 1 x (0.25 µg/ml) or 20 x (5 µg/ml) the MIC. The latter concentration was chosen as it resembles a therapeutic concentration in humans (41). CIP was added 1 h prior or 1 hour after infection. At 2 and 4 h post infection, an effect could only be observed were 5 µg/ml CIP were added 1 h prior to infection (Figure 4), resulting in a reduction of 0.87 and 1.34 log CFU/well respectively compared to the control without CIP. After 24 h post infection a clear influence of the antibiotic could be seen in all samples that have been incubated in presence of CIP. At that time, a reduction of the intracellular bacteria of approx. 1.32 log CFU/well and 2.48 log CFU/ml was observed for the

low CIP concentration (0.25 µg/ml) and the high CIP concentration (5 µg/ml), respectively, compared to the control. Overall, CIP reduced but did not eliminate the amount of intracellular bacteria in low and high concentration.

Spontaneous mutation frequency. As rifampicin resistance is conferred by single point mutations in *rpoB,* it is a good indicator for determining spontaneous mutation rates in bacteria (213). The initial MICs to rifampicin of all isolates were similar and ranged from 8 to 16 µg/ml. The mutation frequencies to 100 µg/ml rif of the quinolone resistant and susceptible *Salmonella* isolates are shown in Figure 5. Surprisingly, the three human isolates of *S.* Infantis exhibited an elevated mutation frequency that was approx. 6.2 fold higher than the average and significantly different from most of the other isolates for 134 and 132 ($P < 0.05$). The average mutation frequency from all quinolone resistant isolates was 3.7×10^{-09} and could not be distinguished from susceptible isolates or isolates with another resistance pattern regardless if the mutation frequencies of *S.* Infantis were included or excluded from the average. The spontaneous mutation frequency for human isolates (n=28) including *S.* Infantis was 1.9 times higher than for food isolates (n=4). Excluding *S.* Infantis, the human isolates showed only a 1.2-fold higher mutation frequency than the food isolates. We verified the method by showing that the hypermutator control strain *E. coli* CSH115 had an approx. 100 fold increased mutation frequency compared to *E. coli* ATCC 25922.

Mutation frequency after macrophage infection and ciprofloxacin treatment. Whether macrophage infection and the subsequent exposure to quinolones has an influence on the CIP mutation frequency of quinolone (CIP and nalidixic acid) sensitive bacteria was determined by adding CIP in different concentrations to the extracellular medium at 1 h post infection. For comparison, the effect of direct exposure to a subinhibitory concentration of CIP on the mutation frequency of these bacteria was determined. The phagocytic cell lysates were transferred to LB medium and grown for 16 h where the total counts amounted to approx. 10^9 CFU/ml. This corresponds to approx. 11 generations considering that the macrophage lysates contained approx. 10^6 CFU/ml of bacteria cells. The cells that experienced a reduction in their susceptibility to CIP might have had a longer generation time than the other cells. As we do not know the precise length of this generation time, we could not calculate the original amount of CIP adapted cells within the macrophages at 4 h post infection However, we used the non-pretreated control culture as a baseline (Table 2) and subtracted the baseline frequencies from the mutation frequencies that resulted from the indicated treatment (Figure 6). The CIP MICs prior to infection were 0.008 µg/ml for the strain no. 2 and 0.016 µg/ml for

the strain no. 109, 101 and 109 Δ*recT*::km^R. The latter strain was a mutant included to analyze the influence of *recT* on the intracellular emergence of mutations. Overall, growth was only observed on selective media containing CIP in concentrations of 0.032 (all strains) and 0.064 µg/ml (only strain 2). If not otherwise indicated, reduced CIP susceptibility stands for growth on 0.032 µg/ml CIP. The frequency of cells with reduced CIP susceptibility was highest for multidrug resistant isolate no. 2 under all investigated conditions. In the control cultures pretreated only with culture medium, the frequency was approx. 6.5-fold higher for strain no. 2 than for the other strains (no. 109 and 101) (Table 2). The direct exposure to subinhibitory concentrations of CIP (0.5 x the MIC) generated the highest mutation frequencies (except for strain 101). Within macrophages, the frequency of reduced CIP susceptibility was generally low in absence of CIP (except for no. 109) and in presence of low concentrations of CIP (2 x MIC) but it was higher than in the control culture without any pre-treatment (except for strain no. 2). The infection of macrophages and the subsequent exposure to high concentration of CIP (20 x MIC) resulted on average in a 3-fold higher mutation frequency than when only low concentrations of CIP were added. Similar results could be obtained for colonies of strain no. 2 selected on 0.064 µg/ml CIP (Figure 6). Interestingly, compared to its wildtype, the mutant Δ*recT*::km^R showed increased frequencies of CFUs with reduced CIP susceptibility only when pre-treated with CIP. The mutant showed a 2.7-fold higher mutation frequency after infection of macrophages and subsequent CIP exposure (0.32 µg/ml) and a 2.3-fold elevated frequency when directly exposed to sub-inhibitory concentration of CIP. These results were however not statistically significant (P = 0.23 and P = 0.56, respectively).

Discussion

It has been suggested that the acquisition of antibiotic resistance, particularly quinolone resistance, is associated with an increase in virulence and/or fitness of the pathogen (29) but the underlying bacterial and host factors are largely unknown. Mechanisms that lead to the evolution of more virulent and quinolone resistant *Salmonella* are also not clear. Using cell cultures, we assessed the capability of ciprofloxacin (CIP) resistant *Salmonella* to adhere and invade intestinal epithelial cells and to survive in macrophages in absence and presence of CIP. Furthermore, we determined spontaneous mutation frequencies (to rifampicin) of CIP resistant *Salmonella* under non-selective conditions. Finally, we assessed the frequency at

which CIP sensitive *Salmonella* isolates can acquire a reduced CIP susceptibility after different pretreatment (macrophage infection and CIP exposure).

Quinolone resistant *Salmonella* did not show an increased invasion or adhesion capability to epithelial cells compared to *Salmonella* being susceptible or resistant to other antibiotics. Interestingly, considerable differences could be detected within isolates of serovar *S.* Derby and *S.* Saintpaul and among isolates of the *S.* Typhimurium phagetype DT104. Even though these bacteria adhered equally well to the epithelia cells, they seem to be impaired in their ability to trigger SPI-1 (*Salmonella* pathogenicity island 1) mediated invasion of host cells (21). This supports other studies that have observed that the disease outcome of non-typhoidal *Salmonella* in humans is divers even within strains of the same serovar and that it does not only depend on the host immune system but also on the virulence potential of the bacterial strain (20,27,34,65).

The intracellular counts within macrophages short after phagocytosis (2 h post infection) were equal for all the test strains, also for those that appeared to have a low ability to invade epithelia cells. Unlike non-phagocytic cells, *Salmonella* can enter macrophages by SPI-1-dependend and independent mechanisms (24). At 4 h post infection, intracellular levels of *Salmonella* with low level CIP resistance seemed to have slightly more increased in macrophages than CIP sensitive isolates but this was without statistical significance. However, At 24 h post infection, a clear difference could be observed for *S.* Infantis, where the CIP resistant isolate (no. 134) showed 1.4 times more intracellular bacteria than the CIP susceptible one (no. 97). The latter isolate (no. 97) was also impaired for growth in LB broth and might rather lack a general fitness factor than a macrophage survival factor. Growth in LB was also reduced for MR *S.* Typhimurium DT104 (no. 2) but that was not reflected during growth in macrophages. A study from Wang et al. (60) showed contradictory results as they observed a clear decrease of CIP resistant *Salmonella* in their ability to invade and grow host cells (macrophages and epithelial cells). In this study, they focused on *in vitro* selected CIP resistant strains with high MICs. However, there is evidence that *in vitro* selected mutants have a reduced fitness compared to naturally resistant strains (11,12). In our study, we used quinolone resistant field isolates which might explain why we did not observe a fitness loss in these isolates.

The epidemiological evidence of more severe illness in patients infected with quinolone resistant *Salmonella* led us hypothesize that quinolones could also be directly involved in pathogenicity, e.g. by inducing virulence factors. Overall, the presence of CIP in low (1 x

MIC; 0.25 µg/ml) and therapeutically relevant concentrations (20 x MIC; 5 µg/ml) (41) seemed to have an inhibitory effect on macrophage survival of CIP resistant *S.* Typhimurium DT104 (MIC = 0.25 µg/ml) after 24 h post infection. CIP accumulates mainly in the cytosol of the macrophages and reaches only poor concentrations in the organelles such as the phagosome (56). During a *Salmonella* infection the phagosome is modified by the bacteria and transformed into the *Salmonella* containing vacuole (SCV) at approx. 1 h post infection (24). The SCV seemed to shield the bacteria from the antibiotic as a reduction could not be observed within the first 4 h of infection. Only when 5 µg/ml CIP was added 1 h prior to infection a reduced uptake followed by a decrease of the intracellular bacteria already during the first 4 h of infection could be demonstrated. The high antibiotic concentration (20 x MIC) eliminated a major part of the extracellular bacteria before invasion and presumably caused injury to many bacteria that resulted in a reduced ability to survive the macrophage defense. The general decrease of S. Typhimurium DT104 observed after 24 h could be the result of phagocyte lysis and bacterial release into the extracellular medium leading to the elimination by CIP. However, even therapeutic concentrations of CIP failed to eliminate all bacteria as we were able to recover 3.84 log CFU/well of intracellular bacteria when CIP was added 1 h before infection and 4.38 log CFU/well when CIP was added 1 h after infection. It has been shown elsewhere that the addition of a higher CIP concentration (20 µg/ml) to the extracellular medium reduces the number of intracellular *Salmonella* for 2 log after 1 h of incubation (26). In another study, ofloxacin has been shown to be more effective in killing intraphagocytic *Salmonella* in a concentration corresponding to 10 x MIC of the respective test strains (14). After 8 h post infection the intracellular bacteria were completely eliminated. Interestingly, it has been observed for *Staphylococcus aureus* and *E. coli* that quinolones trigger the expression of phage encoded virulence factors (30,47). From the results of our study, we cannot conclude that virulence in *Salmonella* is altered in presence of quinolones.

Low and high level resistance to fluoroquinolones is most often caused by point mutations (29) implicating that the evolution of quinolone resistance may be driven by an increased bacterial mutation rate. In our study, the spontaneous mutation frequencies did not correlate with the resistance pattern of the *Salmonella* isolates. This is in contrast to other studies were elevated spontaneous mutation frequencies have been observed for quinolone resistant *Salmonella* and *E. coli* (1,39). However, in the study of Allen and Poppe (1), the control group of susceptible *Salmonella* isolates was very small. In our experiment, the control group and the test group of quinolone resistant isolates were equal in size and comparable, at large. Our results suggest that in a static environment and absence of any selective pressure,

quinolone resistant *Salmonella* do not seem to mutate more often than sensitive ones. Even though constitutive mutator phenotypes have been reported to have a selective advantage during infection in mice and subsequent antimicrobial treatment (23), they also quickly acquire mutations that are not beneficial (22). In contrast, we observed that human isolates exhibited slightly higher spontaneous mutation frequencies than food isolates pointing out that the level of mutation frequency may differ depending on the circumstances. The successful pathogen might possess a temporary elevated mutation rate that is restricted to the environments where genetic variations are needed in order to survive, such as during infection (38).

Quinolone resistance in *Salmonella* has shown to evolve *in vivo* and in several cases were this has been reported, the original susceptible bacteria were related to severe illness (mild gastroenteritis are rarely treated with antibiotics) (37). This could suggest that more virulent *Salmonella* are more likely to become quinolone resistant as they are more likely to come in contact with the antibiotic during infection. Invasive strains are suggested to persist within macrophages (15) which are known to induce mutations in intracellular bacteria (50) and to accumulate fluoroquinolones (56). Thus, we were interested in the impact of macrophages in presence and absence of CIP on the frequency of quinolone susceptible isolates to acquire CIP resistance (or a reduced susceptibility to CIP). We could show that this frequency was increased for most strains after infection of macrophages for 4 h followed by subculturing in non-selective LB medium. All investigated *Salmonella* strains could grow on a CIP concentration of at least 2 x their MIC. Strain no. 2 could also grow on a concentration corresponding to 8 x its MIC. The molecular mechanisms leading to this MIC shift have not been investigated but it is probably the consequence of point mutations as mechanisms associated with gene regulation are unlikely to persist after growth in non-selective LB medium. In *Salmonella*, point mutations in gyrA are thought to cause a minimal MIC of 0.12 µg/ml but point mutations at an MIC of 0.064 have been reported (49). When CIP was added to the extracellular medium, the final vacuolar CIP concentration in the macrophage was unknown. However, it seemed to have an effect on the bacteria as the addition of high CIP concentrations (corresponding to 20 x MIC) at 1 h post infection increased markedly the frequency of intracellular bacteria with reduced CIP susceptibilities. Lysis of the phagocytes and the release of the bacteria into the extracellular medium until 4 hours post infection are unlikely. Thus the CIP mutations were presumably generated within the macrophages. This is presumably the result of the bacterial SOS response which is activated inside macrophages and in presence of quinolones and which protects the bacteria from lethal DNA damages on

the expenses of a transient elevated mutation rate (17,58,64). It is possible that the observed reduction in the CIP susceptibility predisposes the bacteria to become resistant to higher concentration when survival in macrophages is prolonged. In contrast to the intracellular bacteria, were growth was expected to be comparably weak, the bacteria exposed directly to subinhibitory concentrations of CIP for 3 h were able to proliferate indicating that mutation frequencies in replicating populations are higher.

RecT (SG1183) is encoded within a phage operon including the gene for the exoribonuclease VIII which are both thought to be involved in recombination and repair of DNA (51). In presence of CIP, *recT* seemed to be involved in reducing the emergence of cells with reduced CIP susceptibilities. In contrast, *recT* did not seem to have an effect when CIP was absent indicating that it is induced during the quinolone mediated replication arrest. This is backed by the findings that bacteriophage genes are induced in presence of quinolones (30,47). The results are conflicting with a previous study where *recT* seemed to be involved in reducing mutation rates under non-selective conditions (Manuscript II). In this study *recT* was more frequently found in *Salmonella* isolates that were resistant to multiple antibiotics and/or quinolones. It seems surprising that quinolone resistant strains apparently harbor a gene that reduces mutations. However, during a systemic spread, the bacteria may remain for a long time within macrophages and might be exposed to prolonged CIP treatment. In these bacteria *recT* might contribute in reducing the number of too many detrimental mutations. Thus, the bacteria carrying this gene can survive and profit from non-lethal mutations generated within macrophages.

To conclude, we were not able to show a difference between quinolone resistant and sensitive *Salmonella* in their ability to adhere and invade epithelial cells and to survive and grow in macrophages. Thus, cell cultures might not be applicable to indirectly investigate the virulence potential of quinolone resistant *Salmonella.* Alternatively, the invasion of epithelial cells and macrophages survival are not important factors for virulence of quinolone resistant *Salmonella*. These bacteria were also not more predisposed to mutate under static non-selective conditions. However, we observed an increased frequency in the reduction of the ciprofloxacin susceptibility after macrophage infection which was enhanced in presence of ciprofloxacin. *Salmonella* that are more invasive may persist within macrophages and have a higher chance to encounter ciprofloxacin, frequently used to treat systemic infections. Our results suggest that such conditions enhance the adaptation of *Salmonella* to ciprofloxacin. To prevent that more invasive *Salmonella* develop quinolone resistance during infection, it

should be ensured that the antibiotic efficiently eliminates *Salmonella* in the SCV of macrophages and the simultaneous administration of SOS inhibitors should eventually be considered.

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Tables and Figures

Table 1 (next page): Isolates used in the different experiments of this study and their categorization based on the resistance pattern

Antibiotics: cip, ciprofloxacin; str, streptomycin; tet, tetracyclin; nal, nalidixic acid; ffn, florfenicol; sul, sulfonamides; neo, neomycin; smx, sulfamethoxacole; amp, ampicillin; chl, chloramphenicol; spe, spectinomycin; tmp, trimethoprim; gen, gentamicin; aug2, amoxicillin + clavulanat (2:1), kan, kanamycin. Other abbreviations: Sp., spontaneous; adh/inv, adhesion and invasion; MP, macrophage; [1]The classification of the isolates according to the resistance pattern: Q, resistant to at least one fluoroquinolone; MR, resistant to four or more antibiotics; R, resistant to less than four antibiotics; S, senstitive. [2]If nothing is indicated, the MIC has not been determined. Ref (references): [3]42, [4]Manuscript I, [5]Manuscript II, [6]61, [7]18, [8]43, [9]54, [10]19. + A plus indicates that the isolate has been used in this experiment

No.	Strain	Source	C[1]	Resistance pattern	Cip MIC (μg/ml)[2]	Experiments: Sp. mutation frequency	Adh/Inv of Int407	MP survival: Without cip	MP survival: With cip	Adaption to cip	Growth in LB/DMEM	Ref.
	E. coli ATCC 25922					+						
	E. coli CSH 115					+						3
26	S. Derby	pork	S				+					4
13	S. Derby	human	S				+					4
119	S. Hadar	human	R	str, tet		+						
122	S. Hadar	human	Q	cip, nal, str, tet	0.25	+						
121	S. Hadar	human	Q	cip, nal, (ffn), str, tet	1	+						
120	S. Hadar	human	R	str, tet		+						
127	S. Infantis	pig	S				+					
97	S. Infantis	pig	MR	tet, sul, str, neo, amp		+	+	+			+	
134	S. Infantis	human	Q	cip, nal	0.25	+	+	+			+	
135	S. Infantis	human	Q	(chl), cip, nal, neo, smx, spe, str, tet, tmp	0.25	+						
132	S. Infantis	human	S			+						
39	S. Newport	human	S			+	+					4
35	S. Newport	human	Q	amp, cip, gen, nal, smx, spe, str, tet	0.5	+	+					
44	S. Newport	human	Q	cip, nal	0.25	+	+					4
21	S. Newport	pig	Q	cip		+						
40	S. Newport	human	S			+						5
117	S. Newport	human	Q	amp, chl, ffn, nal, cip, neo, smx, tet, tmp	0.12	+						
114	S. Newport	human	R	smx, str, tet		+						
118	S. Newport	human	Q	amp, (aug2), chl, cip, ffn, nal, neo, smx, tet, tmp	0.25	+						
84	S. Newport	pig	Q	cip		+						
113	S. Newport	human	S			+						
138	S. O:4,12; H:i: - DT120	human	MR	amp, smx, str, tet			+					
137	S. O:4,5,12; H:i:- DT193	human	MR	amp, (aug2), smx, str, tet		+						
136	S. O:4,5,12; H:i:- NT	human	Q	amp, cip, nal, smx, str, tet	0.12	+	+					
34	S. Saintpaul	human	Q	cip, nal	0.12	+	+	+			+	5
41	S. Saintpaul	human	MR	apr, chl, gen, smx, spe, tet, (ffn), (str)		+	+					4
27	S. Saintpaul	human	S			+	+	+			+	4
22	S. Saintpaul	cattle	Q	amp, gen, nal, spe, str, sul, tet, cip	0.25	+						
	S. Typhimurium 4/74						+					
	4/74 *invH* 201::Tn*phoA*						+					6
109	S. Typhimurium DT104	human	R	smx, spe, str	0.016	+	+	+		+	+	5
	109 Δ*recT* ::km^R			smx, spe, str, kan	0.016					+	+	5
101	S. Typhimurium DT104	human	S		0.016					+		
94	S. Typhimurium DT104	human	Q	amp, (aug2), chl, cip, ffn, nal, smx, spe, str, tet	0.25	+						
2	S. Typhimurium DT104	human	MR	spe, (ffn)	0.008	+	+	+		+	+	7, 4
28	S. Typhimurium DT104	human	Q	amp, chl, cip, ffn, nal, smx, spe, str, tet, (aug2)	0.25	+	+	+	+		+	5
1	S. Typhimurium DT104	human	Q	nal, amp, chl, str, tet, cip, aug2, smx, spe	0.12	+	+	+			+	8, 4
48	S. Typhimurium DT104	pig	MR	sul, tet		+						
53	S. Typhimurium DT104	pork	Q	amp, chlo, cip, flo, nal, spe, str, sul, tet	0.25	+						
77	S. Typhimurium DT104	pork	R	spe, str, sul		+						
62	S. Typhimurium DT104	pork	MR	amp, chl, spe, str, sul, tet		+						
17	S. Typhimurium DT104	pork	R	spe, str, sul		+						4
16	S. Typhimurium DT12	pork	S				+					4
4	S. Typhimurium DT12	human	S				+					9
6	S. Typhimurium U292	human	S				+					10
68	S. Typhimurium U292	pig	S				+					
42	S. Virchow	human	S			+	+					4
25	S. Virchow	pig	Q	nal, cip	0.5	+	+					
43	S. Virchow	human	Q	cip, spe, str	0.5		+					5
86	S. Virchow	human	Q	amp, cip, nal	2	+						
88	S. Virchow	human	Q	cip	0.5	+						

Table 2: Spontaneous frequency of the occurrence of cells with reduced susceptibility to ciprofloxacin

	Frequency per 10^8 CFU			
Strain no.	0.032 µg/ml cip (SEM)		0.064 µg/ml cip (SEM)	
109	36	(6)		
ΔrecT::km^R	15	(8)		
101	29	(5)		
2	210	(161)	17	(11)

DMEM was inoculated with 10^6 CFU/ml and subsequently incubated at 37 ºC and 5 % CO_2. After 4 h, the culture was diluted 1:10 in LB and grown at 37 ºC for 16 h. The culture was plated on ciprofloxacin containing agar and on non-selective agar. SEM, standard error of the mean of three independent replicates.

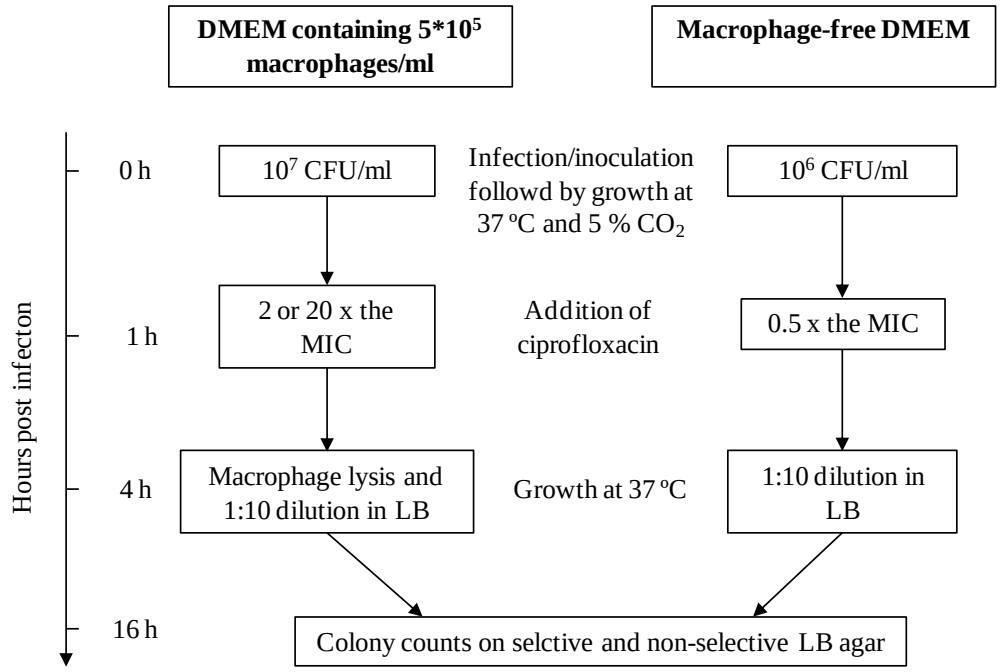

Figure 1: Experimental workflow. Macrophages (RAW264.7) were infected with bacteria and subsequently treated with different concentrations of ciprofloxacin. For comparison, macrophage-free culture medium (DMEM) was inoculated with the same bacteria and subsequently incubated with subinhibitory concentrations of ciprofloxacin. Both assays included a control without antibiotics. To amplify the bacteria within the macrophages and of the macrophage-free medium, the lysates/cultures were transferred to LB and incubated for 16 h before plating on non-selective and selective agar (0.032, 0.064 and 0.128 µg/ml ciprofloxacin).

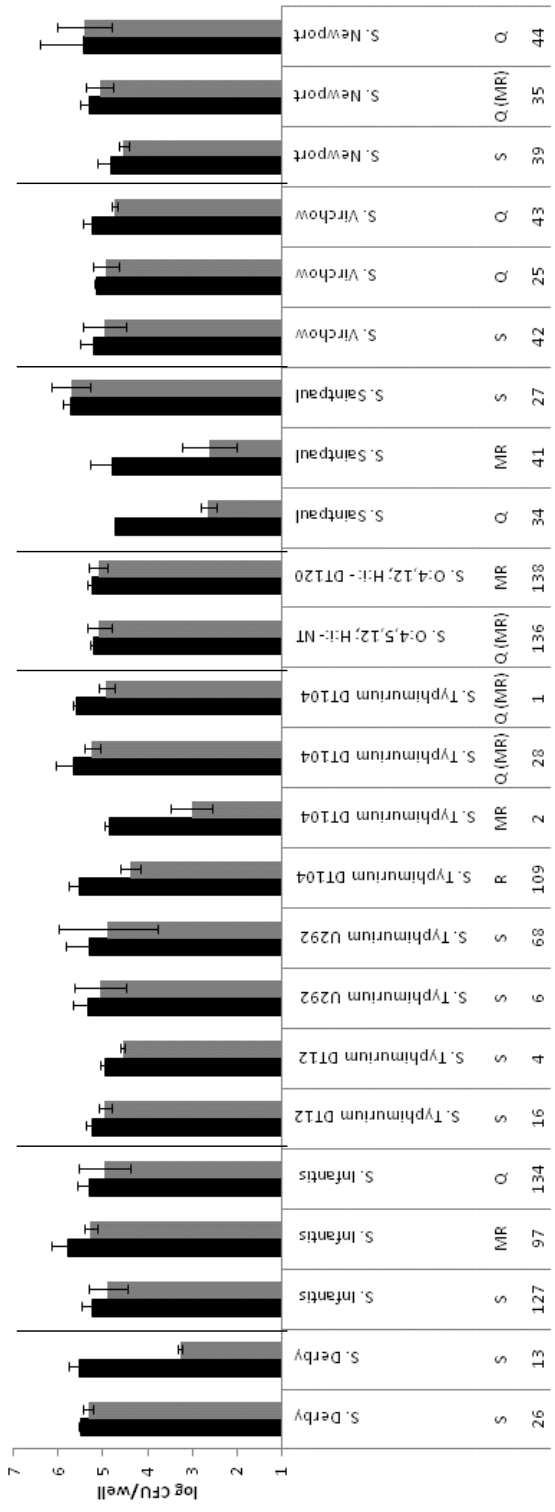

Figure 2: The number of cell associated *Salmonella* in a gentamicin protection assay using the human epithelia cell line Int407. Bacteria that adhered to the epithelia cells within 1 h post infection are shown by the black columns (log CFU/well). The bacteria that invaded within 3 h post infection are shown by grey columns (log CFU/well). Error bars represent standard errors of the mean (SEM) of three independent experiments. The classification of the isolates according to the resistance pattern: Q, resistant to at least one fluoroquinolone; MR, resistant to four or more antibiotics; R, resistant to less than four antibiotics; S, fully susceptible.

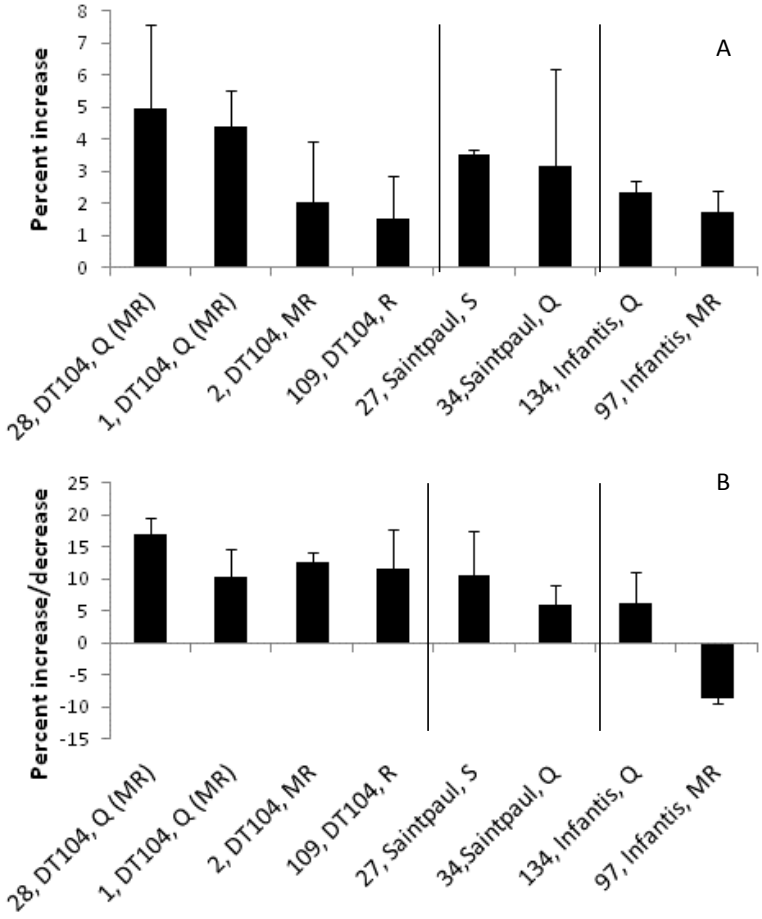

Figure 3: Percent increase or decrease of the intraphagocytic *Salmonella* at 4 h (A) and 24 h (B) post infection compared to 2 h post infection using the macrophage cell line RAW264.7. Error bars represent standard errors of the mean (SEM) of three independent experiments. The classification of the isolates according to the resistance pattern: Q, resistant to at least one fluoroquinolone; MR, resistant to four or more antibiotics; R, resistant to less than four antibiotics; S, fully susceptible. DT104, *S.* Typhimurium DT104.

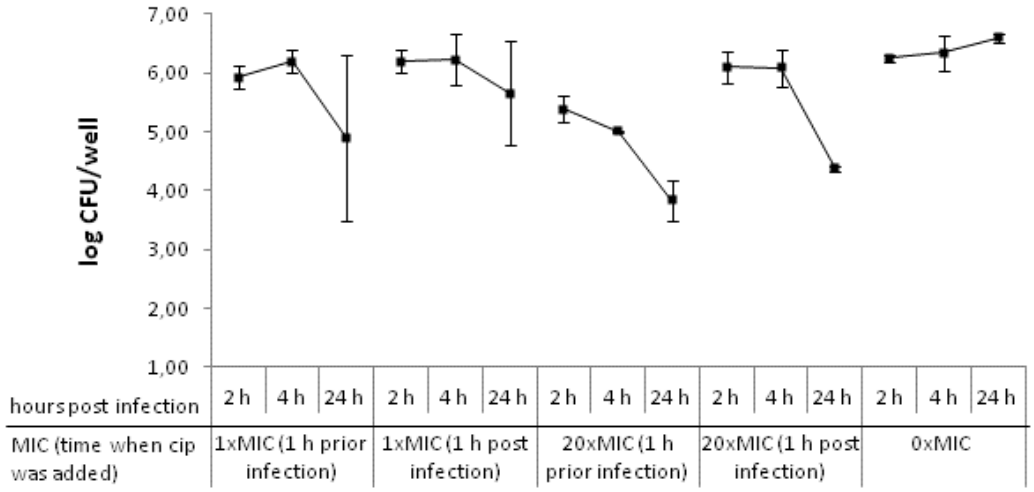

Figure 4: Intracellular counts (log CFU/well) of *S.* Typhimurium DT104 (isolate no. 28) within macrophages (RAW264.7) in absence (0xMIC) or presence of ciprofloxacin (CIP) in a concentration of 1xMIC (0.25 µg/ml) or 20xMIC (5 µg/ml). CIP was added either at 1 h before or after infection. Error bars represent standard deviation (SD) of two independent experiments.

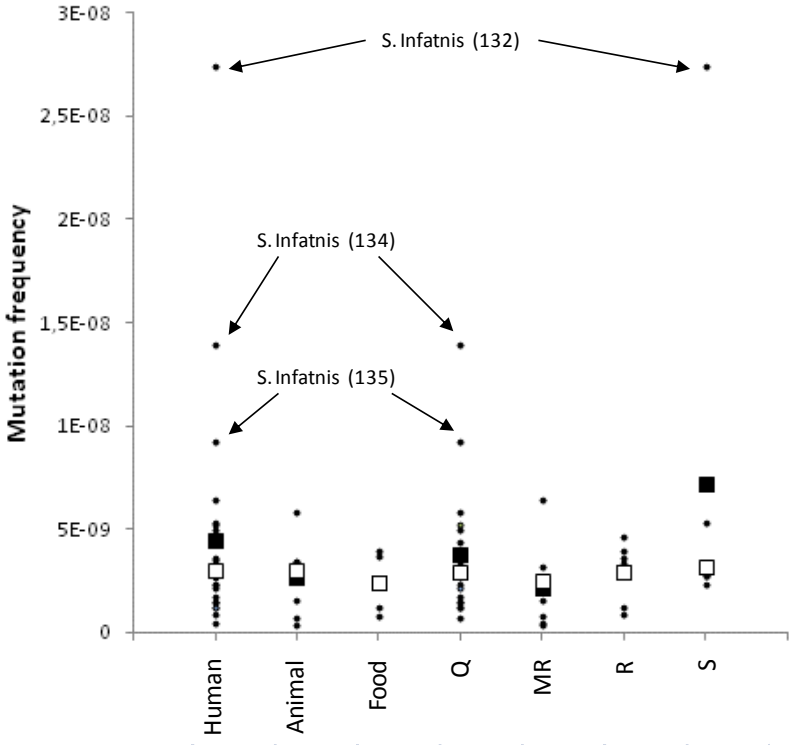

Figure 5: Mutation frequencies (small dots) of 38 *Salmonella* isolates classified after their source (human, animal, food) and resistance pattern into Q (resistant to at least one fluoroquinolone), MR (resistant to four or more antibiotics), R (resistant to less than four antibiotics) and S (fully susceptible). The average mutation frequency by source or resistance pattern is shown by rectangles including (black rectangles) or excluding (white rectangles) the *S.* Infantis isolates (no. 132, 134, 135 and 97).

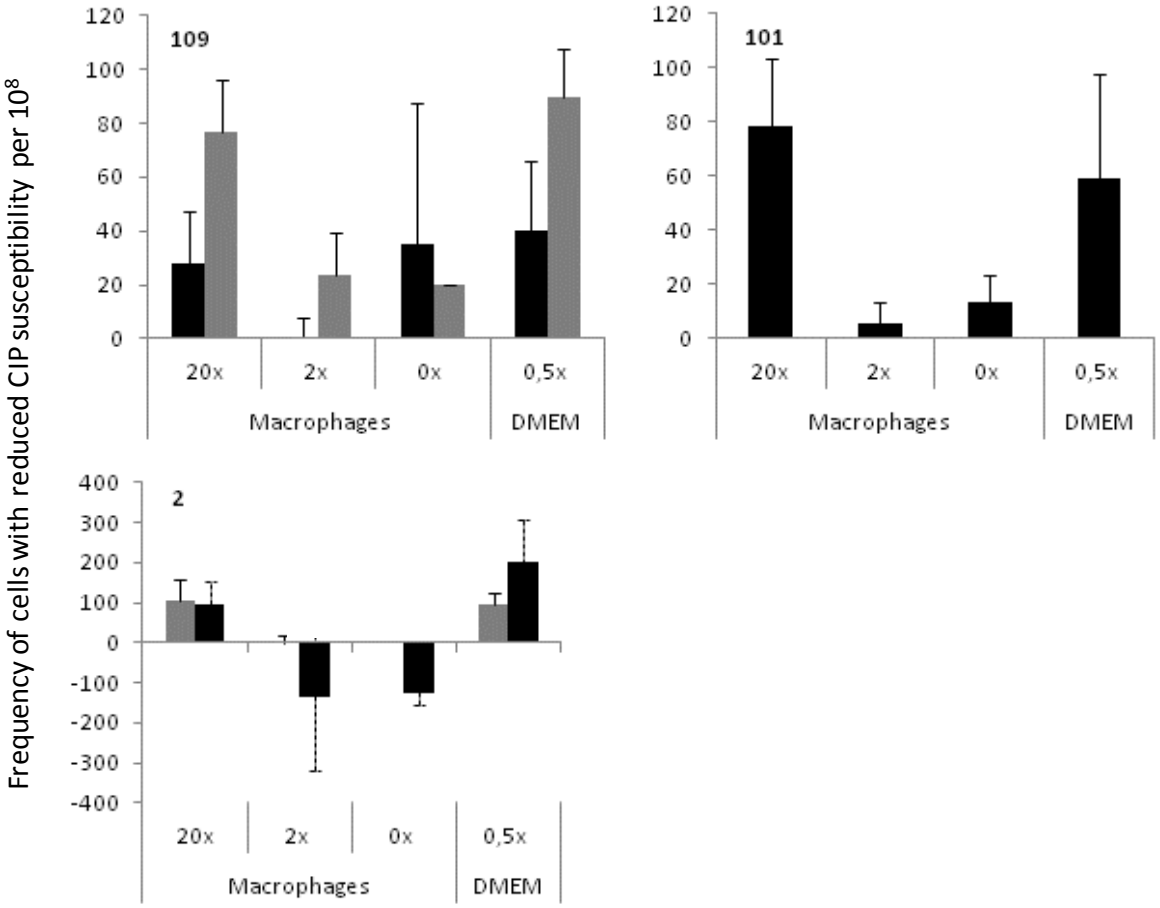

Figure 6: Frequencies of the occurrence of cells selected on 0.032 (strains no. 109, 101 and 2) and 0.064 (only strain no. 2) µg/ml ciprofloxacin (CIP) after different pre-treatments of the cultures (X-axis): after infection of macrophages and subsequent incubation with CIP in a concentration corresponding to 20 x, 2 x or 0 x the MIC and after incubation in macrophage-free medium (DMEM) in presence of subinhibitory concentrations of CIP (0.5 x the MIC). The frequencies after the indicated treatments are subtracted by the frequencies obtained from the control cultures pre-treated with DMEM in absence of antibiotics (baseline). For strain no. 109 the frequencies of the wildtype (black columns) and the isogenic mutant Δ*recT*::km^R (grey columns) are shown. For strain no. 2, the frequencies selected on 0.032 (black columns) and on 0.064 µg/ml cip (grey columns) are shown. Error bars represent standard errors of the mean (SEM) of three independent experiments.

Appendix

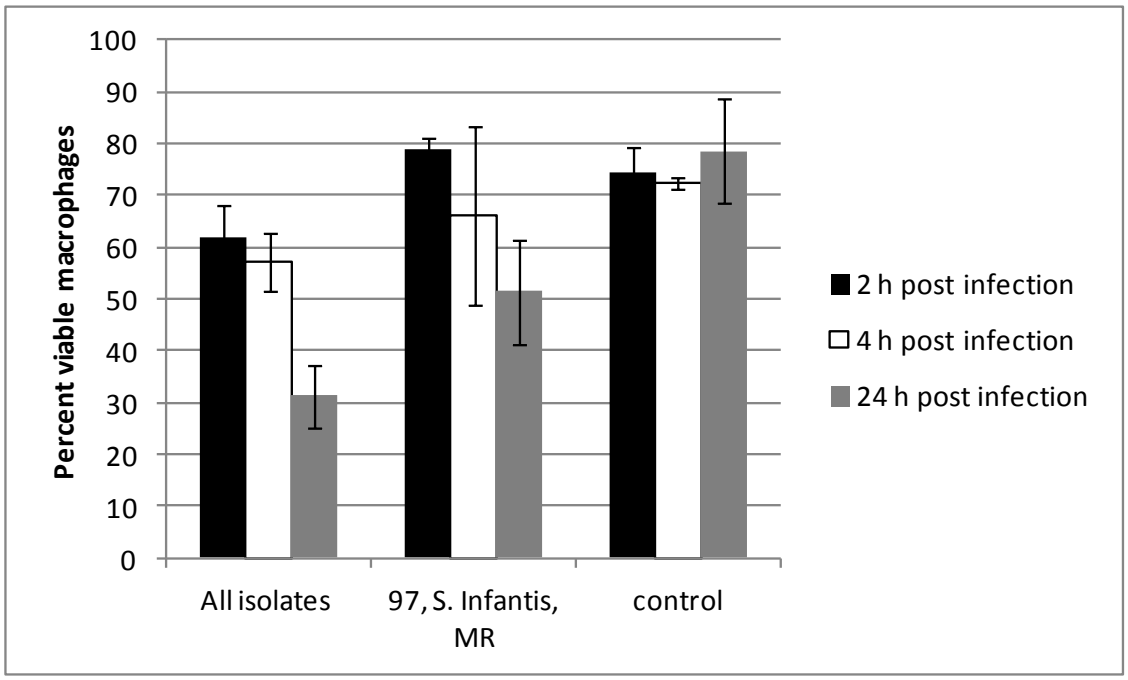

Figure A1: The percentage of viable macrophages infected with different *Salmonella*. Trypan blue staining was used to color dead macrophages. The control was inoculated with PBS instead of *Salmonella*. Q, resistant to at least one fluoroquinolone; MR, resistant to four or more antibiotics; R, resistant to less than four antibiotics; S, fully susceptible. The isolates investigated include *S.* Typhimurium DT104 (2 x Q, MR, R), *S.* Saintpaul (S, Q) and *S.* Infantis (Q); Error bars represent SEM of two independent experiments.

Table A1 (next page): Genes dominating in fully susceptible *Salmonella* isolates determined by comparative genomic hybridization

The numbers of isolates where the respective genes were present determined by array-based genomic hybridization are shown; Q, resistant to at least one fluoroquinolone; MR, resistant to four or more antibiotics; R, resistant to less than four antibiotics; S, fully susceptible. [1]The percentage indicates the relative proportion of the single genes within the given gene group compared to the total number of genes dominating sensitive isolates; grey highlighted numbers indicate if a gene dominates in sensitive non-Typhimurium or Typhimurium isolates

Gene name	Function and other gene	Genetic element	Typhimurium (n=16)				Non-Typhimurium (n=13)			
			Q (n=2)	MR (n=2)	R (n=2)	S (n=10)	Q (n=4)	MR (n=1)	R (n=1)	S (n=7)
Mobile genetic elements					24%[1]				0%	
PSLT062		plasmid pSLT	0	0	1	8	0	0	0	0
PSLT063			0	0	1	6	0	0	0	0
PSLT068	putative ParB-like nuclease domain		0	0	1	8	0	0	0	0
PSLTtraD	conjugative transfer: DNA transport		0	0	1	6	0	0	0	0
SBG0903	hypothetical phage-related protein		0	0	2	8	3	0	0	2
SL1950	bacteriophage terminase (large subunit)		0	0	0	8	1	0	0	0
SL2590	phage replication protein P		0	0	1	8	2	0	0	1
SLP2_0032	plasmid encoded putative antirestriction protein		0	0	0	6	0	0	0	1
Genes with other function					61%				25%	
STM0176 (*stiB*)	fimbrial chaperone, starvation-stress protein (101)		0	0	1	7	1	0	0	4
STM2617	antiterminator-like protein		0	1	1	10	3	0	0	0
STY0060 (*yaaF*)	putative nucleoside hydrolase (IUNH family)		0	0	1	9	3	1	1	7
STY0076 (*carA*)	carbamoyl-phosphate synthase small chain		0	0	0	7	3	0	0	5
STY0321	Rhs-family protein		0	1	0	10	1	0	0	2
STY0746 (*kdpB*)	potassium-transporting ATPase B chain		1	0	1	10	4	1	1	6
STY0823 (*hutU*)	urocanate hydratase		0	0	1	8	2	1	0	5
STY1138 (*hpcD*)	5-carboxymethyl-2-hydroxymuconate delta- isomerase		0	0	1	8	4	1	1	6
STY1658 (*add*)	adenosine deaminase		1	1	1	5	0	0	1	5
STY2378 (*stcD*)	putative outer membrane lipoprotein		0	0	1	8	2	1	0	3
STY2485 (*napA*)	probable nitrate reductase	nap operon	0	0	1	10	3	1	0	7
STY2487 (*napF*)	ferredoxin-type protein NapF		0	0	1	10	2	1	1	7
STY2492 (*yojL*)	thiamine biosynthesis protein		0	0	0	6	2	0	1	5
STY2665 (*cysZ*)	putative sulfate transport protein CysZ		0	0	1	6	3	0	0	4
STY2698 (*eutG*)	putative alcohol dehydrogenase		0	0	1	7	3	1	0	6
STY2771 (*ndk*)	nucleoside diphosphate kinase		0	0	2	9	3	1	1	6
STY2774	putative anaerobic reductase component		0	0	1	8	2	0	0	4
STY4440 (*pspG*)	phage shock protein G		0	1	0	10	2	0	0	6
STY4451 (*ssb*)	single-strand DNA-binding protein		0	0	1	6	3	1	1	6
STY4188 (*yhjK*)	phosphodiesterase		0	0	1	9	2	0	1	7
STY4715 (*amiB*)	N-acetylmuramoyl-L-alanine amidase		0	0	1	7	4	1	1	4
Genes with unknown function:					15%				75%	
SG0300	pseudogene		2	2	1	6	0	0	1	6
SG2538			0	0	1	6	1	0	1	4
STM0769			0	0	1	7	3	0	0	6
STY0288			2	2	1	5	0	0	0	5
STY2660			0	0	2	7	4	0	1	6
STY3542			0	0	0	6	2	1	0	4
STY4411			0	2	1	5	0	0	0	5
STY4452			0	0	1	8	3	1	1	6

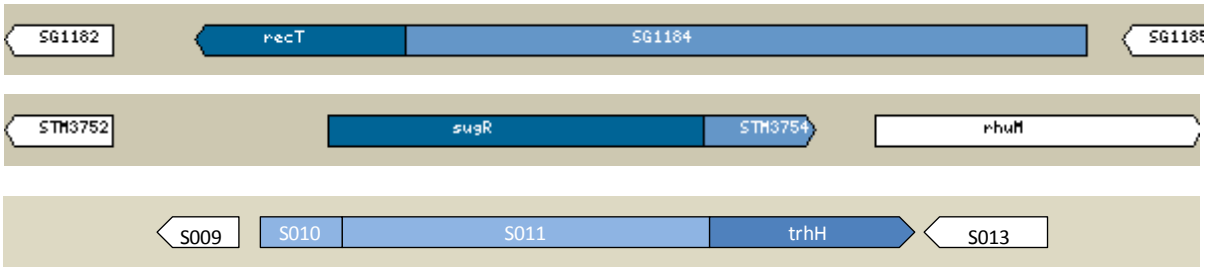

Figure A2: Predicted operons of the genes *recT* (SG1183) in *S.* Gallinarum 287/91 (NCTC 13346) and *sugR* (STM3753) in *S.* Typhimurium LT2 (ATCC 700220) using Microbes Online Operon Predictor (www.microbesonline.org) and *trhH* (AF261825_S012) in the *Salmonella* genomic island 1 (accession no. AF261825) as described by Boyd et al. (33). The respective genes are displayed in dark blue, other operon genes in light blue and adjacent genes in white. The arrowhead indicates the transcription direction.

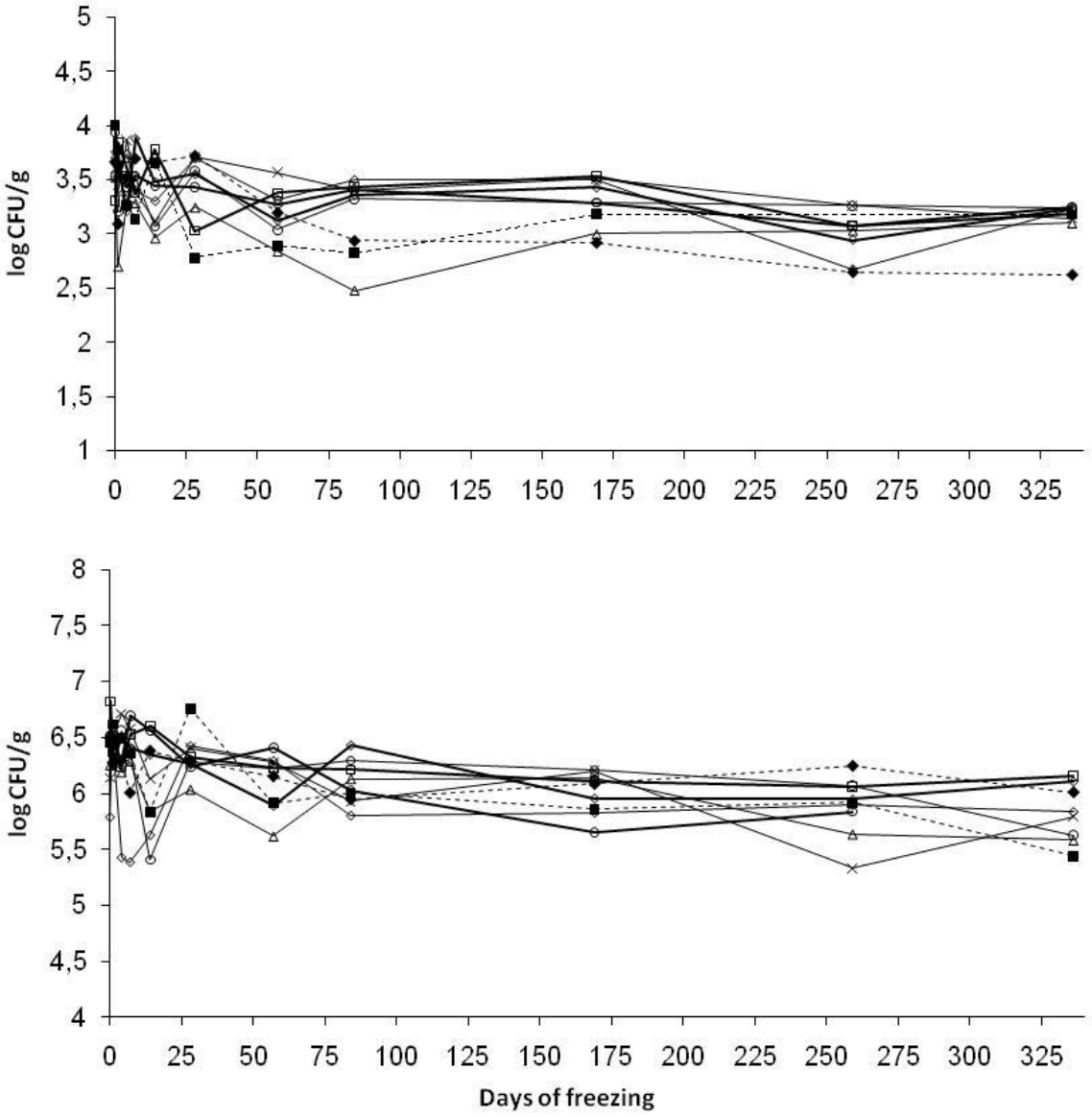

Figure A3. Effect of freezing (-18°C) on colony counts of *Salmonella* in minced pork; A: inocula of 10^{-6} CFU/g; B: inocula of 10^{-3} CFU/g; dashed lines indicate successful *Salmonella* types (01 DT104, 02 DT104, 03 DT120); thin lines represent non-successful *Salmonella* types (04 DT12, 09 Derby, 10 Infantis); bold lines represent outbreak strains of 2008 (05 DT135, 06 U969, 07 U288); Standard deviations based on 2 replicates ranged from 0,23 – 0,51 log CFU/g (not shown).