Stress response and virulence in *Salmonella* Typhimurium: a genomics approach

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Dit onderzoek is uitgevoerd binnen de onderzoekschool VLAG

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Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
Prof. Dr. M. J. Kropff,
in het openbaar te verdedigen
op dinsdag 16 januari 2007
des namiddags te half twee in de Aula

Hermans, Armand Petrus Henricus Maria
Stress response and virulence in *Salmonella* Typhimurium: a genomics approach - 2007

Thesis Wageningen University, Wageningen, The Netherlands – with summary in Dutch – 182 p.

Keywords: *Salmonella* serovar Typhimurium / stress response / virulence / prophages / transcriptomics

ISBN: 90-8504-572-X

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ABSTRACT

Since 1995 the number of infections caused by multiple-antibiotic-resistant *Salmonella* serovar Typhimurium phage type DT104 isolates has increased in many parts of the world. Several hypothetical reasons have been proposed to explain this increase. The multiple-antibiotic-resistance and also increased virulence and/or survival of stressful conditions have been mentioned.

The current molecular biology research has been focused on the antibiotic resistance genes. In our research, we isolated two novel *Salmonella* serovar Typhimurium DT104-specific DNA fragments which are associated with virulence. One fragment was identical to *irsA*, which is suggested to be involved in macrophage survival and the other fragment was homologous to HldD, an *Escherichia coli* O157:H7 protein involved in a specific lipopolysaccharide (LPS) assembly pathway. While analyzing all novel fragments obtained, we were able to identify a DT104-specific prophage (= bacteriophage integrated into the bacterial genome) remnant that we designated prophage ST104B and that harbors the HldD homologue. We observed that the presence of the genes coding for multiple-antibiotic-resistance, the HldD homologue containing ST104B prophage remnant and phage subtype DT104L revealed to be common features of the most emerging variant within the group of different *Salmonella* serovar Typhimurium DT104 isolates.

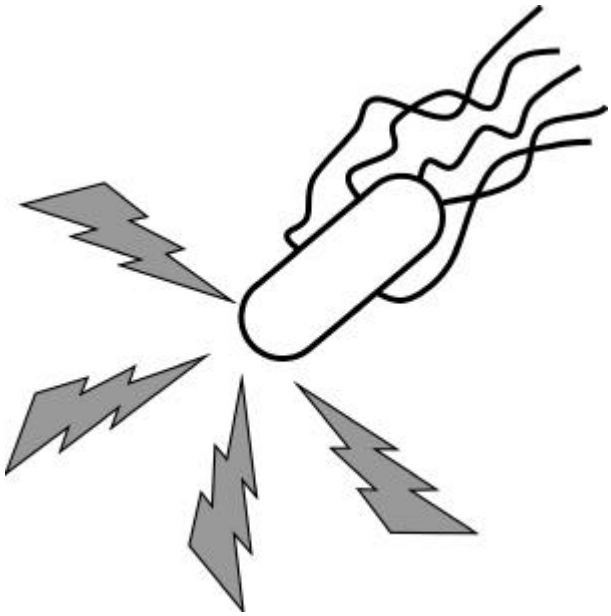
Furthermore, a thematic oligo microarray was developed for *Salmonella* serovar Typhimurium DT104 to be able to study expression of 425 genes involved in stress response and virulence. This microarray was used to measure gene expression in the course of time during growth of a *Salmonella* serovar Typhimurium DT104 wild type strain and its *luxS* deletion mutant, since LuxS might play a role in stress response and virulence. Expression of stress response and virulence genes in the course of time appeared to be largely growth-phase-dependent. The most prominent effect of deleting the *luxS* gene was the increased expression of 15 LPS synthesis and assembly genes at the end-exponential growth phase. Notably, the *luxS* deletion mutant showed higher *in vitro* adhesion and invasion capacity into human epithelial cells, although the expression levels of invasion genes appeared to be similar to that in the wild type. We concluded that the loss of *luxS* results in overexpression of LPS genes and most likely also the LPS molecules, thereby affecting *in vitro* virulence characteristics of this DT104 *luxS* deletion mutant.

In addition, gene expression was measured and compared for a *Salmonella* serovar Typhimurium DT104 wild type strain cultured under aerobic and anaerobic conditions and subjected to heat, oxidative, and acid stress. Stress genes such as the RpoS and PhoPQ regulon, chaperones and universal stress proteins were mainly induced or repressed in a similar manner in both aerobically or anaerobically grown cells when subjected to the different stresses. Furthermore, the virulence(-associated) LPS, PhoPQ, Spv, SPI-1, and SPI-2 encoded genes were differentially regulated by the different stresses. The thematic microarray developed allows assessment of the impact of stresses and combinations thereof on the expression of stress and virulence genes for *Salmonella* serovar Typhimurium DT104.

Finally, the results of our molecular biology research enlarges the current understanding of characteristics of emergence of *Salmonella* serovar Typhimurium DT104 by harboring additional virulence factors or by surviving stress conditions.

Chapter 1

Introduction and outline of the thesis



INTRODUCTION

The foodborne pathogen *Salmonella*.

Salmonellae are Gram-negative, rod shaped, facultatively anaerobic, nonspore-forming, usually motile bacteria (Fig. 1). In general, salmonellae are able to infect a wide range of hosts from cold-blooded animals to humans resulting in diseases ranging from mild diarrhea and gastroenteritis to severe systemic infections such as typhoid fever. Some *Salmonella* types can infect a wide variety of animals, while others only one. For example, the serovars Typhimurium and Enteritidis infect humans as well as chickens and mice. Other serovars are host-adapted, infecting only a few species such as serovar Dublin which primarily infects cattle and serovar Choleraesuis which primarily infects swine. Finally, some serovars are host-specific, infecting only one single animal host, such as serovar Typhi that only infects humans and serovar Pullorum that only infects chickens. Although *Salmonella* serovars share some virulence factors needed for intestinal infections, unique virulence factors among serovars also occur that are responsible for host specificity and specific disease symptoms (28).

Salmonellae infections in humans are generally foodborne but can also be contracted through contact with infected animals. Food products from farm animals such as poultry, pigs, and cattle have been identified as an important source of human salmonellae infections (81). Estimations are made that worldwide each year up to 1.3 billion cases of acute gastroenteritis and diarrhea are caused by non-typhoid salmonellosis, resulting in 3 million deaths annually. Furthermore, about 16 million cases of typhoid fever occur each year with a fatal outcome of around 0.6 million. Although fatal salmonellae infections mainly occur in developing countries, mortality due to acute salmonellae infections also has an important impact in industrialized countries. Especially children and elderly people have a higher risk of dying because of the dehydrating effects of diarrhea (39, 60). In The Netherlands approximately 50,000 infections with salmonellae occur annually (83).

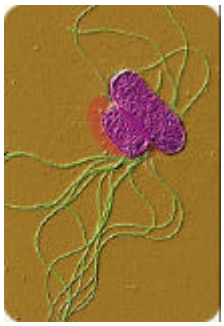


FIG. 1. Transmission electron micrograph (false coloured) of *Salmonella* serovar Typhimurium by P. Gunnig and R. Bongaerts IFR Norwich (http://www.foodandhealthnetwork.com/predictive.html)

Salmonellae taxonomy.

To identify salmonellae among the large number of different bacteria occurring in the environment, several biochemical reactions are performed. The following general reaction pattern represents salmonellae apart from a few exceptions. Salmonellae are able to ferment and form gas on glucose and produce H_2S. Lactose and sucrose are not fermented and urea is not hydrolyzed. They form lysine-decarboxylase and do not form indole and acetyl-methylcarbinol. Finally, salmonellae do not show ß-galactosidase activity (26, 54). Based on differences in these biochemical reactions (the few exeptions) and surface proteins, salmonellae can be divided into the two species *Salmonella enterica* and *Salmonella bongori* (Fig. 2), and subsequently *S. enterica* into six subspecies (ssp.): ssp. *houtenae*, ssp.

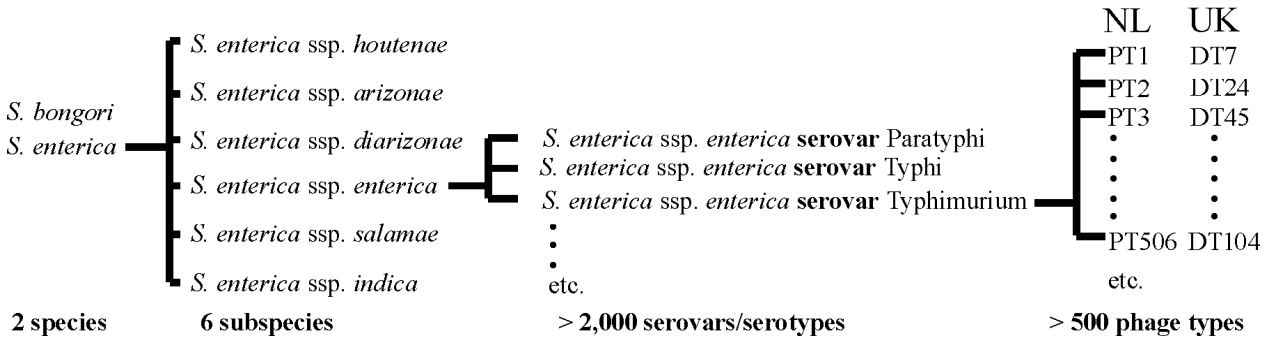


FIG. 2. Schematic representation of salmonellae classification and diversity.

arizonae, ssp. *diarizonae*, ssp. *salamae*, and ssp. *indica*. In addition, more than 2,000 serovars can be assigned to ssp. *enterica*. Examples of well known serovars are Paratyphi, Typhi and Typhimurium (9, 66).

Salmonellae classification is mainly based on the biochemical results for species and subspecies recognition. Additional classification for the different serotypes is based on the immunoreactivity of two surface structures, the O and H antigen, by performing agglutination assays using antisera that react with groups of related antigens or a single antigen. The Gram-negative cell wall is composed of a thin, inner layer of peptidoglycan and an outer membrane consisting of molecules of phospholipids, lipopolysaccharides (LPS), lipoproteins and surface proteins (Fig. 3). The O antigen is a polymer of carbohydrate O subunits (polysaccharide) that is the outermost component of lipopolysaccharide. Natural occurring variation in the sugar components of the O subunit of specific salmonellae results in variation in O antigen immunoreactivity, resulting in different O serogroups. The H antigen represents the filamentous portion of the bacterial flagella (as depicted in Fig. 1). The H antigen can vary in the middle region of the protein subunits called flagellin, which is surface-exposed. *Salmonella* expresses two different H antigens, which are encoded by two different genes, referred as Phase 1 and Phase 2 H antigen (20).

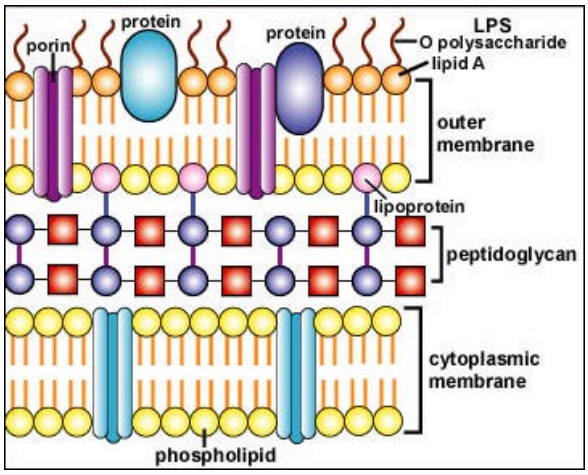


FIG. 3. Structure of a Gram-negative bacterial cell wall. http://www.cat.cc.md.us/courses/bio141/lecguide/unit1/prostruct/u1fig10b.html

To distinguish the different epidemiological phenotypic subdivisions of a serotype, for example within *Salmonella* serovar Typhimurium, phage-typing was introduced by Callow in 1953 resulting in the currently used phage-typing Anderson scheme of 1977 (5) that can distinguish 207 phage types. Different bacteriophages (bacterial viruses) are added separately to a certain isolate of *Salmonella* serovar Typhimurium resulting in lysis or no-lysis of the bacterial cell for each bacteriophage. The pattern of lysis correlates to a phage type number. Lysis of the cell is dependent on the variation of surface-exposed proteins, similar to the serotyping. Normally, a bacteriophage enters the bacterial cell,

multiplies and lyses the cell. The reason that some isolates are sensitive and others are resistant to certain bacteriophages, is not fully understood, although the presence of inactive bacteriophages that are integrated into the genome of *Salmonella* (called prophages) plays a major role. For example, a prophage in the genome that encodes a non-prophage gene such as a bacterial outer membrane protein can change the recognition of the cell for a bacteriophage. An overview of phage and bacterium interactions is shown in Fig. 4. In The Netherlands a different set of bacteriophages is used resulting in a Dutch phage-typing system which differs from the global English phage-typing system and can distinguish more phage types (Max Heck, RIVM, The Netherlands, personal communication).

On a chromosomal DNA level, the different *Salmonella* serovars share 90% DNA content based on DNA hybridization analysis (24), which was also observed by DNA sequencing analysis (28). Most *Salmonella* genes are also present in the nearest family member *Escherichia coli*. Approximately, 80% to 85% identity is found between corresponding genes of both species (14, 24, 50). Comparison of different *Salmonella enterica* genome sequences demonstrates a high degree of genetic exchange. The bacterial chromosome is continually evolving under the influence of (genetic) selection, the integration of mobile genetic elements and genetic recombination. Bacteriophages, plasmids and transferable elements such as integrons are the common types of mobile genetic elements responsible for chromosome plasticity in *Salmonella* (28), which may affect stress survival and/or virulence potencies of specific strains.

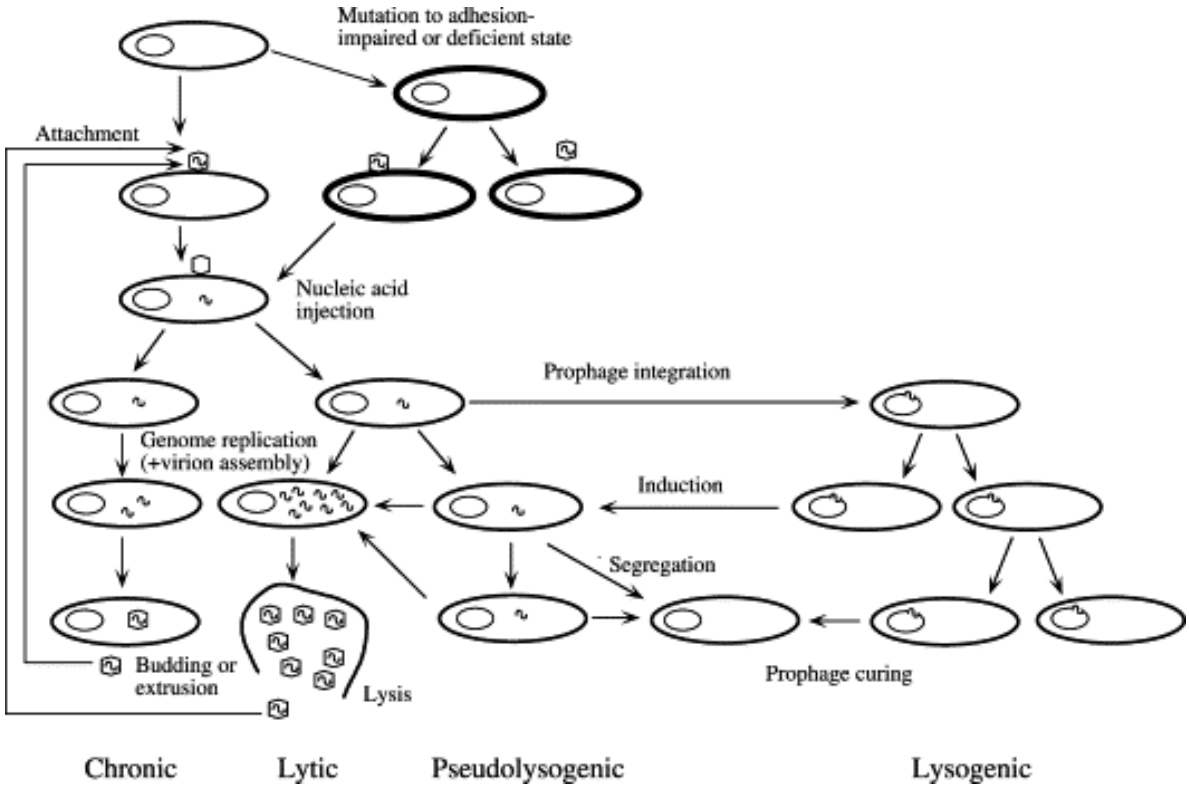


FIG. 4. Types of phage life cycles. The model is adopted from (85).

Salmonella serovar Typhimurium DT104.

The *Salmonella* type studied in this thesis is the multiple-antibiotic-resistant *Salmonella* serovar Typhimurium phage type DT104, which can be subdivided in the two phage types PT506 and PT401 using the Dutch phage-typing system. *Salmonella* serovar Typhimurium is the most common serovar causing gastrointestinal salmonellosis in humans. *Salmonella* serovar Typhimurium is often found in food products. Consuming these products can lead to an infection, causing illnesses like vomiting, diarrhea and stomachaches (6). Consumption of food of animal origin is the main route for transmission of gastrointestinal salmonellosis, and accordingly the most important perpetrator in industrialized countries (60). In developed countries antibiotics are used for example for treatment of bacterial infections but also in animal feed to promote animal growth. Substantial evidence has been presented that the excessive use of antibiotics has led to resistance within the bacterial population (15) and the emergence of multiple-antibiotic-resistance pathogens such as *Salmonella* serovar Typhimurium DT104. *Salmonella* serovar Typhimurium DT104 was first recognized in the early 1980s in the UK and infections concerning DT104 were found to be widespread through Western and Eastern Europe, North America and the Middle East. With the exception of a small outbreak in Scotland in the mid 1980s, there were no human isolates reported until 1989 (78) and in the 1990's DT104 was recognized as an internationally important human and animal pathogen. The occurrence of *Salmonella* serovar Typhimurium DT104 human isolates in the Netherlands among all *Salmonella* isolates and among *Salmonella* serovar Typhimurium isolates, is shown from 1990 to 2005 in Fig. 5 (81, 82). After a peak in 2001, the specific DT104 type is currently decreasing. However, a large DT104 outbreak was reported in October 2005 in The Netherlands with a three-fold higher phage type DT104 occurrence than previously found. Again 42.5 % of all serovar Typhimurium isolates were typed as DT104 in 2005, which is similar to the numbers determined in 2001 (71).

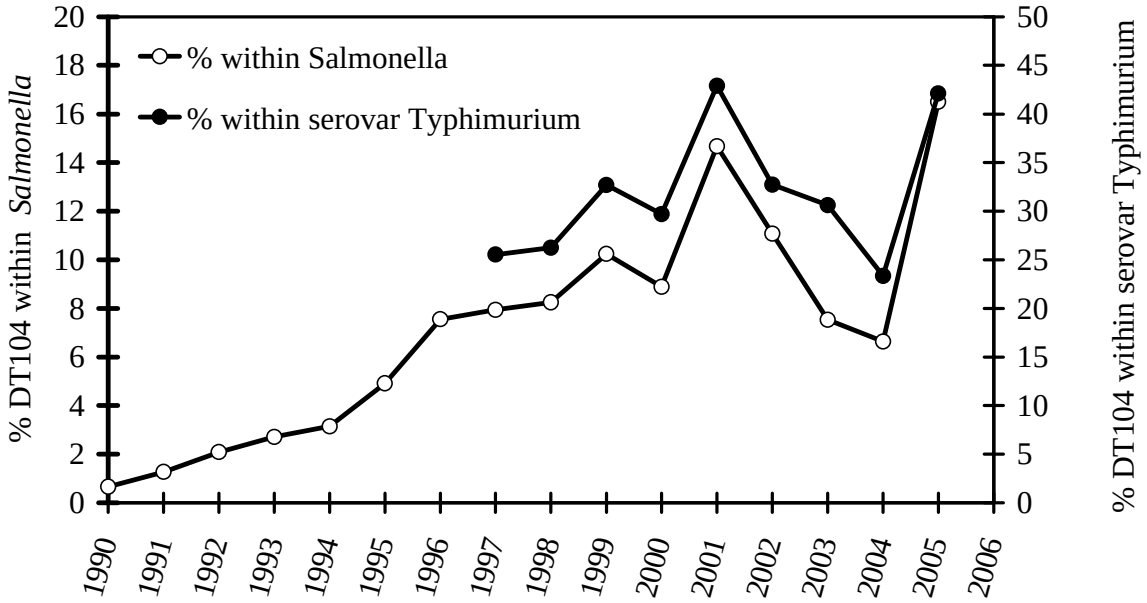


FIG. 5. Occurrence of *Salmonella* serovar Typhimurium DT104 (= PT401 and PT506) human isolates in The Netherlands ((81, 82), http://www.rivm.nl/infectieziektenbulletin)

Early isolates from *Salmonella* serovar Typhimurium DT104 were resistant to the five antibiotics: ampicillin, chloramphenicol, streptomycin, sulfonamide, and tetracycline, referred to as the penta-resistance type ACSSuT. The five corresponding resistance genes are clustered on the genome of *Salmonella* serovar Typhimurium DT104 on integron-, phage-, and plasmid-like structures, called *Salmonella* genomic island one (SGI-I) (37, 43, 79, 80). In later years. DT104 isolates were found that contained also resistance to trimethoprim and ciprofloxacin, (82), and more recently isolates were found that showed resistance against nine antibiotics (43).

In addition to multiply-antibiotic-resistance (19), *Salmonella* serovar Typhimurium DT104 may have higher tolerance or may adapt better to heat and acid than other *Salmonella*, which could offer an additional explanation for the increase in the number of infections i.e. the emergence of this pathogen (43). An infection with *Salmonella* serovar Typhimurium DT104 was reported to be more severe than infections with other *Salmonella* serovars. More often blood was found in the feces, more hospitalization was needed and the mortality of an infection with *Salmonella* serovar Typhimurium DT104 was higher (82). In contrast, invasion studies by others demonstrated that DT104 showed similar invasion and attachment capacities as other common *Salmonella* serovars and phage types (4, 19, 43, 78). Recently, differences in invasion capacity were observed for DT104 cells of different growth stages. Invasion into human epithelium Caco-2 cells (*in vitro*) was the highest at the end of the exponential growth phase. Mid-exponential and early-stationary phase cells were around 10-fold less invasive and overnight grown cells of a significant period of time in stationary growth were around 25-fold less invasive. *In vivo* experiments using rats revealed also that exponential phase DT104 cells were significantly more virulent than overnight grown stationary phase cells (P. Berk, unpublished).

Stress response.

Foodborne pathogens such as *Salmonella* serovar Typhimurium DT104 suffer from a variety of stresses in their ecological niches and during pathogenic infections (depicted in Fig. 6). Foodborne pathogens can nestle in food, which is for example refrigerated, heated, salted and/or pickled. Upon entering the human body, pathogens may also encounter several stresses, including acid stress during stomach passage. In the intestine, bacteria encounter high concentrations of organic acids, due to the degradation of fats (fatty acids) and carbohydrates. In addition, *Salmonella* serovar Typhimurium can multiply inside macrophages, where the bacterial cells are subjected to toxic substances and low pH values. Selective environmental adaptation or evolution of microorganisms by mutations or horizontal gene transfer could be responsible for new strategies of stress resistance. This selection can be provided by the mild food preservation techniques used nowadays. Exposure to mild stress may affect adaptation of *Salmonella* serovar Typhimurium DT104 to survive more extreme stresses. Accordingly, mild food conservation techniques could play a role in the emergence of specific *Salmonella* strains. However, at present no evidence has been reported to support this hypothesis.

The stress of starvation for essential nutrients is the most common kind of stress. Especially the absence of an energy-yielding carbon source leads to a range of changes in cellular metabolism and patterns of gene expression in the bacterium (33). The genetic and

physiologic changes that the bacterium undergoes in response to starvation-stress are referred to as the starvation-stress response (SSR). Several loci of the SSR stimulon have been identified in *Salmonella* Typhimurium. The majority of loci identified, are under positive control of the *rpoS*-encoded sigma factor RpoS, also referred to as sigma S factor or σ^S (see below). In order to survive, *Salmonella* must be able to sense, respond and adapt to changing environments (16). Thus the SSR allows the bacteria to survive periods of starvation. In addition, induction of the SSR, particularly by carbon source starvation, also increases bacterial resistance to a number of other environmental stresses (70), called cross-protection. Another frequently encountered and potentially harmful stress is low pH. Acid stress is described to be the combined biological effect of low pH and weak (organic) acids present in the environment (1). Acidification of food is an ancient but still used technique in food preservation and protection against pathogens. However, facultative intracellular pathogens such as *Salmonella* tolerate periods of low pH, which they also encounter during pathogenesis in the stomach and macrophage phagolysosomes (10). Adaptation to low pH even proved to be essential for *Salmonella* to reach full pathogenicity (65).

Salmonella serovar Typhimurium, like other enteric bacterial pathogens such as *Escherichia coli*, harbors all kinds of stress adaptation or survival mechanisms to deal with these environmental stresses (reviewed in reference 67). Typical genes can be induced under specific or all stress conditions. These genes are coding mainly for transcriptional stress regulator proteins that can activate other genes necessary to adapt or survive the stress condition. Several important transcriptional stress regulators are listed in Fig. 6 as regulons. The important regulator RpoS regulates a large number of genes in response to stress upon entry into the stationary growth phase or other stresses and is therefore referred to as a

environment	stress factor	regulons induced	results
out of host 1	cold, low nutrients	*rpoS, csp*	general stress resistance
stomach	extreme acid pH	*rpoS, fur, ompR phoP*	PhoP induced, bile resistance induced RpoS induced, short chain fatty acids (SCFA) resistance induced
duodenum	bile	*phoP*	membrane modifications, invasion suppressed
ileum	decreased O_2 supply	*fnr, arcA*	switch from aerobiosis to anaerobiosis
	SCFA	*rpoS*	acid-induced cross-resistance to SCFA, SCFA induced cationic antimicrobial peptides (CAMP) resistance
	bacteriocins	*???*	???
	competitive flora, quorum sensing	*sdiA, luxS*	virulence regulation, acid stress?
epithelium	CAMP	*phoP*	LPS modifications, resistance to macrophage CAMPs
out of host 2	cold, low nutrients, aerobiosis	*rpoS, csp, fnr, arcA, oxyR, soxRS*	

FIG. 6. Flow chart of the different stresses experienced by *Salmonella* when colonizing a susceptible host. Flow of infection is downward (arrow-indicated). Experiencing one form of stress always makes *Salmonella* of increased resistance to the stress likely to be encountered during the next step of infection, e.g., acid stress increases *Salmonella* resistance to bile. Adopted from (67).

general stress regulator (reviewed in reference 41 and 48). The transcriptional regulators Fnr, OxyR, and SoxRS are involved in oxidative stress response. The regulation of stress response genes by these regulators under certain stress conditions is very complex and will be exemplified by discussing the acid stress response mechanism in more detail.

When *Salmonella* is exposed to acid stress several genes are induced that code for the transcriptional regulators RpoS, PhoP, and Fur. Subsequently, these regulators induce the expression of other stress genes coding for so-called acid shock proteins (ASPs) as shown in Fig. 7. These regulators can be induced by invasion of protons (H^+) into the bacterial cell. PhoP is induced by the presence of organic acids outside the cell. The regulators induce several ASPs, and also a set of proteins that result in decarboxylase activity. Decarboxylases neutralize the intra cellular pH by removal of H^+. For example H^+ are consumed in the conversion of arginine into agmatine, when agmatine in exchange with arginine is co-transported into the cell (10). Other ASPs repair or protect macromolecules. For example RpoS induces Dps, which binds to DNA in order to protect the DNA from degradation (21, 38).

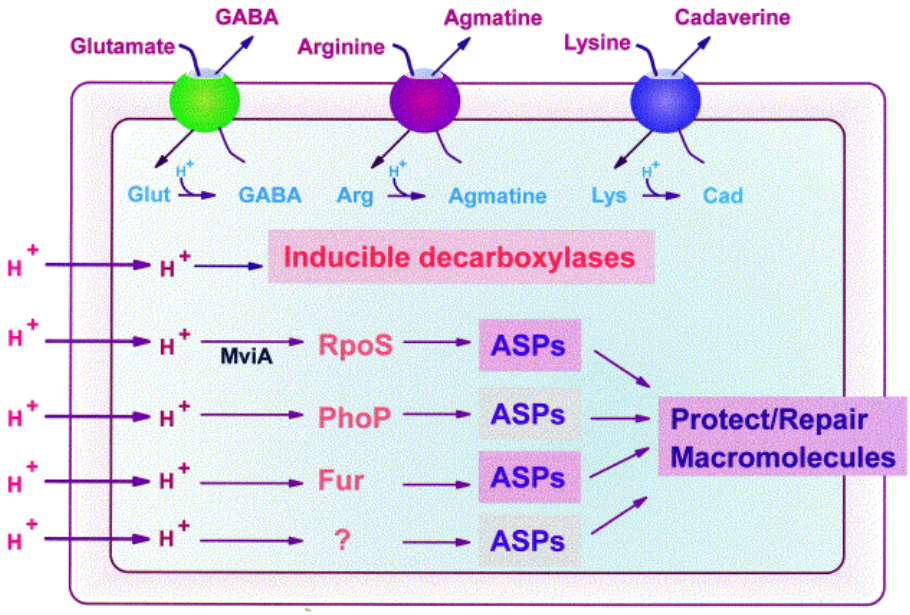


FIG. 7. Schematic drawing of acid induced pathways and induction of decarboxylases. Adopted from (10).

In addition to induction of the stress response via transcriptional stress regulators, the regulators themselves are also regulated via the stress (for example H^+ for acid stress) in a sophisticated manner. The regulation of the important general stress regulator RpoS will be described in more detail. Regulation of σ^S occurs at nearly every theoretically possible level (Fig. 8). For *rpoS* expression, it has been reported that the expression can be stimulated by reduced growth rate (45, 46, 58, 76), although abrupt arrest of growth, such as in response to sudden glucose starvation, only weakly increases *rpoS* transcription (45, 46). Furthermore the *rpoS* translation to σ^S is stimulated (i) by high osmolarity (56), (ii) during growth at moderately low temperatures (20 °C) (69), (iii) on reaching a certain cell density (around 1.5 x 10^8 cells/ml) during growth in minimal glucose medium (45, 46), and (iv) in response to acidity if cells are transferred from pH 7 to pH 5 in rich medium (42). Furthermore, σ^S degradation (proteolysis) is also controlled by different stresses. In cells growing on

minimal medium, σ^S is degraded with a half-life between 1 min and a few minutes (45, 55). However, in response to stresses such as starvation (45, 75), hyperosmolarity (56), heat shock (55), or acid (11), σ^S proteolysis is considerably reduced or even completely inhibited, resulting in high levels of σ^S accumulated in the cell. Nevertheless, the kinetics of this stabilization can vary between different stresses. For example, on hyperosmotic shift, σ^S is strongly stabilized within a few minutes (56), while after heat shock it can take somewhat more time (55), indicating that the level of σ^S is regulated in a similar manner by the different stresses, although the regulatory mechanisms involved are likely to be different. Finally, σ^S revealed to be controlled by a complex signal transduction network whose redundancy, additiveness, and internal feedback regulatory loops are crucial for its signal-integrative power (reviewed in reference 42).

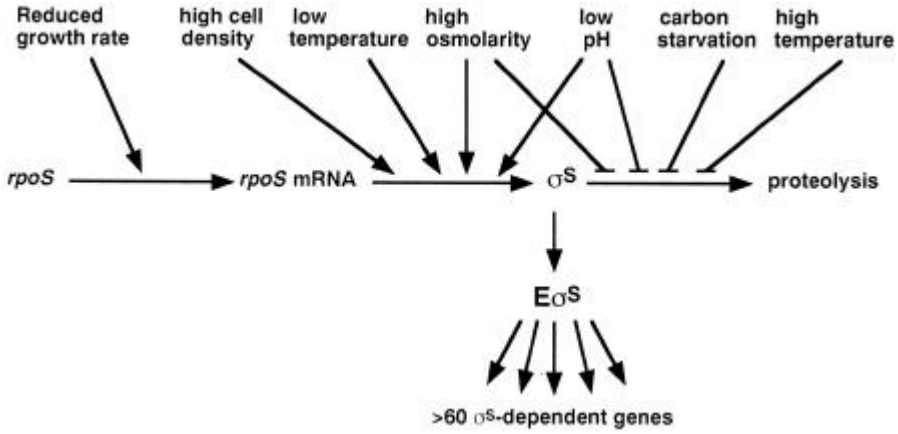


FIG. 8. Various levels of σ^S regulation are differentially affected by various stress conditions. An increase of the cellular σ^S level can be obtained either by stimulating σ^S synthesis at the levels of *rpoS* transcription or translation or by inhibiting σ^S proteolysis. Adopted from (42).

Stress response and virulence.

Salmonella serovar Typhimurium possesses the ability to escape immune responses and overcome body defenses such as low pH. In order to become pathogenic and cause illness it must survive subsequently the acid barrier of the stomach, volatile fatty acids, bile and low oxygen in the small intestine, competition with the gut flora, the physical barrier of the epithelial cells, and acidic pH of the phagosomes (see Fig. 6). Amongst other mechanisms, *Salmonella* serovar Typhimurium uses its acid tolerance systems (Fig. 7) to survive these acidic host environments. Several of the stress response regulators mentioned above have been also described as virulence factors (see Table 1) because stress response proteins are involved in survival of stressful conditions both inside and outside the host. Deletion (inactivation) of the stress regulator PhoP (51, 52) or RpoS (23, 30) resulted in attenuated strains, while strains remained virulent if the stress regulators Fnr (22, 68), Fur (36), OxyR (61), or SoxRS (31, 62) were deleted. An important regulator of both stress adaptation and virulence is RpoS. Already an attenuated RpoS production, caused by a mutation in the *rpoS* gene of a virulent strain, renders it unable to develop a full acid tolerance response and reduces the virulence potential (47). In addition, *Salmonella* serovar Typhimurium contains a plasmid, pSLT, on which a considerable number of virulence genes are present (*spv* operon). These virulence factors are controlled by RpoS (35, 87). The *spv* region appears to promote the survival and rapid growth of *Salmonella* in the host, thereby increasing virulence, but its exact role in this process awaits further study (49).

Full virulence of *Salmonella* requires multiple virulence factors. Around, 4% of the *Salmonella* serovar Typhimurium genome is required for fatal infection of mice, which

corresponds to 200 virulence genes. The need for such a large number of virulence factors is thought to reflect the complex interactions of *Salmonella* with the host. The genes responsible for these interactions are found on plasmids or within the chromosome as units of one, or a few virulence genes (islets) or as large cassettes composed of a series of genes and operons (pathogenicity islands) (49). In the genus *Salmonella*, five *Salmonella* pathogenicity islands (SPIs) are identified so far (39, 49). Some other SPIs have been identified but not characterized yet. Only *Salmonella enterica* ssp. *enterica* has a full complement of the genes of all five pathogenicity islands (63). These SPIs have a lower GC content than the rest of the chromosome and are often inserted into tRNA genes. Therefore, SPIs are likely acquired via horizontal gene transfer from phages or plasmids of unknown origin (49).

TABLE 1. Stress regulators and their relationship to virulence in *Salmonella enterica*.

Protein	Function	Virulence of the mutant[a]
ArcAB	Anaerobiosis/aerobiosis	V
ClpP	Heat shock protease	A
DnaK/DnaJ	Heat shock chaperone	A
Fnr	Anaerobiosis/aerobiosis	V
Fur	Acid pH, oxidative and nitrosative stress	V
GroEL/ES	Heat shock chaperone	?
HtrA	Heat shock protease	A
LuxS	Quorum sensing	V
OmpR/EnvZ	Osmotic shock, acid response	A
OxyR	Oxidative and nitrosative stress	V
PhoPQ	Acid pH, bile salts, CAMP	A
RelA/SpoT	Stringent response	A
RpoE	Extracytoplasmic shock	A
RpoH	Heat shock	?
RpoS	Acid pH, SCFA resistance	A
SdiA	Quorum sensing	V
SoxRS	Oxidative and nitrosative stress	V

[a] A, attenuated; V, virulent; ?, unknown/uncertain. Adopted from (67).

Three SPIs are known to be under control of the stress response regulator PhoPQ (2, 49). In this way virulence is regulated via a mainly acid stress response mechanism regulator, indicating that virulence is induced by acid. These SPIs encoding proteins that are responsible for the invasion of the gastrointestinal epithelium and for survival and replication in host phagocytes (39, 49) might also be activated outside the host in specific food-related stresses, such as acid. In addition, Fur might perform also a role in virulence because Fur mutants are more acid sensitive than wild type strains (86). Finally, a stress tolerant strain of *Salmonella* serovar Enteritidus PT4 revealed to be more virulent in mice and more invasive in chicken than a stress sensitive strain (44). Apparently many similarities between gene expression of stress and virulence genes in *Salmonella* serovar Typhimurium occur. These systems themselves are influenced by environmental stress conditions. Therefore, it is suggested that the ability to sense and adapt to environmental stresses is essential for efficient survival and infection of the host.

Finally, in several other bacterial species AI-2/*luxS*-mediated regulation has been observed on biofilm production, motility, iron acquisition, or virulence factors (reviewed in

reference 88). *Salmonella* serovar Typhimurium contains two possible cell-to-cell signaling systems that are also mentioned in Fig. 6 as possibly important transcriptional stress response regulators: i) the signal receptor SdiA, although the corresponding signal-generating enzyme was not found and SdiA only detects and responds to signals generated by other microbial species ii) the signal-generating component LuxS that mediates synthesis of autoinducer-2 (AI-2) in response to cell density and AI-3 of which the information is rather limited. (reviewed in reference 3). AI-2 revealed to be produced from the mid to the end-exponential growth phase, where its highest level is reached, followed by degradation in the stationary growth phase (72). The only genes currently known to be regulated by AI-2 in *Salmonella* are the *lsr* operon genes encoding an uptake and modification system for AI-2 (3, 73, 74).

Horizontal gene transfer.

Horizontal gene transfer of mobile genetic elements such as bacteriophages or plasmids is an important mechanism for salmonellae to acquire DNA. This mechanism can lead to genomic differences within salmonellae on DNA sequence level but can also interfere with phenotypic characteristics resulting in for example different serovar or phage types in the taxonomy scheme of salmonellae (Fig. 3). In addition, virulence factors were acquired via horizontal transfer, such as the SPIs coding for invasion and replication functions for infection and the plasmid that contains the virulence *spv* operon. Furthermore, adaptation to stress conditions might be explained by gaining new traits via horizontal gene transfer. Finally, *Salmonella* serovar Typhimurium DT104 has acquired the genes for multiply-antibiotic-resistance via integron-, phage-, and plasmid-like structures.

Only in recent years and mainly due to an increase in the numbers of whole genome sequences, it became clear that numerous virulence factors were prophage (= bacteriophage integrated into the genome) encoded in pathogens (Table 2). For example the *Shiga* toxins encoding genes *stx1* and *stx2* of *E. coli* are prophage located and different superoxide dismutase genes (*sodC*) that are necessary for *E. coli* or *Salmonella* to cope with stressful environments within the infected host (13). Experiments demonstrated that prophage-located virulence genes have played a decisive role in the emergence of *Salmonella* serovar Typhimurium. From comparison of closely related bacteria it appears that prophage sequences frequently account for a major proportion of the differences between the genomes (18).

Bacteriophages are bacterial viruses. Transduction is the term used to designate bacteriophage-mediated transfer of DNA from one cell to another cell. The DNA is carried in a virion and introduced in the cell the same way as viral DNA. In generalized transduction any gene may be transported while in specialized transduction only DNA located near the end of the prophage can be transported (12). In the bacterial cell two

TABLE 2. Common themes among bacteriophage-encoded virulence factors

Protein	Gene	Prophage	Bacterial host
Extracellular toxins			
Shiga toxins	*stx1*, *stx2*	H-19B	*E. coli*
Enterohaemolysin	*hly2*	ΦFC3208	*E. coli*
Cytolethal distending toxin	*cdt*	Unnamed	*E. coli*
Proteins altering antigenicity			
Glucosylation	*rfb*	ε^{34}	*S. enterica*
Glucosylation	*gtr*	P22	*S. enterica*
Effector proteins involved in invasion			
Type III effector	*sopE*	SopEΦ	*S. enterica*
Type III effector	*sseI* (*gtgB*)	Gifsy-2	*S. enterica*
Type III effector	*sspH1*	Gifsy-3	*S. enterica*
Enzymes			
Superoxide dismutase	*sodC*	Sp4, 10	*E. coli O157*
Superoxide dismutase	*sodC-I*	Gifsy-2	*S. enterica*
Superoxide dismutase	*sodC-III*	Fels-1	*S. enterica*
Neuraminidase	*nanH*	Fels-1	*S. enterica*
Serum resistance			
Outer Membrane Protein	*bor*	λ	*E. coli*
Outer Membrane Protein	*eib*	λ-like	*E. coli*
Others			
Virulence	*gtgE*	Gifsy-2	*S. enterica*
Antivirulence	*grvA*	Gifsy-2, Fels-1	*S. enterica*

Selection from (13, 17).

responses can follow infection (Fig. 4.). A lytic response, in which the viral DNA is replicated and packaged into virus particles, leading to the lysis of the host cell. In a lysogenic or temperate response, the DNA is integrated in the host cell DNA. In this way the phage DNA can be replicated together with the host cell DNA for many generations. It is commonly anticipated that a bacteriophage senses the physiological state of its host bacterium, which in turn influences its decision to induce a new replication cycle or to stay silent (18). A bacterial cell with integrated phage DNA is called a lysogen. When a lysogen contains a bacteriophage that stays quiescent in the bacterial DNA this bacteriophage is called a prophage (12). Prophages are, however, not a passive genetic cargo of the genome but are likely to be active players in cell physiology. The integration of a bacteriophage into the host chromosome is assumed to occur by site-specific recombination mechanisms that recognize both phage and host attachment sites. These sites share a homologous sequence. The crossover occurs within the identity segment and once integrated, the prophage is flanked by duplicate copies of the homology. A number of phages integrate within host structural genes. The attachment site of these phages contains the 3'end of the target gene so that it replaces the portion of the gene displaced by the integration. The consequence of this mechanism is that the target gene remains intact, and the prophage is flanked by duplications of the 3'end of the gene. Both protein and tRNA genes are used for attachment (7). Temperate phages encode functions that increase the fitness of the lysogen. Due to the selective value of these postulated phage genes, the lysogenic cell will be maintained or even be over represented in the bacterial population. An obvious selective advantage for the lysogenic host is the protection of the lysogen against phage infection by phage repressor

proteins and superinfection exclusion genes of the prophage. Bacteria are confronted with a dilemma: bacteriophages are a threat to their survival and at the same time they are an important tool for the acquisition of genes for increased survival in their ecological environment or to survive in new environments (18).

An additional gene located on a prophage which does not have a phage function but may act as fitness factors for the bacterium, such as the genes depicted in Table 2, is termed a “moron”. Theoretically, the moron is thought to enhance the bacterial virus (bacteriophage) replication when residing as a prophage in the bacterial chromosome. This enhancement is indirect since the moron-encoded function enhances the fitness of the bacterium and improves the fitness of the bacteriophage only passively via its propagation within the bacterium. To provide a benefit for the bacterium, a moron must fulfill several requirements. (i) The moron has to be useful in the ecological niche of the bacterium, either beneficial in the bacterium’s “old” niche; in its sudden changing "old" niche; or to conquer a new niche. (ii) The expression of the moron function must be coordinated and well controlled with the functions of the host bacterium. The moron has to be expressed under very specific conditions to be uniquely beneficial. (iii) In some cases the moron function relies on the proper function of and interaction with other bacterial factors. For example, in Gram-negative bacteria, extracellular enzymes or toxins requiring a specialized export apparatus will be functional only if the bacterium provides the proper transport systems. (17)

Functional genomics.

Recent technical developments and mainly triggered by the availability of whole genome sequences resulted in high-throughput methods to analyze multiple features of an organism in one glance. For example instead of only measuring gene expression of one or a few genes, nowadays hundreds of genes or even all genes present on the genome of an organism can be measured simultaneously. Detecting on a large scale the presence of known genes from a sequenced organism in another non-sequenced organism is called genomics. Measuring gene expression on a large scale is named transcriptomics. Similar approaches for proteins or metabolites are called proteomics and metabolomics. If genomes of different organisms are compared, this approach is called comparative genomics. Also on transcript, protein and metabolite level comparisons can be made between organisms.

The development of the microarray technology has increased research on detecting large numbers of genes and expression of these genes. The microarray is a relatively new technology that allows monitoring expression and regulation of many, and in some cases all of the genes of an organism. Unlike traditional techniques, in which researchers chose one or two genes and searched for the conditions under which their expression level changed, the use of microarrays allows scientists to determine how expression of large quantities of genes change as a function of time, environmental conditions, and genetic composition of cells (57). Thus, one of the strengths of transcriptomics is the ability to completely define regulons and their regulatory networks.

For *Salmonella* serovar Typhimurium, transcriptomics studies have been triggered by the publication in November 2001 of the complete genome sequence of *Salmonella* serovar Typhimurium Lilleengen type 2 (LT2), which is an attenuated laboratory strain. The number

of genes located on its 4,847 kilobases (kb) genome was estimated around 4,500 and 108 for the 94 kb virulence plasmid (50). More recently, other virulent *Salmonella* serovar Typhimurium strains are (being) sequenced by the Sanger Institute, resulting in publicly available (un)finished but not yet annotated genome sequences (http://www.sanger.ac.uk/Projects/Salmonella), including the strains SL1344, D23580, and a phage type DT104 and DT2 strain. *Salmonella* serovar Typhimurium transcriptomics started with in-house developed microarrays from the groups of Jay Hinton in the UK and Michael McClelland in the USA. However, only recently *Salmonella* serovar Typhimurium microarrays are available commercially from Qiagen and Sigma-Genosys. Most of the *Salmonella* serovar Typhimurium transcriptomics research was focused on virulence topics or stress response in relation to host conditions such as cationic antimicrobial peptides, bile, hydrogen peroxide and temperature shift from 25 ^{o}C to 37 ^{o}C and is reviewed by Thompson *et al.* (77). All of the transcriptomics work was performed with either *Salmonella* serovar Typhimurium LT2, ATCC14028(s) or SL1344. The SL1344 strain is an interesting virulent strain to study because this strain and also many isolates of the phage types DT49 and DT204 contains the virulence factor SopE on a prophage called SopΦ. Moreover these SopE containing isolates emerged in the 1970s and 1980s (32, 40, 53) However, no transcriptomics research has been performed on the more recently emerging DT104.

Treatment with 1 μg/ml of the cationic peptide polymyxin revealed that 4.5% of the genes and putative genes identified in the sequenced LT2 strain were induced (>3.5-fold) and 3.4% repressed (<3-fold) (8). Within the macrophage 20.6 % of the genes changed in expression (>2-fold) (29). In response to 3% bile 2.3% were induced and 2.9 repressed (>3-fold) (64). When the expression of swarming (0.6% agarose medium) and non-swarming (liquid medium) cells were compared this revealed that 19.9% were induced and 11.7% repressed (84). Finally, a temperature shift from 25 ^{o}C to 37 ^{o}C resulted in 11.9 % of the genes to change in expression (59). A more detailed analysis of what genes where induced or repressed under these various conditions resulted in the following. Cationic peptide treatment induced genes of the RpoS and PhoP regulon and repressed SPI-1 located and flagella genes (8). The macrophage environment induced virulence genes of the *Salmonella* virulence plasmid and SPI-2, while SPI-1, LPS, surface structures, cell motility and secretion encoding genes were repressed (29). Bile treatment repressed expression of SPI-1, motility and flagella encoding genes (64). When cells swarm, SPI-1, LPS and iron regulated genes are induced, while SPI-2 is repressed (84). The temperature shift induced SPI-1, flagella and chemotaxis encoding genes (59). Finally, hydrogen peroxide treatment induced SoxSR, LPS and SPI-2 genes, while phoPQ and SPI-1 genes were repressed (34).

The above mentioned transcriptomics work revealed many different effects of the various conditions. However, comparison between the different studies remains difficult because different media, strains, microarrays, data normalization etc. have been used. Standardization of the microarray experiments at the experimental design stage, such as similar culture medium, RNA isolation and using DNA as reference sample will improve the possibility of comparing different studies (77). Furthermore, several studies measured gene expression in the course of time, while others studied an effect only at mid-exponential growth. Sometimes, a gene expression effect might only be observed in a specific growth phase and only measuring expression at mid-exponential would not be sufficient. Finally, culturing the cells anaerobically or aerobically could also have large effects on gene

expression under the various conditions mentioned above. However, a detailed (comparative) analysis of the performance of *Salmonella* serovar Typhimurium under aerobic and under anaerobic conditions has not been performed up to now.

Concluding remarks.

Traditional conservation techniques such as sterilization, freezing and pickling to control biological safety hazards in food are severe and can have major effects on for example the flavor and texture of the food. Therefore preservation techniques are becoming milder in answer to consumers' demands for higher quality and less heavily processed and preserved food. These milder food-processing methods consist of mild heating, high-pressure treatments, and vacuum or oxygen-depleted packaging. These methods have demonstrated to maintain the quality and texture of the food better than the traditional methods. Due to these mild techniques pathogens can survive more easily (1). Moreover, stress adaptation of food pathogens may affect their virulence because of better survival of the stresses encountered in the host during infection including passage of the stomach and the gastro-intestinal tract. The adaptation of these food pathogens to environmental stresses such as the food preservation-related stresses is considered to be an increasingly important area of microbiology (27). *Salmonella* serovar Typhimurium DT104 possesses the ability to survive mild environmental stress and to adapt to more severe stresses (25). Thus milder food conservation techniques might also enable *Salmonella* serovar Typhimurium DT104 to adapt to mild stress. Whether this also leads to the occurrence of stable highly stress resistant and/or more virulent isolates remains to be established.

Many genes are involved in the various stress response mechanisms underlying stress adaptation and survival. To be able to study and analyze all these genes, a transcriptomics approach by studying gene expression of multiple genes simultaneously would be suitable. Therefore, the microarray technology was used. In the transcriptomics work presented in this thesis, a thematic microarray was used that represented known and putative genes involved in stress response and virulence mechanisms for *Salmonella* serovar Typhimurium. This thematic stress response and virulence microarray was based on the publicly available genome sequence of the LT2 strain (50). Because the sequence of LT2 was used to measure gene expression in *Salmonella* serovar Typhimurium DT104, additional unknown stress response and virulence present in the genome of DT104 could be missed and sequences of particular genes might be different between both strains. However, strategies can be developed to overcome these experimental problems, for example by genomic subtractive hybridization to identify DT104-specific genes. In addition, only studying expression of a selection of genes i.e. genes fitting within a specific theme, instead of the whole genome, may also have significant advantages including more easy data handling. Another advantage of using thematic microarrays are the lower costs, which makes this technology also available for research groups that study organisms that are not commercially interesting enough for companies to develop whole genome microarrays. Thematic microarrays are therefore ideally suited for assessment of gene profiling under a wide range of conditions.

OUTLINE OF THIS THESIS

This thesis primarily aims at analyzing global expression of stress response and virulence genes under various food-related stresses that *Salmonella* serovar Typhimurium DT104 has to cope with. Adaptation to one stress condition may result in an increased resistance to other stress conditions, a situation referred to as cross-protection. In addition, adaptation to stress may also affect the virulence potency of pathogens. Since the genome sequence of *Salmonella* serovar Typhimurium LT2 is available, this information was used to select stress and virulence genes for inclusion on a thematic array to study gene profiling in *Salmonella* isolates focusing mainly on DT104. A genomic subtractive hybridization was performed between *Salmonella* serovar Typhimurium LT2 and *Salmonella* serovar Typhimurium DT104 to search for novel *Salmonella* serovar Typhimurium DT104-specific genes to be included on the array (**Chapter 2)**. The *Salmonella* serovar Typhimurium DT104-specific genes obtained, that were often of bacteriophage origin, were further analyzed and a PCR-based method was developed, as described in **Chapter 3,** to study the distribution of prophages ST64B, ST104, ST104B, Gifsy-1, Gifsy-2, Fels-1, and Fels-2 among different *Salmonella* serovar Typhimurium isolates. To be able to study expression of multiple stress response and virulence genes for *Salmonella* serovar Typhimurium DT104, a thematic stress response and virulence microarray was developed and assessed, as described in **Chapter 4**. The microarray developed was applied for comparative transcriptome analysis of *Salmonella* serovar Typhimurium DT104 and its *luxS* deletion mutant at various phases of growth **(Chapter 5)**. Deletion of *luxS* prevents production of autoinducer-2, a cell-to-cell signaling molecule playing a role in quorum sensing and stress response regulation (72). **Chapter 6** describes the gene expression analysis of *Salmonella* serovar Typhimurium DT104 exposed to food and/or virulence-related stresses such as heat, hydrogen peroxide and acid, both under aerobic and anaerobic conditions. Finally, **Chapter 7** summarizes the research described in this thesis, the main conclusions and future perspectives.

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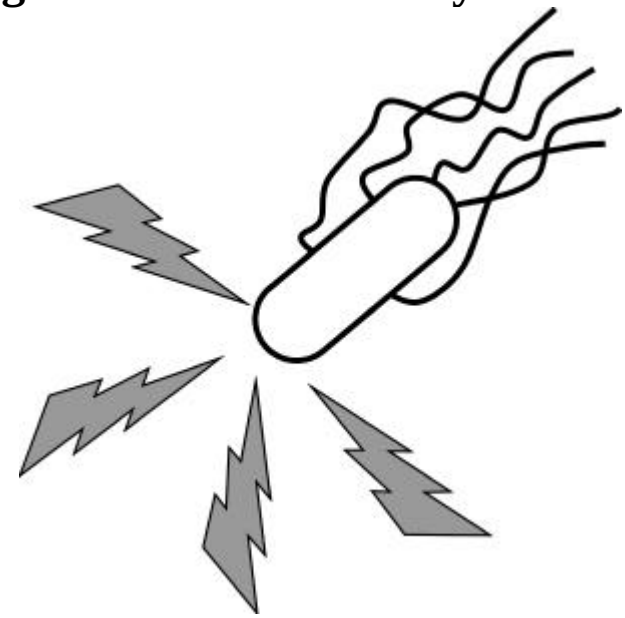
Chapter 2

Identification of novel *Salmonella enterica* serovar Typhimurium DT104-specific prophage and non-prophage chromosomal sequences among serovar Typhimurium isolates by genomic subtractive hybridization



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Published in: Applied and Environmental Microbiology, 2005, 71:4979-4785

ABSTRACT

Genomic subtractive hybridization was performed between *Salmonella enterica* serovar Typhimurium LT2 and DT104 to search for novel *Salmonella* serovar Typhimurium DT104-specific sequences. The subtraction resulted mainly in the isolation of DNA fragments with sequence similarity to phages. Two fragments identified were associated with possible virulence factors. One fragment was identical to *irsA* of *Salmonella* serovar Typhimurium ATCC 14028, which is suggested to be involved in macrophage survival. The other fragment was homologous to HldD, an *Escherichia coli* O157:H7 lipopolysaccharide assembly-related protein. Five selected DNA fragments - *irsA*, the HldD homologue, and three fragments with sequence similarity to prophages - were tested for their presence in 17 *Salmonella* serovar Typhimurium DT104 isolates and 27 non-DT104 isolates by PCR. All five selected DNA fragments were *Salmonella* serovar Typhimurium DT104-specific among the serovar Typhimurium isolates tested. These DNA fragments can be useful for better detection and typing *Salmonella* serovar Typhimurium DT104.

INTRODUCTION

During the past decades, *Salmonella enterica* subsp. *enterica* serovar Typhimurium (*Salmonella* serovar Typhimurium) infections have increased in many parts of the world. In particular, the multiple-antibiotic-resistant *Salmonella* serovar Typhimurium phage type DT104 has been identified as an emerging pathogen (9, 12, 24). For example, for human isolates in The Netherlands, the percentage of *Salmonella* serovar Typhimurium DT104 increased from 7 % of total *Salmonella* serovar Typhimurium isolates in 1990 to 1995 to 29% in 1996 to 2001, respectively (27). *Salmonella* serovar Typhimurium DT104 is multiply antibiotic resistant via a 43-kb *Salmonella* genomic island I (SGI-I), containing phage- and plasmid-related genes, and five antibiotic-resistance genes to ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline (3, 5, 25).

Pathogens can acquire horizontally transferable genetic elements such as plasmids, genomic islands, and prophages, which often contain virulence factors. For example, the acquisition of virulence factors located on prophages can play an important role in the emergence of specific pathogens (4, 11). Various virulence factors located on transferable elements have been described for *Salmonella* serovar Typhimurium for example, *Salmonella* serovar Typhimurium LT2 contains a *Salmonella* virulence plasmid, *Salmonella* pathogenicity islands, and Gifsy and Fels prophages (15). In addition, strain-specific virulence factors located on prophages have been described for several *Salmonella* serovar Typhimurium strains. Phage Fels-1 of *Salmonella* serovar Typhimurium LT2 carries *nanH* and *sodCIII*, phage Gifsy-3 of *Salmonella* serovar Typhimurium ATCC 14028 encodes *pagJ*, and phage SopEΦ of *Salmonella* serovar Typhimurium SL1344 contains *sopE* (8, 16). Two prophages (PDT17 and ST104) have been identified in *Salmonella* serovar Typhimurium DT104 (22, 23), although no virulence association has been reported. In addition, a *Salmonella* serovar Typhimurium DT104-specific DNA fragment has been identified which is homologous to genes encoded by *Escherichia coli* O157:H7 prophages (14, 20).

The objective of the present work was to identify and characterize *Salmonella* serovar Typhimurium DT104-specific sequences, which may lead to the identification of novel virulence factors. Therefore, a genomic subtractive hybridization (2, 6, 7, 17) was performed between *Salmonella* serovar Typhimurium LT2 and DT104.

MATERIALS AND METHODS

TABLE 1. *Salmonella* serovar Typhimurium isolates used in this study

Phage type[a]	Strain no.	Isolation source[b]
ARS	435	turkey
	444	human
DT104	7945	pig
	ATCC BAA-188	human
	so-2945	human
	so-3633	cacao
	stm 911	human
	stm 327	helva dessert
LT2 (=DT4)	ATCC 700720	-
	286 (ATCC 29946)	-
ND	275 (ATCC 13311)	human feces
	375	-
	389	-
	390	-
OS	254	-
	255	-
	256	-
	257	-
PT3	322	meat
	419	chicken
PT10	323	-
PT296	413	pig
	462	human
PT301	416	pig
PT350	412	pig
	445	meat
PT353	414	pig
PT401	408	pig
	411	pig
	420	-
	461	human
PT506[c]	406	human
	410	pig
	418	dairy cow
	427	human
	433	human
	436	pig
	443	chicken products
	448	human
	451	pig
	454	human
	455	human
PT507	452	pig
PT510	415	pig

[a] PT, phage types according to the Dutch phage-typing system. ND, not determined.
[b] -, isolation source unknown.
[c] PT506 is typed as DT104 in the English phage-typing system.

Bacterial strains and growth conditions.

The bacterial strains used in this study are listed in Table 1; they included isolates from the strain collections of RIKILT, the Dutch National Institute of Public Health and the Environment (RIVM), the Norwegian Institute of Public Health (isolates 911 and 327) and the American Type Culture Collection (ATCC). All isolates were stored at -80^{o}C in brain heart broth (Merck, Darmstadt, Germany) plus 50% glycerol (Merck). The isolates were grown overnight in brain heart broth (Merck) at 37^{o}C without shaking.

The Dutch phage-typing system for serovar Typhimurium was gauged in 1997 and 1998 against the English-phage typing system and showed no clear one-to-one relationship. The following relationships between the two phage-typing methods were applied to the phage types mentioned in this paper: the Dutch atypically reacting strains (ARS) correspond with ARS in the English system; the nontypeable strains (OS) correspond with OS; PT10 with DT3; PT296 with DT12; PT3 with DT41, DT1, and DT12; PT301 with DT52; PT350 with DT193; PT353 with DT194; PT401 with DT193, DT104, and DT120; PT506 with DT104; PT507 with DT208; and PT510 with DT208 (W. van Pelt, personal communication).

Subtractive hybridization library construction.

First, genomic DNA was extracted from *Salmonella* serovar Typhimurium DT104 strain 7945 (tester) and strain LT2 (driver) by using a genomic DNA wizard kit (Promega, Madison, Wis.). Subtractive hybridization was carried out using the PCR-Select Bacterial Genome Subtraction kit (BD Clontech, Palo Alto, CA), as recommended by the manufacturer. In addition, glycogen (2 μg/μl; SEQ DTCS kit; Beckman Coulter, Princeton, NJ) was added during the precipitation step after the *RsaI* digestion to increase the precipitated DNA yield. The PCR products obtained at the end of the subtraction procedure were ligated into the pGEM-T Easy vector (Promega). The subtractive hybridization library was constructed by transforming the ligation mixture to XL2-Blue ultracompetent *E. coli* cells (Stratagene, La Jolla, CA) with ampicillin (50 μg/ml) and isopropyl-β-D-thiogalactopyranoside (IPTG)-5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) selection and screening on Luria-Bertani Lennox agar plates (Difco, Detroit, Mich.), as described by the supplier. Individual colonies (n = 192) were picked and grown overnight at 37^{o}C in Luria Bertani Lennox broth (Difco) with ampicillin (50 μg/ml) selection. Plasmid DNA was isolated using a miniprep plasmid isolation kit (QIAGEN, Valencia, CA).

DNA sequencing and analysis.

DNA sequencing was performed on a capillary sequencer (Beckman Coulter) using the CEQ DTCS kit (Beckman Coulter) according to the supplier's instructions. The sequence reactions were initiated by using the forward primer M13. The sequences obtained from the clones were analyzed using BLASTN and BLASTX through the databases mentioned in the next section. The BLASTN or BLASTX hit with the highest similarity was picked and, if possible, linked to functionality. The unique fragments obtained that showed no similarities to the already known *Salmonella* serovar Typhimurium DT104-specific SGI-I (GenBank accession no. AF261825 (3)), were additionally sequenced twice in both directions (M13 forward and M13 reverse primer).

Nucleotide databases used.

The following databases were used to analyze the sequences of the subtraction library: (*i*) GenBank at the National Center for Biotechnology Information (NCBI); (*ii*) the *Salmonella* genomes of the microbial-genomes database (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi) at NCBI (the finished genomes of *Salmonella enterica* serovar Paratyphi A ATCC 9150, *Salmonella enterica* serovar Typhi CT18, *Salmonella* serovar Typhi Ty2, and *Salmonella* serovar Typhimurium LT2 and the unfinished genomes of *Salmonella enterica* serovar Dublin, *Salmonella enterica* serovar Enteritidis PT8 strain LK5, *Salmonella* serovar Typhimurium DT104, *Salmonella* serovar Typhimurium SL1344, *Salmonella* serovar Paratyphi B strain SPB7, and *Salmonella bongori* 12149); (*iii*) the DNA fragment databases of *Salmonella* serovar Enteritidis PT4, *Salmonella enterica* serovar Gallinarum 287/91, *Salmonella* serovar Typhimurium DT104, *Salmonella* serovar Typhimurium SL1344, and *S. bongori* 12419, of which the sequence data of which were produced by the *Salmonella* spp. Sequencing Group at the Sanger Institute and can be obtained from ftp://ftp.sanger.ac.uk/pub/pathogens/Salmonella; and (*iv*) the DNA fragment databases of *Salmonella* serovar Paratyphi A ATCC 9150 and *Salmonella enterica* subsp. *diarizonae* serovar 61:1,v:1,5,(7) ATCC BAA-639, of which the sequence data of which

were produced by the Genome Sequencing Center, at the Washington University School of Medicine and can be obtained from http://genome.wustl.edu/blast/client.pl

Detection of genomic DNA fragments by PCR.

Primer sets were designed (Gene Runner, version 3.05) to detect five DNA fragments selected from the fragments obtained from the subtractive hybridization library (see Table 3): two fragments with non-prophage sequence homology (fragments 117 and 144) and three fragments homologous to prophage sequences found only in *Salmonella* serovar Typhimurium DT104 and not in other *Salmonella* genomes (fragments 84, 168, and 180). The two non-prophage fragments (fragments 117 and 144) were named *irsA* and HldD homologue according to their homology to *irsA* of *Salmonella* serovar Typhimurium ATCC 14028 and HldD of *E. coli* O157:H7, respectively. In addition, one control primer set was used to detect a DNA fragment (orf STM1056) in the Gifsy-2 prophage which should be present in all *Salmonella* strains. An overview of the primers used and the expected amplicon sizes is shown in Table 2.

The primers (Isogen, Maarssen, The Netherlands), at a 0.2 nM concentration, were combined with about 1 to 10 ng DNA template and amplified with Taq polymerase (Invitrogen, Carlsbad, CA). After an initial denaturation at 95^{o}C for 3 min, the samples were subjected to 30 cycles of 95^{o}C for 30 s, 60^{o}C for 60 s, and 72^{o}C for 45 s, followed by a final 7-min incubation at 72^{o}C. Samples were fractionated by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. All PCRs were performed four times for each *Salmonella* serovar Typhimurium isolate shown in Table 1.

TABLE 2. Overview of the PCR primers used for the PCR detection of genomic fragments

PCR fragment	Primer name	Sequence (5'-3')[a]	Amplicon size [bp]
HldD homologue	hldDF	ACAATGCTTTCGAACCTGATGGGC	510[b]
	hldDR	CCATCGCTTCAATTGCAACCATGC	
irsA	irsAF	ATTCAGGCTGCGCTCCGTCTTTAC	416[b]
	irsAR	CTTAATGAGGCGGCGGAACAGTAC	
Fragment 84	frag-84F	ATGGCACAACCACTCAGTAATCCG	372[b]
	frag-84R	AGATGGCATCGGCGTAGTTATGAG	
Fragment 168	frag-168F	CATCCATTACCGGGTTGTCCATCC	492[b]
	frag-168R	CGCGTTTAACGGTACAGATGGTGG	
Fragment 180	frag-180F	CCATAATAGGAAGCATTGCGTGAG	476[b]
	frag-180R	ATGCCATCTAGCTATCTCTGCGAC	
Gifsy-2 control	gifsy-2 RB-F	GGTGGCTAAATGTAAATGACGTGG	488[c]
	gifsy-2 RB-R	TGAGCGAGATCGAGATGAAGCTTG	

[a] All primers were designed and used first in this study.
[b] Amplicon size based on *Salmonella* serovar Typhimurium DT104.
[c] Amplicon size based on *Salmonella* serovar Typhimurium LT2.

Nucleotide sequence accession number.

The nucleotide sequences of the 34 *Salmonella* serovar Typhimurium DT104 fragments that are listed in Table 3, have been submitted to GenBank in numerical fragment order with accession numbers AY462969 to AY463002, respectively.

RESULTS

DNA sequencing and analysis.

The sequence reactions performed on 192 picked colonies of the subtractive hybridization library resulted in 126 different DNA fragments, of which 57 fragments were not found in the *Salmonella* serovar Typhimurium LT2 genome by using BLASTN analysis. After BLASTN and BLASTX analysis through GenBank, these 57 *Salmonella* serovar Typhimurium DT104 fragments were divided into four groups showing sequence similarity to either SGI-I (group A; $n = 23$), phage sequences (group B; $n = 30$), nonphage sequences (group C; $n = 2$), or nonsignificant sequences (group D; $n = 2$). Table 3 shows the BLAST search results for all fragments of groups B to D. The fragments of group A with sequence similarities to SGI-I were not further analyzed, because this *Salmonella* serovar Typhimurium DT104 island had already been sequenced (GenBank accession no. AF261825) (3). The fragments of group B were additionally divided into four phage subgroups showing sequence similarity to either : (i) the *Salmonella* serovar Typhimurium DT64 bacteriophage ST64B, (ii) the *Salmonella* serovar Typhimurium DT104 bacteriophage ST104, (iii) the *Escherichia coli* (STEC) bacteriophage P27, or (iv) other prophages present in the genome of *Salmonella* serovar Typhi CT18, *Salmonella* serovar Typhimurium, *E. coli* K-12, *E. coli* O157:H7 or *Shigella flexneri* 2a.

Although fragments 22, 66, 75, and 84 were not similar to prophage sequences, these fragments were placed in the group of phages (see Table 3) because the adjacent genome regions of the matching BLAST hits were similar to prophage sequences (data not shown). Fragment 117, fragment 158, and all fragments of the phage subgroups i and ii matched to sequences of *Salmonella* serovar Typhimurium origin. In addition, fragment 158 was the only fragment matching to a sequence of phage type DT104 origin that was not located on SGI-I or prophage ST104. All other fragments had not been associated with *Salmonella* serovar Typhimurium before. Notably, the fragments with DNA sequence similarities (phage subgroup i to iii fragments) could be clustered into a subgroup of similar origin, such as bacteriophage ST104, while the fragments with amino acid sequence similarity (phage subgroup iv and groups C and D) could not be clustered into subgroups of similar origin.

The presence of the 34 *Salmonella* serovar Typhimurium DT104 fragments of groups B to D was found, using BLASTN analysis, to be different in available finished and unfinished *Salmonella* genomes (Table 3). Ten fragments were found only in the *Salmonella* serovar Typhimurium DT104 genome, while the other fragments were randomly found in the other *Salmonella* genomes. Among all fragments, three fragments (fragment 62, 66, and 144) were found in a strain of *S. bongori*.

Identification of possible virulence factor candidates.

Based on sequence homology, three *Salmonella* serovar Typhimurium DT104 DNA fragments obtained could be associated with possible virulence factors: fragments 66, 117, and 144. Fragment 66 was similar (91%) to a *Salmonella* serovar Typhi CT18 gene (orf STY1362). This *Salmonella* serovar Typhi CT18 gene is described as being homologous to a putative toxin subunit 1 gene of *Bordetella pertussis* based on the amino acid sequence. However, this gene represents a pseudogene due to at least one frameshift (19). Therefore, it

TABLE 3. BLAST search results for the *Salmonella* serovar Typhimurium DT104 fragments generated by genomic subtraction between the *Salmonella* serovar Typhimurium DT104 and LT2 strains excluding the fragments similar to SGI-I

Group	Fragment no.	Sequence similarity to[a]	Accession no.	Similarity region[b]	% Similarity[c]	Present in *Salmonella*[d]
B) Phage:						
i) ST64B	4	STM bacteriophage ST64B genome: putative DNA methyltransferase	AY055382.1	32607-32895	93	TMsl
	5	STM bacteriophage ST64B genome: putative transcriptional activator	AY055382.1	31896-32507	98	TMsl
	59	STM bacteriophage ST64B genome: hypothetical protein Sb40	AY055382.1	29290-29704	100	Du, E4, TMsl
	95	STM bacteriophage ST64B genome: putative tail proteins Sb22 and Sb23	AY055382.1	16126-16645	97	Du, E4, TMsl
	149	STM bacteriophage ST64B genome: putative DNA methyl-transferase Sb44	AY055382.1	32897-33312	100	TMsl
	155	STM bacteriophage ST64B genome: hypothetical protein Sb46	AY055382.1	33908-34104	98	E4, TMsl
	170	STM bacteriophage ST64B genome: integrase protein Sb28	AY055382.1	21702-22241	99	Du, E4, TMsl
	178	STM bacteriophage ST64B genome: putative tail protein Sb22	AY055382.1	15708-16007	97	Du, E4, TMsl
ii) ST104	2	STM bacteriophage ST104 genome: tailspike protein	AB102868.1	40450-40738	100	Du, Pa, Pb
	17	STM bacteriophage ST104 genome: NinZ	AB102868.1	19809-20268	99	Di, Du
	36	STM bacteriophage ST104 genome: ORF15 (endopeptidase) and ORF19 (lysozyme)	AB102868.1	21707-22377	99	Di, Pa, Pb
	50	STM bacteriophage ST104: tailspike protein	AB102868.1	41078-41391	100	Du, Pa, Pb
	54	STM bacteriophage ST104 genome: Mnt and tailspike protein	AB102868.1	38879-39670	100	Du, Pa, Pb
	78	STM bacteriophage ST104 genome: ORF46, ORF47 and ORF48	AB102868.1	23183-24148	100	Di, Du, Pa, Pb
	98	STM bacteriophage ST104 genome: ORF19	AB102868.1	9548-9791	100	
	101	STM bacteriophage ST104 genome: ORF23 (antitermination protein Q homologue)	AB102868.1	20269-21264	99	Di, Pa, Pb
	110	STM bacteriophage ST104 genome: NinG	AB102868.1	18853-19808	99	Di
	122	STM bacteriophage ST104 genome: SieB	AB102868.1	10671-10897	99	Di, Pa, Pb
iii) P27	8	STY CT18 genome segment 6: putative holin of bacteriophage P27	AL627270.1	15663-15915	94	Pa, Pb, T2, T18, TMsl
	62	STY CT18 genome segment 4: putative holin of bacteriophage P27	AL627268.1	266762-267028	97	Bo, E4, T2, T18
	66	STY CT18 genome segment 6: putative toxin subunit 1 of *Bordetella pertussis*	AL627270.1	14526-15084	91	Bo, Pa, Pb, T2, T18

iv) Others	16	Putative prophage terminase large subunit of a prophage in STY	CAD05440.1	aa 291-453	82 i 89 p	
	22	Unnamed protein product in *Photorhabdus luminescens* subsp. Laumondii TTO1	CAE15767.1	aa1-132	63 i 81 p	
	56	Unknown protein of prophage CP-933U in *E. coli* O157:H7	AAG57029.1	aa23-99	61 i 79 p	
	75	Unnamed protein product in *Photorhabdus luminescens* subsp. Laumondii TTO1	CAE15775.1	aa142-233	65 i 75 p	
	84[e]	Unnamed protein product in *Photorhabdus luminescens* subsp. Laumondii TTO1	CAE15762.1	aa1-113	59 i 78 p	
	100	Exodeoxyribonuclease of Gifsy prophages in STM LT2	AAL19943.1	aa 58-355	46 i 58 p	Ga, Pa, Pb, TMsl
	158[f]	DNA fragment of STM DT104: hypothetical protein YdaU of Rac prophage in *E. coli* K12	AF275268.1	229-516	100	Du, Ga
	168[e]	*S. flexneri* 2a whole genome: putative bacteriophage protein	AE005674.1	2693454-2693818	93	
	180[e]	Hypothetical protein YmfD of prophage e14 in *E. coli* K12	F64858	aa102-216	69 i 82 p	
C) Non-phage:	117[e]	*irsA* gene in STM ATCC 14028	AY328029.1	1627-1948	99	Du, E4, E8, Ga
	144[e]	LPS biosynthesis enzyme HldD (WaaD) of *E. coli* O157:H7	AAC69662.1	aa29-111	75 i 87 p	Bo
D) Not significant:	47	Hypothetical protein in *Caenorhabditis elegans*	CAA88607.1	aa64-216	23 i 41 p	
	64	Hypothetical protein in *Anaeromyxobacter dehalogenans* 2CP-C	EAL78506.1	aa38-155	34 i 55 p	

[a] Abbreviations used: STY = *Salmonella* serovar Typhi and STM = *Salmonella* serovar Typhimurium.
[b] Region of similarity in basepairs or amino acids (aa) based on accession number.
[c] Similarities are based on nucleotide or amino acid sequences. When identities (i) and positives (p) are given, values for positives (similarity based on similar amino acid properties) of =55% are not significant.
[d] Present or partly present, based on >80% similarity by BLASTN analysis, in the *Salmonella* genomes listed: *bongori* 12419 (Bo), *diarizonae* (Di), Dublin (Du), Enteritidis PT4 (E4), Enteritidis PT8 (E8), Gallinarum (Ga), Paratyphi A (Pa), Paratyphi B (Pb), Typhi Ty2 (T2), Typhi CT18 (T18), Typhimurium SL1344 (TMsl). All fragments are present in *Salmonella* serovar Typhimurium DT104.
[e] DNA fragments used for PCR detection of genomic fragments.
[f] DNA fragment described in literature as *Salmonella* serovar Typhimurium DT104 specific (20).

is unlikely that fragment 66 encodes a virulence factor. In addition, four genes encoding the other subunits necessary to form the active *B. pertussis* toxin (18) were not found in the *Salmonella* serovar Typhimurium DT104 genome (data not shown).

Fragment 117 was highly similar (99%) to a part of the *irsA* gene of *Salmonella* serovar Typhimurium ATCC 14028. The *irsA* locus in *Salmonella* serovar Typhimurium ATCC 14028 is described as being involved in macrophage survival (1). Finally, fragment 144 was homologous (75%) to the lipopolysaccharide (LPS) assembly-related protein HldD (formerly named WaaD) of *E. coli* O157:H7, based on the amino acid sequence.

Detection of genomic DNA fragments by PCR.

The presence of five selected DNA fragments - fragment 117 (*irsA*), fragment 144 (HldD homologue), and three fragments homologous to prophage sequences (fragments 84, 168, and 180) - and a Gifsy-2 prophage control fragment was tested among 44 *Salmonella* serovar Typhimurium isolates by PCR (Table 4). The five selected fragments appeared to be present in all 17 *Salmonella* serovar Typhimurium DT104 isolates and absent in all 27 non-DT104 phage type isolates. In addition, the Gifsy-2 prophage control fragment was indeed present in all *Salmonella* serovar Typhimurium DT104 and non-DT104 isolates.

TABLE 4. PCR results for the detection of six genomic fragments in different *Salmonella* serovar Typhimurium isolates

Phage type[a] (no. of isolates)	Presence of absence[b] of:					
	irsA	HldD homologue	Fragment 84	Fragment 168	Fragment 180	Gifsy-2 control
DT104:						
DT104 (6)	+	+	+	+	+	+
PT506 (11)[c]	+	+	+	+	+	+
Non-DT104:						
ARS (2)	-	-	-	-	-	+
LT2 (2)	-	-	-	-	-	+
	-	-	-	-	-	+
OS (4)	-	-	-	-	-	+
PT3 (2)	-	-	-	-	-	+
PT10	-	-	-	-	-	+
PT296 (2)	-	-	-	-	-	+
PT301	-	-	-	-	-	+
PT350 (2)	-	-	-	-	-	+
PT353	-	-	-	-	-	+
PT401 (4)	-	-	-	-	-	+
PT507	-	-	-	-	-	+
PT510	-	-	-	-	-	+

[a] PT, phage types according to the Dutch phage-typing system. ND, not determined.
[b] +, PCR product present; -, PCR product absent.
[c] PT506 is typed as phage type DT104 in the English phage-typing system.

DISCUSSION

The objective of the present work was to identify and characterize *Salmonella* serovar Typhimurium DT104-specific sequences, which may lead to the identification of novel virulence factors. Therefore, a genomic subtractive hybridization was performed between *Salmonella* serovar Typhimurium LT2 and DT104, which resulted in novel DNA fragments not found in *Salmonella* serovar Typhimurium DT104 before. Notably, a large number of fragments were homologous to prophage sequences.

Based on sequence homology, three *Salmonella* serovar Typhimurium DT104 DNA fragments identified were associated with possible virulence factors: fragment fragments 66, 117, and 144. Fragment 66 was homologous to the putative toxin subunit 1 gene of *B. pertussis* found in *Salmonella* serovar Typhi CT18. As mentioned earlier, it is unlikely that fragment 66 encodes a virulence factor because of its similarity to a pseudogene and the lack of other genes in the *Salmonella* serovar Typhimurium DT104 genome necessary to form the *B. pertussis* toxin.

Fragment 117 was highly similar to a part of the *irsA* gene of *Salmonella* serovar Typhimurium ATCC 14028. The *irsA* locus in *Salmonella* serovar Typhimurium ATCC 14028 is described as being involved in macrophage survival (1). In contrast, the *irsA* amino acid sequence is 91% homologous to a CP933R prophage protein of *E. coli* O157:H7 with unknown function (GenBank accession no. AAG56427.1) and 73% homologous to Gifsy prophage proteins (GenBank accession no. AAL19954.1 and AAL21514.1). Due to unknown functionality and homology to common prophage sequences, the virulence association of *irsA* remains to be elucidated.

Finally, the possible virulence factor association of fragment 144, which resulted in homology to HldD of *E. coli* O157:H7, is further analyzed. Recent insight into the *E. coli* O157:H7 LPS assembly showed that HldD, in addition to HldE (formerly named WaaE or RfaE), is involved in the nucleotide-activated *glycero-manno*-heptose biosynthesis for inner core oligosaccharide assembly (13, 26). The HldD homologue found in *Salmonella* serovar Typhimurium DT104 may also be involved in the *glycero-manno*-heptose biosynthesis pathway. Notably, all known *Salmonella* serovar Typhimurium LT2 *waa* genes were also found in *Salmonella* serovar Typhimurium DT104 by using BLASTX analysis (data not shown). Therefore, the HldD homologue will most likely be an additional protein in *Salmonella* serovar Typhimurium DT104. The HldD homologue, as an additional protein for inner core oligosaccharide assembly, may lead to a different inner core structure of the LPS. A different inner core structure can result in a more stable outer membrane, or in an altered host recognition, leading to an altered immune response (reviewed in reference 21), resulting in increased survival and/or virulence. However, more research is needed to assess this role of the HldD homologue in *Salmonella* serovar Typhimurium DT104 virulence.

The five DNA fragments selected from the subtractive hybridization library, fragment 117 (*irsA*), fragment 144 (HldD homologue), and three fragments homologous to prophage sequences (fragments 84, 168, and 180), were *Salmonella* serovar Typhimurium DT104 specific among the tested serovar Typhimurium isolates (Table 4). Notably, in our PCR results, the *irsA* fragment appeared to be *Salmonella* serovar Typhimurium DT104 specific; however, this fragment is also present in the non-DT104 strain *Salmonella* serovar Typhimurium ATCC 14028 (1). Additional BLAST searches revealed that the upstream

DNA regions of *irsA* in *Salmonella* serovar Typhimurium ATCC 14028 and DT104 differ (data not shown). Therefore, the *irsA* fragment tested is not *Salmonella* serovar Typhimurium DT104 specific, but the genome locus may be DT104-specific.

Many DNA fragments obtained in our study were grouped into larger genome fragments, such as SGI-I and the ST64B and ST104 prophages (Table 3). In this and earlier subtractive hybridization studies, almost all differences between closely-related strains were found to be located on large transferable elements such as prophages, plasmids, or fimbrial operons (6, 7, 17). In addition, our PCR results revealed *Salmonella* serovar Typhimurium DT104-specific prophage DNA fragments (Table 4), similar to a previously described *Salmonella* serovar Typhimurium DT104-specific DNA fragment that encodes for *E. coli* O157:H7 prophages homologues (14, 20). These findings lead us to the assumption that several DNA fragments, obtained from our subtractive hybridization, are probably located on a novel *Salmonella* serovar Typhimurium DT104-specific prophage. Matching the fragments obtained to the *Salmonella* serovar Typhimurium DT104 unfinished genome revealed that all fragments of prophage subgroup iv and groups C and D (see Table 3), including *irsA* and the HldD homologue, are clustered (data not shown). This specific prophage may have contributed to the successful clonal expansion of *Salmonella* serovar Typhimurium DT104, as with *Salmonella* serovar Typhimurium DT49 and DT204, which contain phage SopEΦ and emerged in the 1970s and 1980s (8, 10, 16).

In summary, a genomic subtraction is a useful tool for finding strain-specific genes, including possible virulence factor candidates. In addition, the PCR method developed revealed that the *irsA* and HldD homologue fragments and the three prophage fragments 84, 168, and 180 were *Salmonella* serovar Typhimurium DT104-specific among the tested serovar Typhimurium isolates and can be useful for better detection and typing of *Salmonella* serovar Typhimurium DT104.

ACKNOWLEDGEMENTS

This study was supported by The Netherlands Organization for Health Research and Development, “Nutrition: Health, Safety and Sustainability” program.

We are grateful to Annelien Beuling and Sükrü Yigit for technical assistance. The *Salmonella* serovar Typhimurium DT104 food and human isolates were kindly provided by Wim Wannet, National Institute of Public Health and the Environment (RIVM), The Netherlands; Dik Mevius, CIDC-Lelystad, The Netherlands; and Ole Alvseike of the Norwegian Institute for Public Health.

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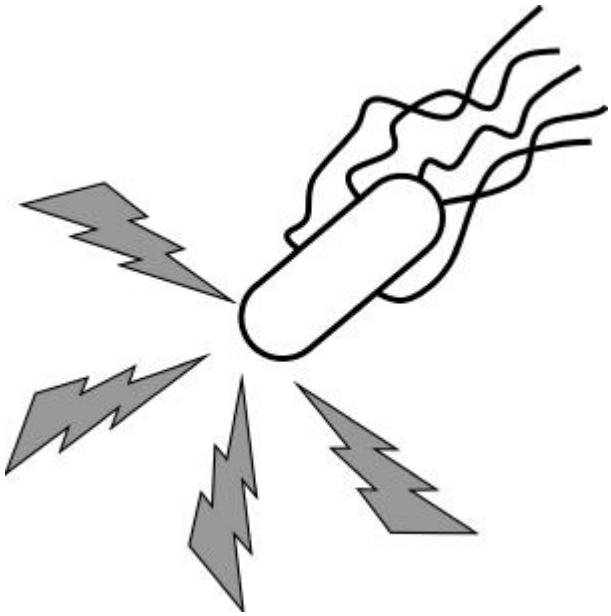
Chapter 3

Distribution of prophages and SGI-1 antibiotic-resistance genes among different *Salmonella enterica* serovar Typhimurium isolates



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Published in: Microbiology, 2006, 152:2137-2147

ABSTRACT

Recently the authors identified *Salmonella enterica* serovar Typhimurium (*Salmonella* serovar Typhimurium) definitive type (DT)104-specific sequences of mainly prophage origin by genomic subtractive hybridization. In the present study, the distribution of the prophages identified, ST104 and ST64B, and the novel prophage remnant designated prophage ST104B, was tested among 23 non-DT104 *Salmonella* serovar Typhimurium isolates of different phage types and 19 isolates of the DT104 subtypes DT104A, DT104B low and DT104L, and the DT104-related type U302. The four *Salmonella* serovar Typhimurium prophages Gifsy-1, Gifsy-2, Fels-1 and Fels-2 were also included. Analysis of prophage distribution in different *Salmonella* serovar Typhimurium isolates may supply additional information to enable development of a molecular method as an alternative to phage typing. Furthermore, the presence of the common DT104 antibiotic resistance genes for the penta-resistance type ACSSuT, aadA2, floR, pse-1, sul1, and tet(G), was also studied because of the authors' focus on this emerging type. Based on differences in prophage presence within their genome, it was possible to divide *Salmonella* serovar Typhimurium isolates into 12 groups. Although no clear relationship was found between different phage type and prophage presence, discrimination could be made between the different DT104 subtypes based on diversity in the presence of prophages ST104, ST104B and ST64B. The novel prophage remnant ST104B, which harbors a homologue of the *Escherichia coli* O157:H7 HldD LPS assembly-related protein was identified only in the 14 DT104L isolates and in the DT104-related U302 isolate. In conclusion, the presence of the genes for penta-resistance type ACSSuT, the HldD homologue containing ST104B prophage remnant and phage type DT104L are most likely common features of the emerging subtype of *Salmonella* serovar. Typhimurium DT104.

INTRODUCTION

Horizontal gene transfer plays an important role in the evolution of bacteria to adapt to changing niches or to exploit new niches. The acquisition of virulence or antibiotic resistance genes via horizontally transferable genetic elements, such as plasmids (conjugation) or bacteriophages (transduction) has resulted in the emergence of a variety of pathogens, (reviewed by (30)). Also, the food-borne pathogen *Salmonella* has acquired a large number of virulence genes via horizontal gene transfer (16). Within the genomes of different *Salmonella enterica* subsp. *enterica* serovar Typhimurium (*Salmonella* serovar Typhimurium) isolates many virulence factors have been reported to be located on prophages, which are bacteriophages integrated into the bacterial genome (2, 12, 14, 18, 25, 26, 39). For example, within the genome of *Salmonella* serovar Typhimurium lilleengen type (LT)2, which is an attenuated laboratory strain and whose genome has been sequenced (24), prophages Gifsy-1, Gifsy-2, Fels-1 and Fels-2 all encode one or more genes that are somehow involved in virulence (reviewed by (7)). These prophages are often identified adjacent to tRNA genes (24), which are hot spots for insertion of transferable elements in general (11, 36), because these genes are highly conserved among bacterial genomes (8, 19). Our study focused on the emerging multiple-antibiotic-resistant *Salmonella* serovar Typhimurium definitive type (DT)104. The isolates of *Salmonella* serovar Typhimurium DT104 that have emerged during the past decades have a core pattern of resistance to the five antibiotics ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline referred to as penta-resistance type ACSSuT (15, 21, 41, 42). The five genes for this penta-resistance type, *aadA2, floR, pse-1, sul1, and tet(G),* are clustered within the genome on the so-called *Salmonella* genomic island I (SGI-I), which also contains horizontally transferable genetic elements such as phage- and plasmid-related genes (5, 6). Based on phage typing for *Salmonella* serovar Typhimurium by the Anderson scheme (1), five subtypes have been described within DT104: DT104A, DT104B, DT04B low, DT104H, and DT104L. Phage type U302 was previously referred to as being DT104-related (6). Interestingly, of 160 DT104 isolates studied, the DT104L subtype isolates harboring penta-resistance have been revealed to be the most frequently occurring subtype, with 40 % of all isolates (23).

The currently used Anderson phage typing scheme uses 34 bacteriophages to distinguish 207 DTs (1) and the pattern of sensitivity of an isolate to these bacteriophages results in a phage type number, such as DT104. However, this typing method has some drawbacks. Several *Salmonella* serovar Typhimurium isolates can not be classified by this method and the method itself requires considerable experience for scoring and to achieve good standardization (20). Furthermore, Schmieger (38) noted that if the original Anderson typing bacteriophages stocks become exhausted, this may be the end of this method. Therefore molecular methods have been suggested to be developed and implemented to replace conventional phage typing (20, 43). The presence of a prophage in the *Salmonella* serovar Typhimurium genome can affect the susceptibility to a bacteriophage resulting in a different phage type, referred to as phage type conversion (29, 34, 43). Analysis of prophage

distribution in different *Salmonella* serovar Typhimurium isolates may supply additional information for such a molecular method for phage typing.

In a previous genomic subtractive hybridization study, we identified novel *Salmonella* serovar Typhimurium DT104L sequences of mainly prophage origin (17). The sequences obtained were similar to those of bacteriophages ST104 (40) and ST64B (27) sequences. Furthermore, a novel prophage remnant was identified, designated prophage ST104B in the current study, which harbors a homologue of the *Escherichia coli* O157:H7 HldD LPS assembly-related protein and the *irsA* gene which is suggested to be involved in macrophage survival (3). The objective of the present study was to investigate the distribution of the prophages ST104, ST104B and ST64B among *Salmonella* serovar Typhimurium isolates of different phage types, with special focus on *Salmonella* serovar Typhimurium DT104 and related types. The four prophages Gifsy-1, Gifsy-2, Fels-1 and Fels-2 that have been described in the sequenced *Salmonella* serovar Typhimurium LT2 strain were also included. Because of our focus on DT104 and the importance of the multi-antibiotic-resistance of this type, all isolates were also tested for the presence of the five SGI-I-located antibiotic resistance genes. Differences in the presence of prophages can be indicative of the virulence potency of a *Salmonella* serovar Typhimurium strain, since many virulence factors have been reported to be located on prophages. Furthermore, these results will help to increase our knowledge of the relationship between prophage presence and conventional phage typing for *Salmonella* serovar Typhimurium.

MATERIALS AND METHODS

Bacterial strains, culture conditions and preparation of genomic DNA.

The *Salmonella* serovar Typhimurium isolates of different phage types used in this study are listed in Table 1 and were obtained from RIKILT Institute of Food Safety, the Dutch National Institute of Public Health and the Environment (RIVM) and the American Type Culture Collection (ATCC). The isolates were typed at the National *Salmonella* Reference Laboratory of the Federal Institute for Risk Assessment, Germany. All isolates were stored at -80 ^{o}C in brain heart broth plus 50 % (v/v) glycerol. The isolates were grown overnight in brain heart broth at 37 ^{o}C without shaking. Genomic DNA was extracted from overnight cultures by using a genomic DNA wizard kit (Promega).

Prophage detection approach.

A PCR method was developed for the detection of prophages Gifsy-1, Gifsy-2, Fels-1, and Fels-2 identified in the attenuated laboratory strain *Salmonella* serovar Typhimurium LT2 (GenBank accession no. NC_003197) (24), and prophages ST104 and ST64B, and the prophage remnant ST104B, referred to as the non-LT2 prophages, which we could identify in *Salmonella* serovar Typhimurium DT104L. The non-LT2 prophages were identified in *Salmonella* serovar Typhimurium DT104L by using the sequences of A) our earlier DT104L genomic subtractive hybridization results (accession no. AY462969 to AY463002)

TABLE 1. *Salmonella* serovar Typhimurium isolates used in this study

Strain no.	Phage type[a]	Isolation source[b]	Resistance pattern[c]	Prophage profile[d]
DT104 and DT104-related				
406	U302	Human	ASu	1
408	DT104B low	Pig		4
410	DT104L	Pig	ACSSuT	1
417	DT104L	Dairy cow	ASu	1
418	DT104L	Dairy cow	ACSSuT	1
420	DT104B low	-		4
427	DT104L	Human	ACSSuT	1
433	DT104L	Human	ACSSuT	10
436	DT104L	Pig	ACSSuT	1
443	DT104L	Chicken products	ACSSuT	1
448	DT104L	Human	ACSSuT	1
451	DT104L	Pig	ACSSuT	1
454	DT104L	Human	ACSSuT	1
455	DT104L	Human	ACSSuT	10
461	DT104B low	Human		4
462	DT104A	Human		2
2945	DT104L	Human	ACSSuT	1
3633	DT104L	Cocoa	ACSSuT	1
7945	DT104L	Pig	ACSSuT	1
Non-DT104				
254	DT194	-		7
255	OS	-		7
256	OS	-		7
257	OS	-		7
275 (ATCC 13311)	DT120	Human faeces		5
286 (ATCC 29946)	DT4 (LT2)[e]	-		9
322	ARS	Meat		6
323	ARS	-		3
375	DT41	-		5
389	DT193	-		8
390	DT193	-		8
411	DT193	Pig		4
412	DT208	Pig	A	7
413	DT208	Pig		5
414	ARS	Pig		2
415	ARS	Pig		5
416	ARS	Pig		7
419	DT1	Chicken		11
435	ARS	Turkey		2
444	DT193	Human		2
445	ARS	Meat		12
452	DT193	Pig		4
ATCC 700720	DT4 (LT2)[e]	-		9

[a] ARS, atypically reacting strain; OS, nontypeable strain.

[b] -, isolation source unknown.

[c] Presence of the following SGI-1 antibiotic resistance genes (resistance to): A, *aadA2* (ampicillin); C, *floR* (chloramphenicol); S, *pse-1* (streptomycin); Su, *sul1* (sulfonamides); and T, *tet(G)* tetracycline. Presence determined as described before (44).

[d] Prophage profile as identified in Table 4.

[e] LT2 is typed as DT4 in the English phage typing system and is the only attenuated laboratory strain listed in Table 1.

(17), B) bacteriophages ST104 (NC_005841) (40) and ST64B (NC_004313) (27) and C) the DT104 genome (NC_004513). The sequence data of the DT104 genome were produced by the *Salmonella* spp. Sequencing Group at the Sanger Institute, Hinxton, UK, and can be obtained from ftp://ftp.sanger.ac.uk/pub/pathogens/Salmonella. A comparison between the genome sequences of LT2 and DT104 at the Gifsy and Fels prophage loci, by using the software package Vector NTI Suite version 5.1 (Invitrogen), revealed that the Fels prophages were absent in DT104. This resulted in the identification of the integration positions of the Fels bacteriophages and the design of primers to test the presence or absence of these prophages (Fig. 1). The Gifsy prophages were found in both LT2 and DT104, therefore primers were designed based only on the annotation of these prophages within the LT2 genome. The genome comparison of both strains also revealed the location of the non-LT2 prophages ST104, ST104B, and ST64B within the DT104 genome (Fig. 1).

For the seven prophages selected, primer sets were designed on the left (LB-F and LB-R) and right (RB-F and RB-R) borders of the prophages and an additional primer set on internal DNA fragments for the non-LT2 prophages ST104 (*cI*), ST104B (HldD homologue) and ST64B (non-coding region). Notably, primer sets for internal DNA fragments of the non-LT2 prophages were designed as an additional control because the prophage border primers were based on the bacteriophage sequences while for the LT2 prophages the primers were based on the actual prophage sequences. Furthermore, no prophage insertion (prophage absent) could be detected by combining the LB-F and RB-R primers for each prophage. An overview of all PCR primers and amplicons used to detect the seven prophages is shown in Table 2 and is schematically presented in Fig. 1. The primers (Isogen) at a 0.2 μmol l^{-1} concentration were combined with 0.2 mmol l^{-1} of each dNTP, 3 mmol l^{-1} $MgCl_2$, 1X PCR reaction buffer and ~ 1 to 100 ng DNA template and were amplified with 1 Unit Taq polymerase (all Invitrogen) in a total volume of 50 μl. After an initial denaturation at 95 ^{o}C for 3 min, the samples were subjected to 30 cycles of 95 ^{o}C for 30 s, 60 ^{o}C for 60 s, and 72 ^{o}C for 45 s, followed by a final 7 min incubation at 72 ^{o}C. The elongation step of 60 ^{o}C was performed for 90 s for the phage ST104B borders primer combination. Samples were fractionated by 2 % (w/v) agarose gel electrophoresis and visualized by ethidium bromide staining. All PCRs were performed at least three times for each isolate.

For several isolates, a weak prophage absent band was observed although the prophages were detected as present (see Fig. 2B and Table 3). Therefore, to exclude for these isolates any DNA contamination by other isolates of the PCRs performed, all PCR materials were tested for contamination, DNA was isolated from single colonies, and colony PCRs were performed directly from 50 single colonies. The PCR materials were all negative and for the PCRs of the single colonies again a weak prophage absent band was observed although the prophages were detected as present. A possible explanation for observing weak prophage absent bands while the prophages were detected as present, will be mentioned in the discussion.

TABLE 2. Overview of the PCR primers used for prophage detection

Prophage detection	Primer name[a]	No.[b]	Sequence (5'→3')	Amplicon size [bp]
Prophage present:				
Gifsy-1	gifsy-1 LB-F	1	GCTAACGAACGGGAAACTTCATAG	506[c]
	gifsy-1 LB-R	2	CTGCTGTTTAGTCAGGACTGACTG	
	gifsy-1 RB-F	3	GCCCGAGGCACGACTTTAGATAAC	359[c]
	gifsy-1 RB-R	4	AACAGACGTTAAGCTCAGAACAGC	
Gifsy-2	gifsy-2 LB-F	5	GCAACGAGTGCAGAACAGGAGAAG	322[c]
	gifsy-2 LB-R	6	AGAGAAGAGCGCAGAACAGGTTTC	
	gifsy-2 RB-F	7	GGTGGCTAAATGTAAATGACGTGG	488[c]
	gifsy-2 RB-R	8	TGAGCGAGATCGAGATGAAGCTTG	
Fels-1	fels-1 LB-F	9	AAGCGCGGCGATATCATTGCTGTG	504[c]
	fels-1 LB-R	10	CCAGTCCCGACATACCTATGCATG	
	fels-1 RB-F	11	GGAGGCTAATCGTGTTTGAGTTTG	407[c]
	fels-1 RB-R	12	ACCACACCGCAATACTCCACGATG	
Fels-2	fels-2 LB-F	13	TAACCTGCTTAGAGCCCTCTCTCC	361[c]
	fels-2 LB-R	14	CTGAACAGAACCCGCTTTAATGGC	
	fels-2 RB-F	15	AACGGCGGAAACATACTGGTACTG	318[c]
	fels-2 RB-R	16	TGAATGAATGTTTGGTGGAGCTGG	
ST104	phageST104 LB-F	17	ATTCCGCTGCGGTTTATGTCAACG	484[d]
	phageST104 LB-R	18	CAAATCACCTGACTGAACATGCTC	
	phageST104 cI-F	19	TGGAACTGGCTGGTATGTCTCAAG	576[d]
	phageST104 cI-R	20	CTCTTTCAATTGGGTCCCAAGCTG	
	phageST104 RB-F	21	GTTCCCATGAATCCCACATACATC	810[d]
	phageST104 RB-R	22	ATTACGCGGGTAGGATCAGAGTAC	
ST104B	phageST104B LB-F	23	GACAGGAAATTACAACGGACGGTG	1299[d]
	phageST104B LB-R	24	ACTCATGCAATCAGGAGAGCTAAC	
	phageST104B hldD-F	25	ACAATGCTTTCGAACCTGATGGGC	510[d]
	phageST104B hldD-	26	CCATCGCTTCAATTGCAACCATGC	
	phageST104B RB-F	27	AGCCGAATAAAGTGGGACTTGTGC	1425[d]
	phageST104B RB-R	28	CATCTATTCTTAAAGGGCAAGGCG	
ST64B	phageST64B LB-F	29	GCGTTTCCCTCACAGCAATTAATC	673[d]
	phageST64B LB-R	30	AAAAGCATGAGGGAAGGTTGTGGC	
	phageST64B int-F	31	CACAACGTAATGATGCTCGCTGGC	527[d]
	phageST64B int-R	32	GGACACTCCGCCAGTAGCTTATTG	
	phageST64B RB-F	33	CTCTTGACTGCACTTTCCACGATC	489[d]
	phageST64B RB-R	34	GGGTTATTTCTTGTGCTTTCCAGG	
Prophage absent:				
Gifsy-1	gifsy-1 LB-F	1	GCTAACGAACGGGAAACTTCATAG	+/-500[e]
	gifsy-1 RB-R	4	AACAGACGTTAAGCTCAGAACAGC	
Gifsy-2	gifsy-2 LB-F	5	GCAACGAGTGCAGAACAGGAGAAG	+/-500[e]
	gifsy-2 RB-R	8	TGAGCGAGATCGAGATGAAGCTTG	
Fels-1	fels-1 LB-F	9	AAGCGCGGCGATATCATTGCTGTG	378[d]
	fels-1 RB-R	12	ACCACACCGCAATACTCCACGATG	
Fels-2	fels-2 LB-F	13	TAACCTGCTTAGAGCCCTCTCTCC	252[d]
	fels-2 RB-R	16	TGAATGAATGTTTGGTGGAGCTGG	

TABLE 2. Continued

Prophage detection	Primer name[a]	No.[b]	Sequence (5'→3')	Amplicon size [bp]
ST104	phageST104 LB-F	17	ATTCCGCTGCGGTTTATGTCAACG	675[c]
	phageST104 RB-R	22	ATTACGCGGGTAGGATCAGAGTAC	
ST104B	phageST104B LB-F	23	GACAGGAAATTACAACGGACGGTG	1207[c]
	phageST104B RB-R	28	CATCTATTCTTAAAGGGCAAGGCG	
ST64B	phageST64B LB-F	29	GCGTTTCCCTCACAGCAATTAATC	558[c]
	phageST64B RB-R	34	GGGTTATTTCTTGTGCTTTCCAGG	

[a] F, forward; R, reverse; LB/RB, left/right prophage border; cI, hldD and int are internal prophage fragments.
[b] Numbers refer to primer numbers in Fig. 1.
[c/d] Amplicon size in *Salmonella* serovar Typhimurium LT2 (NC_003197) / DT104 (NC_004513).
[e] No amplicon size could be calculated when a Gifsy phage was absent, amplicons size revealed to be around 500 bp.

RESULTS

Salmonella serovar Typhimurium LT2 and non-LT2 prophages.

An overview of the genome loci and characteristics of the four *Salmonella* serovar Typhimurium LT2 prophages Gifsy-1, Gifsy-2, Fels-1 and Fels-2 and the three non-LT2 prophages ST104, ST104B and ST64B is depicted in Fig. 1. The loci of the LT2 prophages were derived from GenBank accession no. NC_003197 (24). Bacteriophage Gifsy-1 was integrated into the GTP-binding elongation factor encoding the *lepA* gene of *Salmonella* serovar Typhimurium LT2 (reported in GenBank accession no. AF001386) resulting in prophage Gifsy-1 as indicated, and prophage Gifsy-2 was located between the nicotinate phosphoribosyltransferase-encoding *pncB* and aminopeptidase N-encoding *pepN* genes. Prophage Fels-1 was located between the ORFs STM0892 (*ybjP*, putative lipoprotein) and STM0930 (putative cytoplasmic protein) and prophage Fels-2 between STM2693 (regulatory RNA 10Sa) and STM2740 (phage integrase protein).

A comparison of the *Salmonella* serovar Typhimurium LT2 (NC_003197) and DT104 (NC_004513) genome sequences, by using the previously identified DT104 sequences derived from bacteriophages ST104 (NC_005841) and ST64B (NC_004313), and subtractive hybridization results (AY462969 to AY463002), resulted in the identification of the genome loci and characteristics of the non-LT2 prophages ST104, ST104B, and ST64B. Prophage ST104 was found in *Salmonella* serovar Typhimurium DT104, when compared to the LT2 genome, adjacent to the threonine tRNA gene *thrW*. The integration of bacteriophage ST104 into the DT104 genome resulted in a duplication of the 3' part of *thrW*. Prophage remnant ST104B, which contains an ORF homologous to HldD of *Escherichia coli* O157:H7 involved in LPS assembly, was found adjacent to ORF STM1871 (a putative phage integrase), which is part of the prophage remnant containing the *sopE2*, *pagO* and *pagK* genes. An identical copy of ORF STM1871 was identified on prophage remnant ST104B. Prophage ST64B was located adjacent to the serine tRNA gene *serU*. The genomic integration of the ST64B bacteriophage resulted in a duplication of the 3' part of the tRNA *serU*.

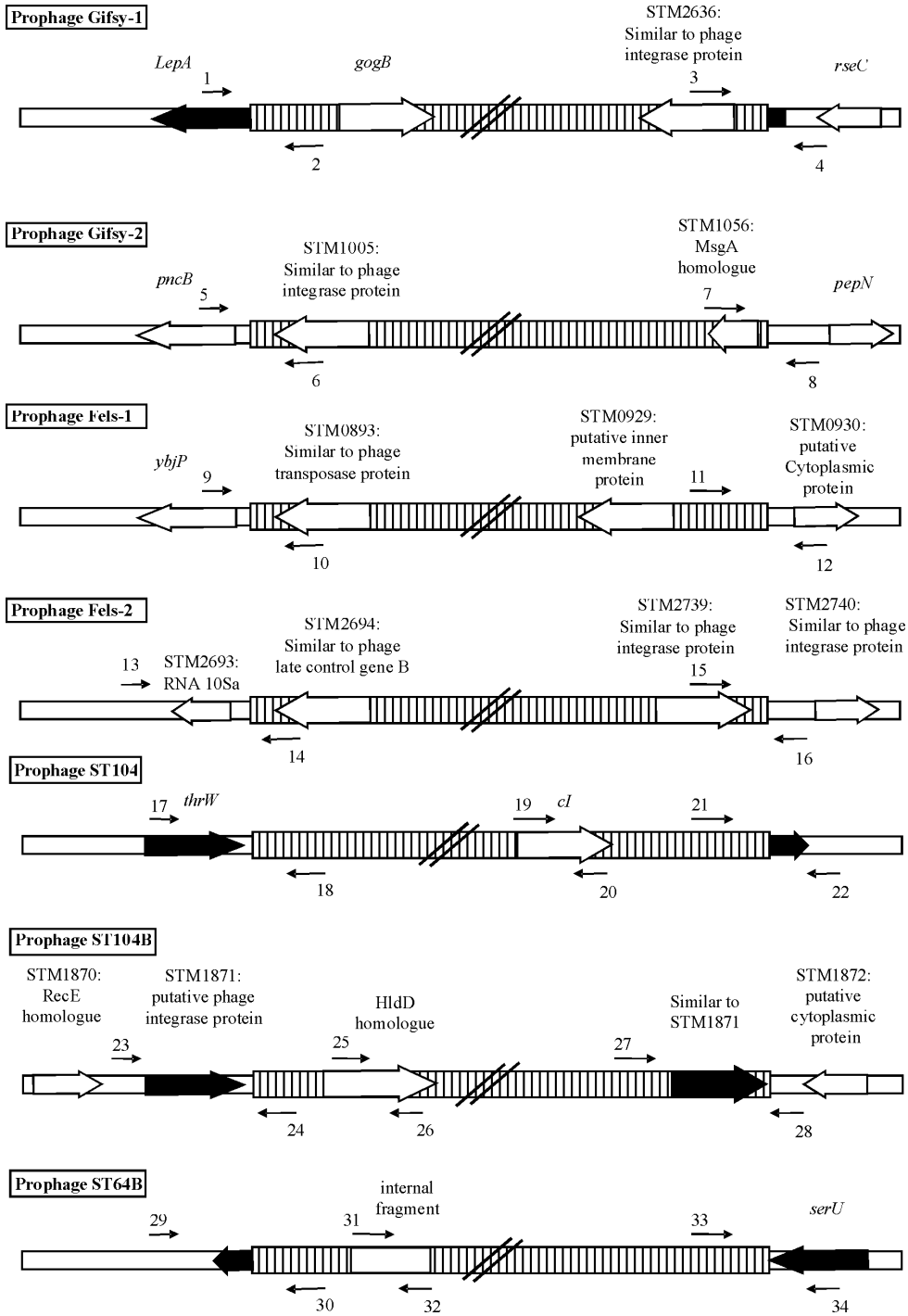

FIG. 1. Overview of the genome loci, characteristics and amplicons for the four *Salmonella* serovar Typhimurium LT2 prophages Gifsy-1, Gifsy-2, Fels-1 and Fels-2, and the three non-LT2 prophages ST104, ST104B, ST64B (not on scale), based on, or derived from the GenBank sequences AF001386, AY462969-AY463002, NC_003197, NC_004313, NC_004513, and NC_005841. The prophages are depicted as larger vertically-hatched regions and the adjacent *Salmonella* serovar Typhimurium LT2 genome segments are depicted as white regions. Arrow boxes denote the direction of transcription of the genes indicated. For prophage Gifsy-1 the black arrow box represents the *lepA* gene which is interrupted due to Gifsy-1 integration. The other black arrow boxes represent genes which are partly duplicated in the *Salmonella* serovar Typhimurium DT104 genome due to prophage integration. The position on the genome of the PCR primers developed is represented by small arrows and the numbers refer to the primer numbers listed in Table 2.

Prophage detection approach.

The PCR primers designed for the detection of the seven prophages (as presented in Table 2 and indicated with arrows in Fig. 1) were initially tested by using genomic DNA isolated from *Salmonella* serovar Typhimurium LT2 strain ATCC 700720 and *Salmonella* serovar Typhimurium DT104L strain 7945. Primer sets were designed to detect the left and right borders for each prophage and to detect internal prophage fragments for the non-LT2 prophages. Furthermore, if the combination of the outer-left and right border resulted in a product, we concluded that the prophage was not inserted into the genome (prophage absent). For example, the results of agarose gel electrophoresis of the PCR products of prophages ST104 (Fig. 2A) and ST64B (Fig. 2B) for the LT2 and DT104L strains are shown, for which left prophage border (LB), right prophage border (RB), internal prophage fragment (*cI* or int.), and outer-left and right prophage border (prophage absent) PCRs were performed. Both prophages were absent from the LT2 strain and present in the DT104L strain, based on the findings that, for LT2, only the prophage absent band was visible on the gels, while for DT104L three dominant bands were obtained, corresponding to the LB, RB and internal prophage fragments, respectively. Notably, an additional weak prophage absent band was observed for prophage ST64B in this DT104L strain (Fig. 2B). The PCR results obtained for these two strains for the seven prophages are presented in Table 3. The Gifsy prophages were detected in both strains, the LT2 Fels prophages only in LT2, and the non-LT-2 prophages (ST104, ST104B, and ST64B) were detected only in the DT104 isolate. Finally, prophages Fels-2, Gifsy-1, and ST64B showed an additional weak prophage absent band although the prophages were detected as present, which was found not to be caused by any contamination (see materials and methods).

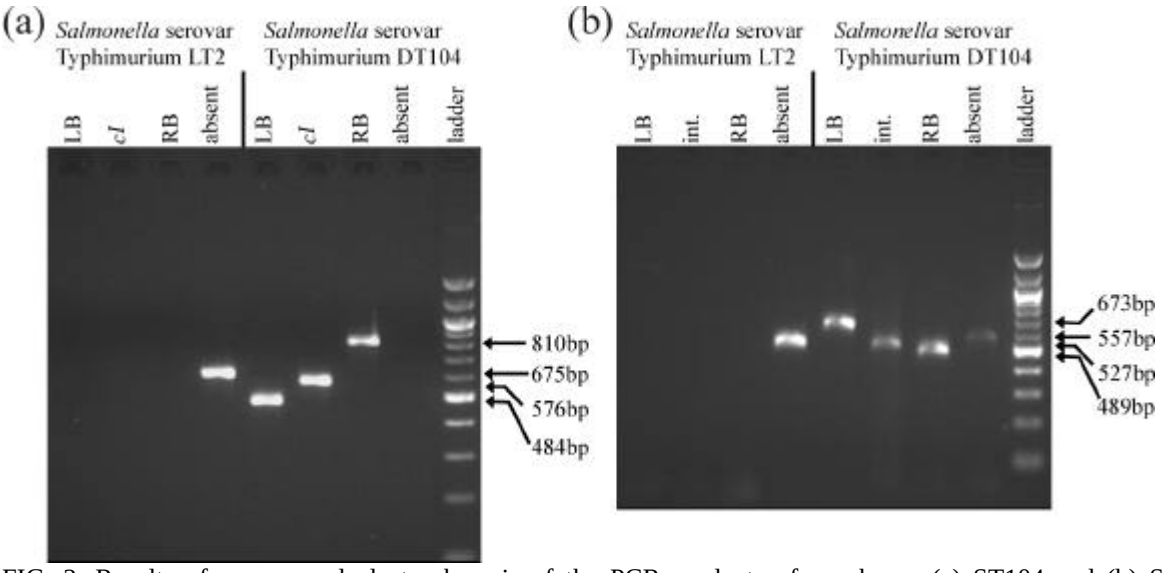

FIG. 2. Results of agarose gel electrophoresis of the PCR products of prophages (a) ST104 and (b) ST64B PCR products for *Salmonella* serovar Typhimurium LT2 ATCC 700720 and *Salmonella* serovar Typhimurium DT104L isolate 7945, where left prophage border (LB), right prophage border (RB), internal prophage fragment (cI or int.), and outer left and right prophage border (prophage absent) PCR fragments are shown. A 100-bp ladder was used as size marker and the PCR fragments lengths are depicted on the right side.

TABLE 3. Results of PCR for the detection of the seven *Salmonella* serovar Typhimurium LT2 and non-LT2 prophages[a]

Amplicon		Phage type, strain no.	
		DT104L, 7945	LT2, ATTC 700720
Prophage Gifsy-1	LB	+	+
	RB	+	+
	Absent	+[b]	+[b]
Prophage Gifsy-2	LB	+	+
	RB	+	+
	Absent	-	-
Prophage Fels-1	LB	-	+
	RB	-	+
	Absent	+	-
Prophage Fels-2	LB	-	+
	RB		
	Absent	+	+[b]
Prophage ST104	LB	+	-
	cI	+	-
	RB	+	-
	Absent	-	+
Prophage ST104B	LB	+	-
	HldD hom.	+	-
	RB	+	-
	Absent	-	+
Prophage ST64B	LB	+	-
	int.	+	-
	RB	+	-
	Absent	+[b]	+

[a] +, present; -, absent; LB/RB, left/right prophage border; Absent, prophage absent and for prophages ST104, ST104B and ST64B an internal prophage fragment (*cI*, HldD hom. or int.) present.
[b] observed as a weak band on agarose gel (see text for explanation)

Prophage distribution among different *Salmonella* serovar Typhimurium isolates.

All prophage detection PCRs, as mentioned above, were performed for the *Salmonella* serovar Typhimurium isolates listed in Table 1. For each isolate, the PCR results were scored on prophage present (+), prophage absent (-) or prophage borders present and internal fragment absent (o), as depicted in Table 4. Prophage Gifsy-1 was present in all isolates except for the two DT104L isolates 433 and 455, and isolates 419 and 445. The Gifsy-2 prophage was present in all isolates. Prophage Fels-1 was only found in four isolates: the two LT2 isolates ATTC 700720 and 286, and the two DT193 isolates 389 and 390. Prophage Fels-2 was found in the two LT2 isolates and nine other isolates of different phage types. Prophage ST104 and ST104B were present in all 14 phage type DT104L isolates and the U302 isolate. All isolates of prophage profiles 1 and 10 that contained the ST104 and ST104B prophages, except the DT104L isolate 417 and the U302 isolate, were of penta-resistance type ACSSuT (depicted in Table 1). In addition, the prophage borders of ST104, without detecting the internal *cI* fragment, were also detected in 20 other isolates belonging to different phage types, including DT104A and DT104B low. Prophage ST64B

TABLE 4. Prophage distribution among different *Salmonella* serovar Typhimurium isolates

Prophage profile	Phage type[*]	Strain no.	Presence or absence[†] of prophage:						
			Gifsv-1	Gifsv-2	Fels-1	Fels-2	ST104	ST104B	ST64B
1	DT104L (12)	410, 417, 418, 427, 436, 443, 448, 451, 454, 2945, 3633, 7945	+	+	-	-	+	+	+
	U302	406	+	+	-	-	+	+	+
2	ARS (2)	414, 435	+	+	-	-	o	-	+
	DT104A	462	+	+	-	-	o	-	+
	DT193	444	+	+	-	-	o	-	+
	DT208	413	+	+	-	-	o	-	+
3	ARS	323	+	+	-	-	o	-	o
4	DT193 (2)	411, 452	+	+	-	-	o	-	-
	DT104B low (3)	408, 420, 461	+	+	-	-	o	-	-
5	ARS	415	+	+	-	-	-	-	+
	DT41	375	+	+	-	-	-	-	+
	DT120	275	+	+	-	-	-	-	+
6	ARS	322	+	+	-	-	-	-	o
7	OS (3)	255, 256, 257	+	+	-	+	o	-	-
	DT194	254	+	+	-	+	o	-	-
	ARS	416	+	+	-	+	o	-	-
	DT208	412	+	+	-	+	o	-	-
8	DT193 (2)	389, 390	+	+	+	+	o	-	-
9	DT4 (= LT2) (2)	286, 700720	+	+	+	+	-	-	-
10	DT104L (2)	433, 455	-	+	-	-	+	+	+
11	DT1	419	-	+	-	-	-	-	+
12	ARS	445	-	+	-	+	o	-	-

[*] no. of isolates between parenthesis.

[†] +, present; -, absent; o, borders present and internal fragment absent.

was present in all 14 phage type DT104L isolates, the U302 isolate and nine other isolates. Also for prophage ST64B, the borders were detected in two additional isolates (322 and 323), although the internal fragment was not detected.

For each isolate, the PCR results for the detection of the seven different prophages were converted into prophage profiles as indicated in Table 4. In total 12 different prophage profiles were identified. Based on the phage typing numbers, several isolates of the same phage type resulted in the same profile, such as the two LT2 isolates resulting in profile 9, the three DT104B low isolates resulting in profile 4 and the 12 DT104L isolates, except for isolates 433 and 455, which lacked prophage Gifsy-1, resulting in profile 1. In contrast, analysis of the five DT193 isolates resulted in more different profiles (profiles 2, 4 and 8) and the two DT208 isolates resulted in profiles 2 and 7.

DISCUSSION

By combining and analyzing *Salmonella* serovar Typhimurium LT2 and DT104 sequences, we were able to identify prophages ST104 and ST64B, and a prophage remnant ST104B in the genome of *Salmonella* serovar Typhimurium DT104. Both prophages, ST104 and ST64B, were found adjacent to tRNA genes which have been commonly observed to be sites for the insertion of transferable elements (11, 36). The HldD homologue, which has been described as a putative virulence factor candidate (17), was located on a prophage-like horizontally transferable genetic element designated prophage ST104B. The presence of virulence factors on such elements has also been described for other *Salmonella* serovar Typhimurium isolates (2, 12, 14, 18, 25, 26, 39). If prophages Gifsy-1, Fels-2, and ST64B were detected in an isolate, shown by strong prophage present bands on the agarose gel, an additional weak prophage absent PCR band was observed. The latter observation may be explained by spontaneous prophage induction, leading to excision of the prophage from the genome, in a small fraction of the stationary phase cells lacking the prophage in their genome (4, 12, 45). In our study, DNA was isolated from overnight grown cultures (stationary growth phase), similar to overnight cultures used for conventional phage typing, and the PCR results may have been obtained from cells with and without the prophages in their genome. For the other prophages no double PCR results were obtained indicating that these prophages were not spontaneously induced or induced at lower, non-detectable levels (4).

The prophage borders of ST104 and ST64B were detected in many isolates, although for some of these isolates the internal fragments of the prophages were not found. In these cases the internal DNA fragment(s) may indeed be absent, resulting in a so-called prophage remnant, but alternatively the internal sequence at this position may be different, which could indicate the presence of a different prophage due to recombinations, rearrangements or deletions, which are common features of prophages (9, 10, 27, 28, 31, 46). Genetic diversity has previously been reported for prophage ST64B. This prophage has been found to be defective in different phage types of *Salmonella* serovar Typhimurium and several strains appeared to contain different sizes of internal prophage fragments (43). In *Salmonella* serovar Typhimurium DT104, active ST64B (13) and ST104 (40) prophages have been reported. The induced ST104 prophage has been isolated only from DT104

isolates. In our study, the internal fragment of prophage ST104 representing *cI* was detected only in the *Salmonella* serovar Typhimurium DT104L isolates and the U302 isolate. Most likely the other isolates contained a remnant or recombination of ST104 because the internal fragment was not detected.

Our study revealed 12 different prophage profiles for the 23 non-DT104 and 19 DT104 *Salmonella* serovar Typhimurium isolates. When the isolates resulting in similar prophage profiles were gauged against their phage type numbers, no clear relationships were found with specific phage types. In some cases, the prophage typing distinguished more groups than conventional phage typing (DT104L or DT193), while in other cases different phage types had a similar prophage profile (profile 2 or 5). Notably, discrimination between DT104A, DT104B low and DT104L isolates could be made based on the presence of prophages ST104, ST104B and ST64B. In earlier studies, the Fels prophages could not be detected whereas the Gifsy prophages could be detected in the genomes of non-LT2 *Salmonella* serovar Typhimurium isolates (33, 35). In our study, more variation in the presence of these LT2 prophages was identified among different non-LT2 isolates. Prophage Gifsy-1 was absent in four isolates and prophage Fels-1 was detected in two and Fels-2 in nine non-LT2 isolates. Although prophage Gifsy-1 was absent in four isolates, both Gifsy prophages can be seen as common *Salmonella* serovar Typhimurium prophages. The prophage remnant ST104B, which harbored the putative virulence factor HldD homologue was detected mainly in phage type DT104L isolates with prophage profile 1 and penta-resistance type ACSSuT (depicted in Table 1). Notably, the antibiotic resistance genes for penta-resistance were detected only in DT104 isolates. The HldD homologue may be involved in LPS assembly in *Salmonella* serovar Typhimurium DT104 resulting in modification of the LPS structure and/or architecture (17). This observation may offer an explanation for the phenotypic gel-based differences described elsewhere in LPS with various DT104 types (22).

This study indicates the potential for molecular typing of *Salmonella* serovar Typhimurium based on prophage sequences. Previously, this potential was also described by using an amplified fragment length polymorphism (AFLP) (20), multilocus sequence typing (MSLT) (37) or microarray-based approach (32). It is well established that phage type conversion can occur when lambdoid bacteriophages integrate into the genome of *Salmonella* serovar Typhimurium (29, 34, 43). In addition, the detection of prophages can also give information about the virulence potenciy of a *Salmonella* serovar Typhimurium strain, since many virulence factors have been reported to be located on prophages (2, 12, 14, 18, 25, 26, 39), and can therefore be useful for risk assessment. The different isolates used in our study may contain as yet unidentified additional prophages or recombinations, called mosaics, of known prophages (9, 10, 27, 28, 31, 46). Our study is believed to be the first to reveal relationship between prophage presence and phage type that may be useful for the development of a molecular method to replace conventional phage typing. However, more genome sequences of other *Salmonella* serovar Typhimurium strains or bacteriophages, combining earlier work (20, 32, 37), and screening of the genomic regions adjacent to tRNAs of new emerging salmonellae as hot spots for bacteriophage integration into the genome (8, 11, 19, 36) will help to gain more insight into the relationship between the presence of prophages and conventional phage typing, and their impact on virulence. Finally, our results suggest that the presence of the genes for penta-resistance type ACSSuT,

the HldD homologue containing ST104B prophage remnant and phage type DT104L are most likely common features of the emerging subtype of *Salmonella* serovar Typhimurium DT104.

ACKNOWLEDGEMENTS

This study was supported by The Netherlands Organization for Health Research and Development, "Nutrition: Health, Safety and Sustainability" program.

The authors would like to thank Ingrid Scholtens, Emmy van de Ven and Sükrü Yigit for technical assistance. We are grateful for the policy of the Sanger Centre to make sequences available before completion. The *Salmonella* serovar Typhimurium isolates were kindly provided by Wim Wannet, RIVM and Dik Mevius, CIDC-Lelystad, The Netherlands. Phage typing was performed at the National *Salmonella* Reference Laboratory of the Federal Institute for Risk Assessment (BfR), Germany. For advice and help about phage typing, thanks are due to Andreas Schroeter and Burkhard Malorny from BfR.

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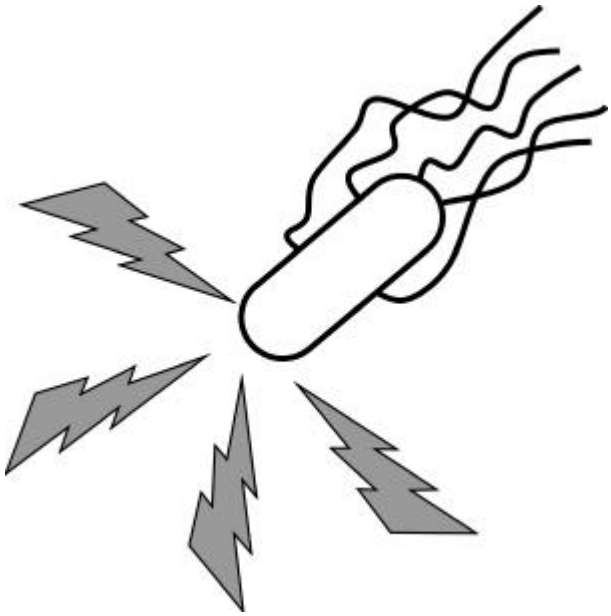
Chapter 4

Development and assessment of a stress and virulence thematic oligonucleotide microarray for *Salmonella* enterica serovar Typhimurium DT104



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In preparation

ABSTRACT

To be able to study stress and virulence aspects of the emerging and multi-antibiotic-resistant foodborne pathogen *Salmonella enterica* serovar Typhimurium DT104, a thematic oligo microarray was developed based on the genome of *Salmonella* serovar Typhimurium LT2 and supplemented with described *Salmonella* serovar Typhimurium DT104 genes. In total, 426 genes were selected and an automated oligo design approach using OligoFaktory resulted in oligos of a similar melting temperature and low cross-hybridization. Genomic DNA hybridizations revealed that all oligos designed based on the LT2 genome sequence were also applicable for DT104. An evaluation of different microarray parameter settings revealed that using epoxy-coated microarrays and sodium phosphate-based spotting buffer resulted in the most sensitive microarrays. The thematic microarrays were also suitable for gene expression studies. Gene expression was measured during growth upon entry into the stationary growth phase at pH 7.0 and pH 5.0 for *Salmonella* serovar Typhimurium DT104. Here, we assessed the thematic microarray by analyzing the expression time patterns of some selected stress and virulence genes: six genes encoding universal stress proteins and paralogues and the five plasmid-located virulence genes *spvRABCD*. The expression of the genes encoding for the six universal stress proteins increased and that of the *spv* virulence genes, except *spvA*, decreased upon entry into stationary phase at both pH conditions. The results for the development and assessment steps mentioned above reveal the applicability of our thematic microarray to study the expression of stress and virulence genes in *Salmonella* serovar Typhimurium DT104.

INTRODUCTION

Worldwide microarray experiments have been initiated by many research groups resulting in an increase of the number of publications including microarray data (11, 12, 21), whereas only limited information has been presented about the development of the microarrays used. To develop microarrays, insight is needed into oligo design (4) and the parameters influencing the hybridization signal, such as spotting single stranded oligonucleotides or double stranded PCR fragments. Furthermore, the oligonucleotide (oligo) length, and the type of microarray glass surface coating or fluorescence Cy-dye label are important parameters (reviewed in 20).

In the work presented, we describe the development and assessment of a thematic oligo microarray allowing expression analysis of stress and virulence genes of the foodborne pathogen *Salmonella enterica* subsp. *enterica* serovar Typhimurium (*Salmonella* serovar Typhimurium) phage type DT104, which is multi-antibiotic-resistant and has been described as emerging during the past decades (3, 7, 18). An oligo-based microarray was developed instead of a PCR product-based microarray because the use of oligos results in lower levels of cross-hybridization combined with higher specificity and better quantitative spotting accuracy (6, 11, 20).

To construct this thematic microarray, first genes have to be selected from the publicly available genome sequence of *Salmonella* serovar Typhimurium LT2 (13) that have been reported in literature to be involved in or related to stress and virulence. Several known *Salmonella* serovar Typhimurium DT104-specific genes also have to be included. In addition, oligos have to be designed for the stress and virulence genes selected. We assessed in silico two different design approaches by studying the melting temperature (Tm) and cross-hybridization characteristics for the oligos obtained. Next, the oligos designed based on the *Salmonella* serovar Typhimurium LT2 genome sequence have to be tested for their applicability for *Salmonella* serovar Typhimurium DT104. Furthermore, an evaluation has to be performed to find out what parameter settings result in more sensitive microarrays, to be able to detect more low-level expressed genes. Finally, gene expression has to be analyzed of some selected stress and virulence genes present on the thematic microarray.

The results for the development and assessment steps mentioned above revealed the applicability of our thematic microarray to study the expression of stress and virulence genes in *Salmonella* serovar Typhimurium DT104.

MATERIALS AND METHODS

Selection of stress and virulence genes for the thematic microarray.

The thematic microarray was developed by selecting genes by a text search through the *Salmonella* serovar Typhimurium LT2 genome (accession no. NC_003197) and virulence plasmid pSLT (accession no. NC_003277) (13). For this text search, the words stress, sigma, shock, stationary, osmolarity, heat, cold, acid, osmotic, response and decarboxylase were used to find stress-related genes and the words virulence, invasion, pathogenicity, lipopolysaccharide and antigen for virulence-related genes. In addition, stress

and virulence-related genes were selected based on their function described in the annotation of the *Salmonella* serovar Typhimurium LT2 genome (13) or in literature, including genes regulated by the stress regulators Ada, SdiA, Fur, OmpR, PhoPQ, RpoE, RpoS, genes that regulate RpoS activity, genes involved in cell structure, two-component system genes and genes differentially expressed in minimal medium (17). Next, genes were selected if located within the same operon (e.g. *inv*, *rfa* or *rfb* genes) or pathogenicity islands (*hil* and *ssa* genes) or regulated by the same regulator as the previously selected genes. Finally, we selected several housekeeping genes as a control of gene expression and some genes encoding putative functions. The list of genes was supplemented with the five antibiotic resistance genes *aadA2, flo, pse1, sul1, tet(G)* and the eight other genes located on *Salmonella* genomic island I (SGI-I) of *Salmonella* serovar Typhimurium DT104 (accession no. AF261825) (1) and the HldD homologue (accession no. AY462995) (5). Finally, the firefly *Photinus pyralis* luciferase gene (accesion no. M15077) was also included to detect the control luciferase mRNA. The 426 stress and virulence genes selected (including the luciferase gene) and their functions are thematically shown in Table S1 as supplementary material of Chapter 4.

Oligonucleotide design for the genes selected.

In total 497 49mer, 50mer and 60mer oligos were designed on the coding sequences of the 426 genes selected. First, 97 50mer and 60mer oligos were designed by using Gene Runner version 3.05 having a GC content of 50% and a melting temperature (Tm) around 79 ^{o}C or 86 ^{o}C for the 50mer or 60mer oligos, respectively . This approach resulted in several possible oligos per gene that were manually checked for cross-hybridization to another position on the genome of *Salmonella* serovar Typhimurium LT2 than the target sequence by using BLASTN and the oligo showing the lowest cross-hybridization was selected. Second, 400 49mer oligos (%GC, 50; Tm, 79 ^{o}C) were designed by using the first prototype of OligoFaktory (Delphi Genetics S.A., Charleroi-Gosselies, Belgium) (16). This latter approach for oligo design resulted in many possible oligos, which were all checked for cross-hybridization by BLASTN in an automated manner. The 426 genes selected and their function, and the 497 corresponding oligos and their characteristics for Tm, oligo length and Gene Runner or OligoFaktory designed are thematically shown in Table S1 as supplementary material of Chapter 4. All oligos were synthesized and modified with a 5'-C6-amine linker by Isogen Life Science, Maarssen, The Netherlands.

Microarray spotting and post-spotting treatment.

The oligos were spotted in 5xSSC spotting buffer or in sodium phosphate-based Nexterion spotting buffer (Schott Nexterion, Jena, Germany) at a 25-30 mM concentration on silylated-coated (Genetix, New Milton, UK) or epoxy-coated (Slide E, Schott Nexterion) glass slides. Two hybridization areas were printed per slide and each oligo was printed twice per hybridization area. After spotting, the silylated slides were treated with sodium borohydride (15) to inactivate free aldehyde groups and to irreversible bind the oligos to the microarray. Furthermore, the silylated slides were blocked by incubating the slides for at least four hours at 42 ^{o}C in hybridization buffer (5xSSC, 0.2% SDS, 5xDenhardt's solution, 50% (v/v) formamide and 0.2 μg/μl denatured herring-sperm DNA) and washed by dipping five times subsequently in two times sterile water and isopropanol and air-dried. The epoxy

slides were after spotting treated for DNA immobilization, washing and blocking as recommended by the manufacturer. Finally, 25 µl Gene Frame windows (Westburg, Leusden,The Netherlands) were fit onto the hybridization areas of the microarrays and covered with cleaned plastic covers (1.5x1.5 cm^2) containing two small pierced holes.

Bacterial strains, growth conditions and sampling.

The bacterial strains used in this study were *Salmonella* serovar Typhimurium LT2 (ATCC 700720) and *Salmonella* serovar Typhimurium DT104 isolates 7945 and so-3633 (obtained from the Dutch National Institute of Public Health and the Environment (RIVM)). All isolates were stored at -80 ^oC in Brain Heart broth (Merck, Darmstadt, Germany) plus 50% glycerol (Merck). For genomic DNA extraction, the isolates were grown overnight in brain heart broth (Merck) at 37 ^oC without shaking.

For RNA expression experiments, LBG media (20g Luria Bertani Lennox broth (Difco, Detroit, Mich.), 5g NaCl and 4.36 g glucose-monohydrate per liter sterile water) were 1/100 inoculated with an overnight culture grown in brain heart broth (Merck) and cultured at 37 °C without shaking. For pH 7.0 experiments 100 mM morpholinepropane-sulphonic acid (MOPS, Sigma-Aldrich, St. Louis, Mo.) was added and for pH 5.0 100 mM morpholinethanesulphonic acid (MES, Sigma-Aldrich) and set at pH 5.0 by adding HCl. The optical density (OD) was measured at 604 nm during the growth experiments. At different time points during a growth experiment, 40 ml culture samples were taken and added to 10 ml of ice-cold mixture of 95% (v/v) 96% ethanol and 5% (v/v) buffered phenol (Invitrogen, Carlsbad, CA). The tubes were centrifuged for 5 min at 1780g at 4 °C.

RNA extraction and DNase treatment.

Total RNA was isolated from the 40 ml culture pellets using TRIzol reagent (Invitrogen) and treated with RQ1 RNase-free DNase (Promega) to remove contaminating genomic DNA, as described by the suppliers. RNase inhibitor RNaseOUT (Invitrogen) was added to avoid RNA breakdown and after 30 min incubation at 37 °C, the RQ1 DNase stop solution was added and the samples were heated and purified with a standard phenol:chloroform extraction and ethanol precipitation, as recommended by the manufacturer. The air-dried RNA pellets were dissolved in 25 µl DNase/RNase-free water, incubated for 10 min at 60°C and the concentration and purity were determined at 230, 260 and 280 nm and by agarose gel electrophoresis (data not shown).

RNA amino-allyl labeling.

To 20 µg of extracted RNA (per hybridization), DNase/RNase-free water (Invitrogen) was added to a final volume of 20 µl and 2 ng control luciferase mRNA (Promega) and 4.5 µg random hexamers (Invitrogen) were added. The RNA was denatured for 10 min at 70 °C, spun briefly and incubated on ice for 10 min. The denatured RNA was converted into amino-allyl labeled cDNA overnight at 37 °C by using 200 Units SuperScript II Reverse Transcriptase (Invitrogen) and adding 4 µl 10x AA-dNTP mixture (5 mM dATP, dCTP, dGTP, 3 mM dTTP (Invitrogen) and 2 mM amino-allyl-dUTP (Sigma-Aldrich)). The cDNA reactions were stopped by adding 1.5 µl 20 mM pH 8.0 EDTA (Merck), treated with 0.1 M NaOH, heated for 10 min at 70 °C and neutralized with 0.1 M HCl for breakdown

unconverted RNA, and ethanol precipitated. Incorporation of fluorescence Cy-dye label is described below.

DNA amino-allyl labeling.

Genomic DNA was extracted from 1.5 ml of overnight cultures by using a genomic DNA wizard kit (Promega, Madison, Wis.), as described by the supplier. To 2 µg of extracted genomic DNA (per hybridization), DNase/RNase-free water was added to a final volume of 20 µl and mixed with 20 µl 2.5x random primers solution (Bioprime DNA labeling system, Invitrogen). The DNA samples were boiled for 5 min and put directly on ice. Additionally, 5 µl 10x AA-dNTP mix (see above) and 40 Units Klenow fragment (Bioprime DNA labeling system, Invitrogen) were added and incubated for 2 hours at 37 °C. The reaction was stopped by adding 5 µl EDTA and ethanol precipitated.

Fluorescence Cy-dye label incorporation.

The air-dried DNA or cDNA amino-allyl labeled cDNA pellets were dissolved in 10 µl 0.1 M Na_2CO_3 (pH 9.3) buffer, incubated for 5 min at 65°C and next a fluorescent Cy-dye (Amersham Biosciences, Piscataway, NJ) was incorporated by adding 10 µl Fluorolink Cy monofunctional dye (1mg Cy-dye in 45 µl DMSO) and incubated in the dark for at least 30 min at room temperature. The samples were Cy5-dye labeled and a reference (a mixture of all RNA time samples or genomic DNA of *Salmonella* serovar Typhimurium LT2) was Cy3-dye labeled. Next, the cDNA reaction mixtures were ethanol precipitated and dissolved in sterile water. All dissolved DNA or cDNA labeled samples were purified to remove uncoupled dyes by using a QIAquick PCR purification kit (Qiagen, Valencia, CA), as described by the supplier. The final pellets were air-dried and dissolved in 17.5 µl hybridization buffer (see above), boiled for 5 min and spun down briefly. Finally, the Cy5-dye labeled samples were mixed (1:1) with the reference Cy3-dye labeled samples resulting in a total volume of 35 µl mixtures.

Microarray hybridization and washing.

The 35 µl mixtures of Cy5/Cy3-dye labeled DNA or cDNA samples were injected into the hybridization areas and the small holes were covered with pieces of plastic cover to prevent evaporation. The slides were incubated for 24 hours at 42 °C in a humid hybridization chamber. After hybridization, the Gene Frame windows were removed and the slides were subsequently first rinsed shortly and second incubated for 5 min in 1x SSC/0.1% SDS, 0.1x SSC/0.1% SDS and 0.1x SSC. The last incubation in 0.1x SSC was for 1 min. Finally, the slides were dried by centrifugation (440g, 2 min, rT).

Microarray scanning and data analyzing.

Microarrays were scanned with the ScanArray 3000 confocal laser scanner (GSI Lumonics, Kanata, ON, Canada) by using a pixel resolution of 10 μm, a Photo Multiplier Tubes value of 90% and the laserpower was set at a level observing no saturated spots. The fluorescent signals per spot and four background areas around each spot were volume measured (sVOL) by using the software package ArrayVision (Imaging Research, St. Catharines, ON, Canada). From these data the signal-to-noise ratios (S/N) were computed for each spot to discriminate true signal from noise as follows: S/N = (fluorescent spot signal - average background signal of four areas surrounding the spot)/(standard deviation of the four background area values). A commonly used threshold value to accurately quantify a signal above the noise is an S/N>3 (19). Prior to normalization, the obtained Cy5 or Cy 3 values that had an S/N= 3 were discarded. For normalization, several parameters are defined: R = Cy5 value of a spot divided by the corresponding reference Cy3 spot value; H = median R value of a hybridization area calculated only from the spots that could be detected in all hybridizations; A = median H value of all hybridization areas; V = median Cy3 hybridization signals per oligo for all hybridization areas. The corrected Cy5 value per spot = R*(A/H)*V. To study patterns of gene expression, the mean corrected Cy5 hybridization signals for different time samples per oligo (duplicate hybridizations and duplicate spots per oligo) and the corresponding standard deviation (STDEV) were plotted on logarithmic scale in the course of time.

RESULTS AND DISCUSSION

Oligo design approach assessment.

The *in silico* melting temperatures (Tm) obtained for the 497 oligos designed are shown in Fig. 1A and 1B. The average Tm computed for the oligos designed by Gene Runner was 79.2 ^{o}C within a range of 17.6 ^{o}C for the 50mer oligos (Fig. 1A left part) and 86.0 ^{o}C within a range of 13.3 ^{o}C for the 60mer oligos (Fig. 1A right part). The average Tm computed for the OligoFaktory designed 49mer oligos was 78.9 ^{o}C within a range of 2 ^{o}C. Furthermore, 93% of the OligoFaktory designed oligos had a stretch <15 nucleotides (nt) that could cross-hybridize to a nontarget sequence (Fig. 1C) and this stretch was around 13nt for all Gene Runner designed oligos (data not shown). The oligos that had a =15nt cross-hybridization stretch might cause significant cross-hybridization according to Kane et al. (8) and He et al. (4). The four oligos in Fig. 1C showing a >18nt stretch of cross-hybridization could be explained that these genes were very homologues.

The smaller Tm variation for the OligoFaktory designed oligos was obtained because this approach results in the generation of many possible oligos which were all checked by the computer in an automated manner for best matching all oligo design selection criteria: 50% GC, Tm around 79 ^{o}C, length of 49nt and low cross-hybridization. In contrast, for the Gene Runner approach only around four to eight oligos designed were checked manually for matching the oligo selection criteria. By using the OligoFaktory approach, oligos of a similar GC amount and Tm were obtained and 91% of the oligos were 49nt in length and

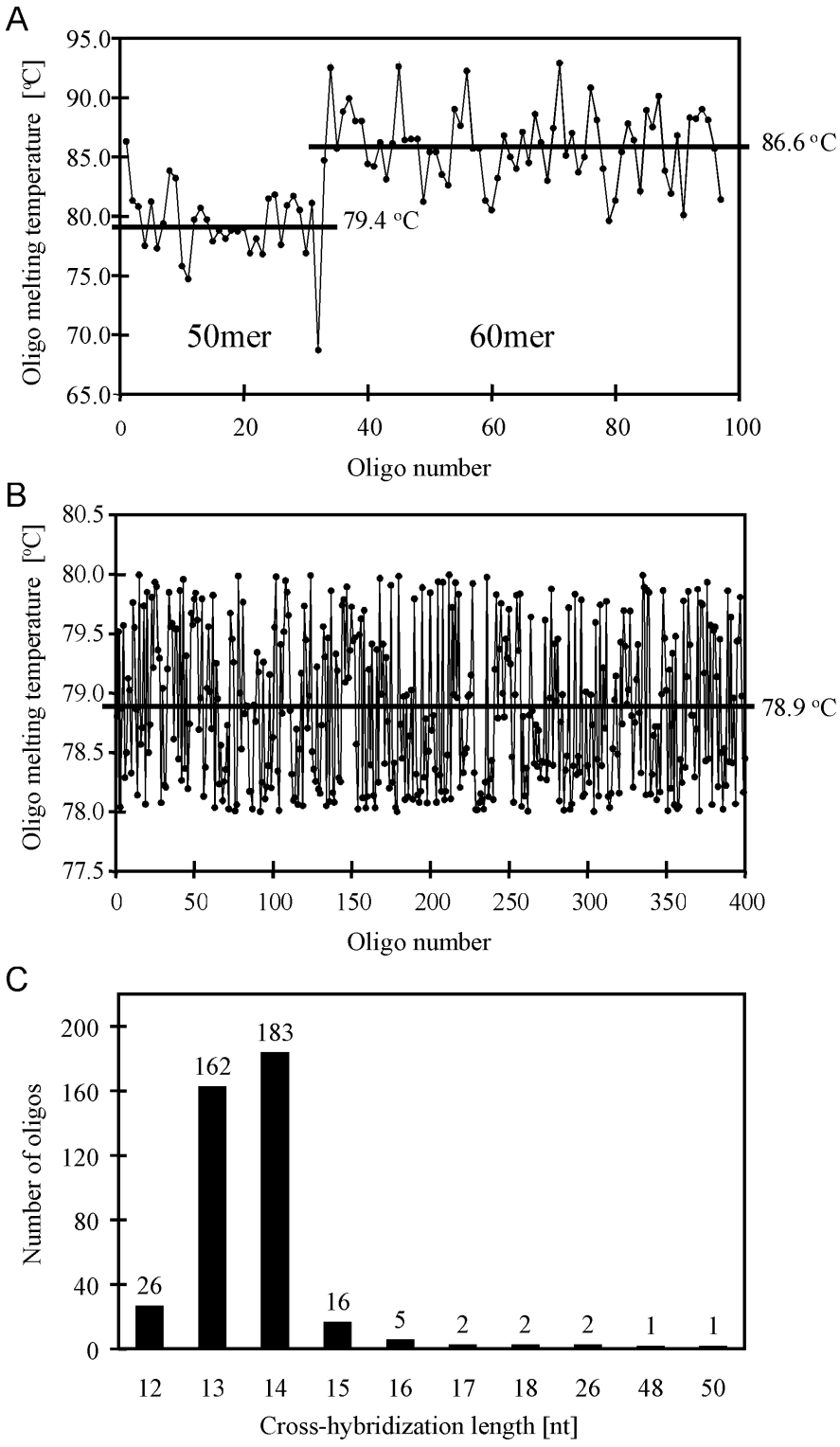

FIG. 1 Overview of the oligo characteristics. Melting temperatures (Tm) for the (A) 97 Gene Runner designed oligos, (B) 400 OligoFaktory designed oligos. (C) The length of cross-hybridization to a nontarget sequence for the 400 OligoFaktory designed oligos.

9% were 50-53nt in length to match the other criteria (data not shown). In contrast, it was hard to be able to design oligos of a suitable length, having a 50% GC content and resulting in an acceptable Tm for the non-automated Gene Runner approach. Therefore, the cross-hybridization stretch was the most important criterion for the Gene Runner designed oligos, explaining the large variation in Tm values as depicted in Fig 1A. Although the *in silico* Tm values of the Gene Runner designed oligos varied more, we observed no differences in hybridization signals from the microarray scans (see Fig. 2) solely based on this variation in the experiments performed.

Finally, it was quite obvious to use the OligoFaktory oligo design approach in future work because the Gene Runner manual approach is hazardous, since only four to eight oligos were subjected to the oligo selection criteria, results in oligos with a large Tm variation, and is time consuming for larger sets of genes.

Applicability of LT2 genome sequence-based oligos for DT104.

Fluorescent Cy5 labeled genomic DNA of *Salmonella* serovar Typhimurium LT2 and DT104 were hybridized to silylated or epoxy-coated microarrays and the scanned microarray images are shown in Fig. 2A and B, respectively. Nineteen nine % of the oligos designed by using the *Salmonella* serovar Typhimurium LT2 genome sequence resulted in positive hybridization signals for both strains. In addition, the only differences between both isolates were the *Salmonella* serovar Typhimurium DT104 antibiotic resistance genes that are additionally depicted in Fig. 2C. Notably, in all experiments performed nine oligos resulted continuously in no-signal (indicated in Table S1 as supplementary material of Chapter 4) and around 2.5% of all spots were missing randomly on microarrays of different spotting runs (data not shown). The obtained DNA hybridization results indicate that the oligos designed based on the genome sequence of *Salmonella* serovar Typhimurium LT2 are also feasible to be able to study gene expression in *Salmonella* serovar Typhimurium DT104.

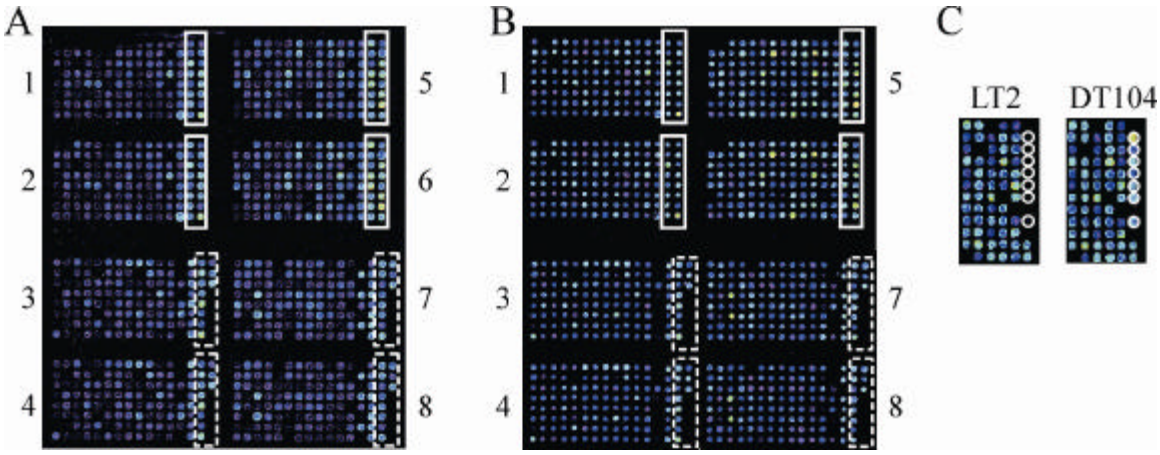

FIG. 2 Examples of scanned microarray images of Cy5 fluorescent labeled genomic DNA of *Salmonella* serovar Typhimurium (A) DT104 hybridized to a silylated-coated microarray and (B) LT2 hybridized to epoxy-coated microarray. Lined and dashed boxes in A and B represent the 60mer oligos. Spotting block 2 is a duplicate of block 1, 4 is duplicate of 3, 6 is duplicate of 5 and 8 is duplicate of 7. (C) Part of the microarray indicating the differences between genomic DNA of *Salmonella* serovar Typhimurium LT2 and DT104. The five antibiotic resistance genes located on SGI-I of *Salmonella* serovar Typhimurium DT104 are depicted by white circles, which represent downward the oligos tet(G)NEW, sul1NEW, tet(G), sul1, pse1, flo, and aada2.

Evaluation of effect of microarray parameters settings on microarray sensitivity.

As mentioned in the introduction, we evaluated how the hybridization signal was influenced by changing several parameter settings to obtain more sensitive microarrays resulting in the detection of more low-level expressed genes. Extracted genomic DNA of an overnight grown culture and extracted RNA of four different time points during growth at pH 7.0 or pH 5.0 (Fig. 3) for *Salmonella* serovar Typhimurium DT104 were hybridized in sixfold (DNA) or threefold (RNA) to silylated or epoxy-coated microarrays. The genomic DNA was Cy3 or Cy5 labeled, a mixture of all RNA time samples of both pH conditions was Cy3 labeled, and a mixture of all RNA time samples of each pH condition was Cy5 labeled. The mean spot and background hybridization signals of all spots per microarray and the corresponding signal-to-noise ratios (S/N), as an indicator for the microarray sensitivity, were computed for each Cy3, Cy5, DNA or RNA sample. The mean of these values for the six (DNA) or three (RNA) microarrays used were computed and plotted (Fig. 4). All Cy5 labeled samples resulted in higher S/N values compared to the Cy3 labeled samples. This indicates an increase of the sensitivity of the microarray when using the Cy5 label. Furthermore, the use of epoxy instead of silylated-coated microarrays resulted in 2 to 5-fold increases of the S/N values; mainly due to lower background signals. Thus, the use of epoxy-coated microarrays in combination with Cy5 label resulted in microarrays that were more sensitive, and presumably will detect more low-level expressed genes.

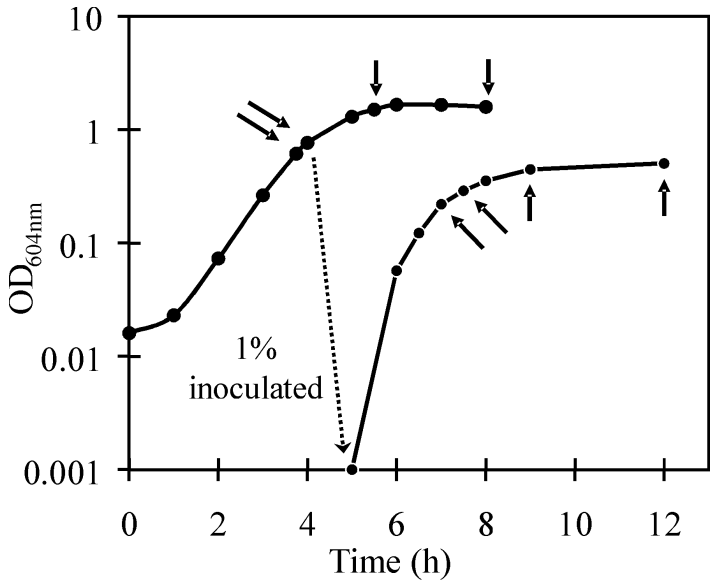

FIG. 3 Growth curve of *Salmonella* serovar Typhimurium DT104 in LBG pH 7.0 (left curve) and LBG pH 5.0 (right curve) medium. Notably, the pH 5.0 culture was inoculated with 1/100 pH 7.0 culture of an OD of 0.75. Samples for RNA extraction were taken at the time points indicated by the arrow.

In addition, an exponential growth phase pH 7.0 RNA sample was hybridized in duplicate to silylated or epoxy-coated microarrays of which the oligos were spotted onto these microarrays in 5xSSC buffer (as used in all former experiments) and additionally this sample was hybridized to epoxy-coated microarrays of which the oligos were spotted in sodium phosphate-based spotting buffer. From these hybridization results, the number of oligos (in %) of which the hybridization signal was a) below background for both duplicate hybridizations, b) above the background for one duplicate hybridization, c) above the noise (S/N>3) for one duplicate hybridization, and d) above the noise (S/N> 3) for both duplicate hybridizations were computed (Fig. 5). The number of oligos resulting in hybridization

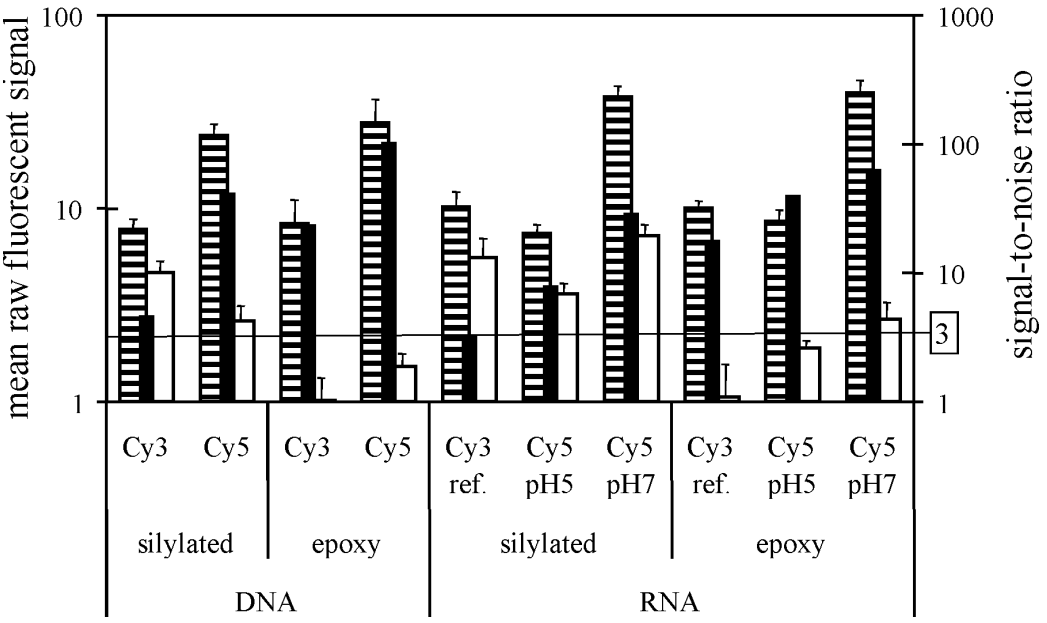

FIG. 4 Mean values of six (DNA) or three (RNA) silylated or epoxy-coated microarrays for the mean raw spot and background fluorescent signal (left axis) of all spots per microarray and the corresponding signal-to-noise ratios (S/N) (right axis). The Cy3 labeled genomic DNA, pH 5.0 cDNA (converted from RNA), or pH 7.0 cDNA, and Cy5 labeled genomic DNA and a mixture of both pH 5.0 and pH 7.0 cDNAs (ref.) of *Salmonella* serovar Typhimurium DT104 were hybridized. Striped bar represents spot value, white bar represents background value and black bar represents S/N values. Horizontal line indicates S/N=3 threshold, which is a commonly used threshold value to accurately quantify a signal above the noise (19).

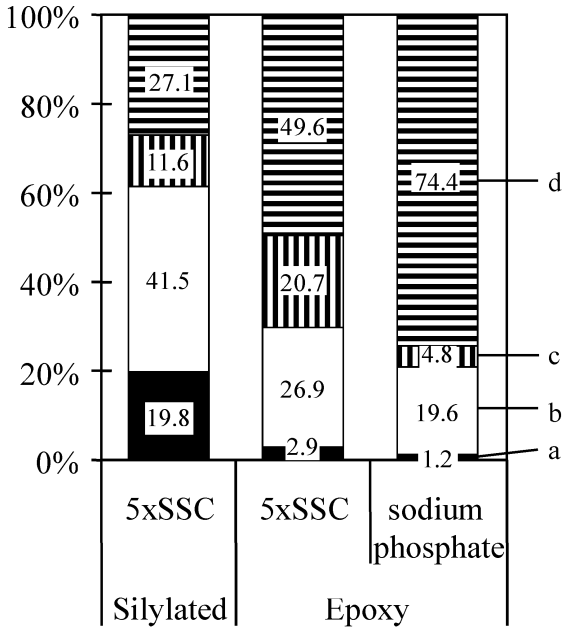

FIG. 5 Number of oligos (in %) of which the hybridization signal was a) below background for both duplicate hybridizations, b) above the background for one duplicate hybridization, c) above the noise (S/N>3) for one duplicate hybridization, and d) above the noise (S/N> 3) for both duplicate hybridizations. These values were computed for an exponential growth phase pH 7.0 RNA sample hybridized to silylated or epoxy-coated microarrays of which the oligos were spotted onto the microarrays by using 5xSSC and additionally epoxy-coated microarrays in combination with using sodium phosphate-based spotting buffer.

signals above the noise (S/N>3) for both duplicate hybridizations (group d) was for silylated-coated slides 27.1% and for epoxy-coated slides 49.6%, if the oligos were spotted in 5xSSC buffer. In addition, if a sodium phosphate-based spotting buffer was used an additional increase to 74.4% was observed of the number of oligos above the noise. The higher numbers for the epoxy-coated microarrays confirm the results depicted in Fig. 4 that the epoxy-coated microarrays were more sensitive. Again, epoxy-coated microarrays were also more sensitive due to lower background signals indicated by the lower numbers for groups a and b. Finally, the largest difference observed between the different spotting buffers revealed to be reproducibility between duplicate hybridizations. The 20.7% of the oligos that resulted in hybridization signals above the noise only in one of the hybridization duplicates (group c) for 5xSSC spotted, epoxy-coated microarrays were above the noise for both duplicates if sodium phosphate-based spotting buffer was used. This observation is most likely explained by better spotting accuracy on epoxy-coated microarrays if oligos are diluted in sodium phosphate-based spotting buffer.

In addition to the computed results above, visual effects of a different oligo length or microarray glass coating on spot signal and shape were observed. Larger spot diameters were observed on silylated-coated microarrays (Fig. 2A) compared to epoxy-coated microarrays (Fig. 2B). Notably, the 60mer oligos (indicated with white boxes in Fig. 2A and B) resulted only on the silylated-coated microarrays in higher signals, which could be explained by the fact that more labeled DNA can bind to longer oligos.

Gene expression time patterns for stress and virulence genes.

The RNA samples obtained from the four different time points at two pH conditions for *Salmonella* serovar Typhimurium DT104 (see above) were hybridized in duplicate to the microarrays and analyzed. Here, we show the gene expression time patterns at both pH conditions for the expression of the universal stress proteins and paralogues (Fig. 6) and the *Salmonella* plasmid virulence genes (Fig. 7). The expression increased upon entry into stationary growth phase for all universal stress proteins and paralogues genes (*uspA*, *uspB*, *uspC*, *uspE*, *uspF*, and *uspG*) under both pH conditions. Notably, the expression of *uspB* at both pH conditions and *uspF* only at pH 7.0 (Fig. 6B) increased until the last time point, while the other *usp* genes for both conditions showed a small decrease towards the last time point. In *Escherichia coli* the exact biochemical functions of the universal stress proteins are still unknown. However, several Usp proteins play a role in protecting the cell against DNA-damaging agents, and it has been suggested that these proteins operate in the same pathway (10). Furthermore, for *Escherichia coli* several Usp proteins have been described to be induced upon entry into the stationary growth phase in rich medium (14). In our experiment, although on gene expression level, we also observed an induction upon entry into the stationary growth phase of the *usp* genes for *Salmonella* serovar Typhimurium DT104 cultured in rich medium at both pH 5.0 and pH7.0. Similar expression patterns for most of the *usp* genes ground the former results that these proteins may operate in the same pathway (10).

In addition, at both pH conditions all *spv* genes except *spvD* showed the lowest expression at the start of the stationary growth phase (Fig. 7). The *spvD* expression

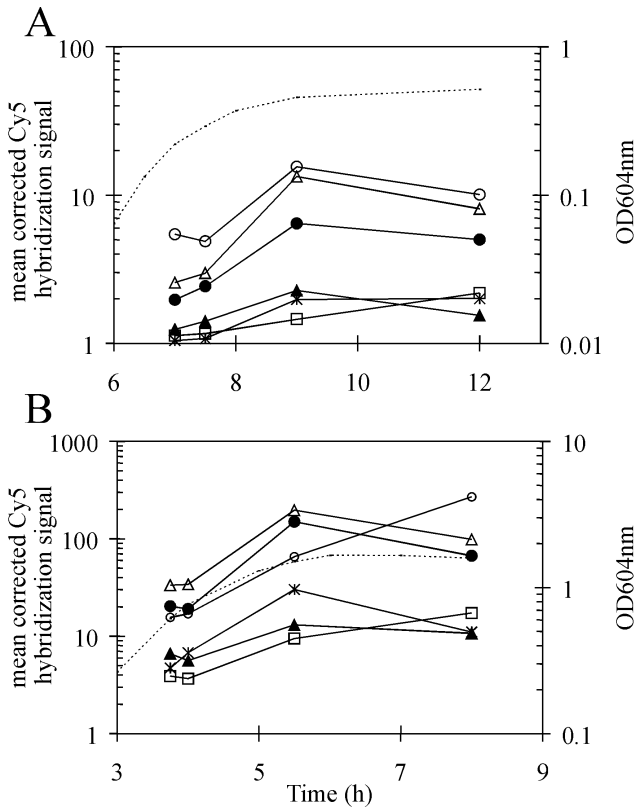

FIG. 6 Expression time patterns of *Salmonella* serovar Typhimurium DT104 during growth in (A) LBG pH 5.0 or (B) LBG pH 7.0 medium for the genes encoding universal stress proteins and paralogues *uspA* (closed circles), *uspB* (open squares), *uspC* (closed triangles), *uspE* (stars), *uspF* (open circles), and *uspG* (open triangles). The dashed line represents the OD_{604nm} during growth.

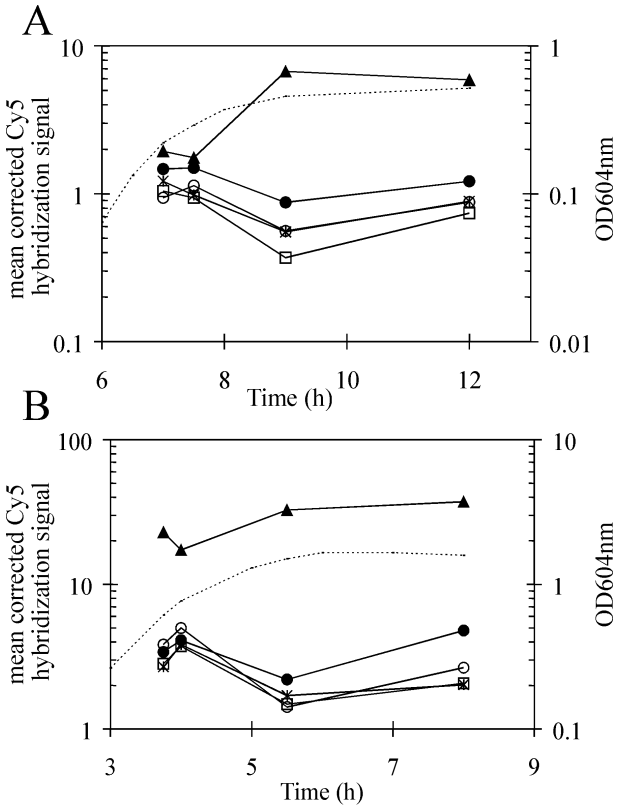

FIG. 7 Expression time patterns of *Salmonella* serovar Typhimurium DT104 during growth in (A) LBG pH 5.0 or (B) LBG pH 7.0 medium for the plasmid-located virulence genes *spvA* (closed circles), *spvB* (open circles), *spvC* (open squares), *spvD* (closed triangles), and *spvR* (stars). The dashed line represents the OD_{604nm} during growth.

rather revealed to be opposite to the other *spv* genes. Our *spv* time expression patterns were not similar to earlier studies with other *Salmonella* strains that showed an increase of all *spv* genes upon entry into station ary growth (2, 9), although we observed such a pattern only for *spvD*. The differences observed might be because these former studies cultured aerobically, and measured the *spv* expression via lacZ fusions instead of culturing anaerobically and measuring the mRNA levels directly as in our experiment.

Finally, we conclude that the thematic microarray developed can be applied to study the expression of stress and virulence genes of *Salmonella* serovar Typhimurium DT104. Furthermore, high-sensitivity microarrays can be obtained by spotting the oligos in sodium phosphate-based spotting buffer on epoxy-coated microarrays allowing the detection of more low-level expressed genes.

ACKNOWLEDGEMENTS

This study was supported by the Netherlands Organization for Health Research and Development, program “Nutrition: Health, Safety and Sustainability”.

We are grateful to Jeroen van Dijk, Alexander Elferink, Angela van Hoek, Jasper Kieboom, Maarten Mols, and Stijn van der Veen for their assistance and fruitful discussions. Furthermore, we would like to thank Marco de Boer of Isogen Life Science for his support and advice during microarray construction.

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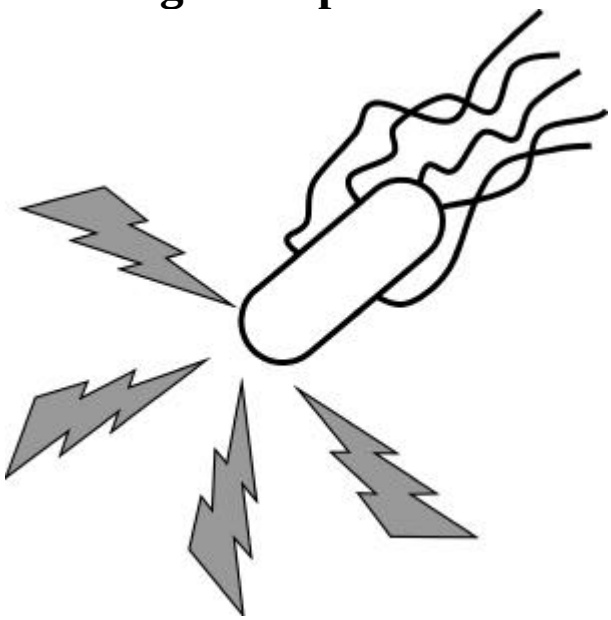
Chapter 5

Comparative transcriptome analysis of stress and virulence genes in *Salmonella enterica* serovar Typhimurium DT104 wild type and its *LuxS* deletion mutant at various growth phases



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Submitted

ABSTRACT

Transcriptome comparisons were performed between *Salmonella* serovar Typhimurium DT104 wild type and its *luxS* deletion mutant at various stages of growth in LB medium by using a thematic microarray containing stress and virulence genes. Expression of these genes in the wild type appeared to be largely growth-phase-dependent, with the highest expression levels observed in the stationary growth phase for genes under control of the general stress sigma factor RpoS, and for genes encoding universal stress proteins. The *Salmonella* pathogenicity island 1 (SPI-1) located invasion genes showed the highest expression in the end-exponential growth phase which correlates with the higher Caco-2 invasion capacity of the cells from this growth phase, as compared to that of cells from other growth phases.

Transcriptome analysis of the *luxS* deletion mutant revealed increased expression of 15 lipopolysaccharide (LPS) synthesis and assembly genes at the end-exponential growth phase. Concomitantly, a higher gene expression was observed for the periplasmic stress sigma factor RpoE that is known to control expression of several of these LPS genes. Other genes encoding chaperones and the heat stress sigma factor *rpoH* that are under control of RpoE were also induced. Notably, the *luxS* deletion mutant showed higher adhesion and invasion capacity into Caco-2 cells, although the expression levels of the SPI-1 invasion genes appeared to be similar to that in the wild type. Thus the loss of *luxS* results in increased transcription of LPS genes conceivably resulting in an overexpression of LPS molecules, thereby affecting *in vitro* virulence characteristics of the DT104 *luxS* deletion mutant.

INTRODUCTION

The multiple-antibiotic-resistant *Salmonella enterica* subsp. *enterica* serovar Typhimurium phage type DT104 (*Salmonella* serovar Typhimurium DT104) was first identified in the UK in 1984 (52). Since then, it has been generally recognized as an emerging food-borne pathogen (21, 28, 51, 54). *Salmonella* serovar Typhimurium, including phage type DT104, contains general and specific stress adaptation and survival mechanisms to cope with a variety of food-related stresses such as cold, heat, salt, or acid. In addition, some of these mechanisms may contribute to *Salmonella* serovar Typhimurium virulence where they assist in the survival of for example acidity during stomach passage (reviewed in reference 41).

Several stress response and virulence genes have been described for *Salmonella* serovar Typhimurium or other enteric pathogens to be controlled by the growth state of the cells. For example, the important stress regulator RpoS, regulates a large number of genes in response to a variety of stresses including entry into the stationary phase of growth (for reviews see (26, 32)). In addition, RpoS controls expression of the plasmid encoded virulence genes *spvRABCD* of which the SpvA and SpvR proteins have been detected only in the end-exponential and stationary growth phase (4, 24). Growth-phase-dependent expression has also been described for genes located on the *Salmonella* pathogenicity island 1 (SPI-1). SPI-1 contains genes encoding virulence factors specifically involved in the invasion of the gastrointestinal epithelium (25, 34). Highest expression of SPI-1 genes and corresponding protein levels were found at the end-exponential growth phase (43).

In addition to growth-phase-dependent signaling systems induced by a variety of stresses including pH extremes and nutrient depletion, cell-to-cell communication for sensing cell density (quorum sensing) may play a role in the activation and coordinate expression of specific (sub)sets of genes. Quorum sensing systems present in *Salmonella* serovar Typhimurium have recently been reviewed (1, 2, 57) and include the three systems reffered to as *sdiA*, *luxS* (AI-2) and *luxS* (AI-3). The signal receptor encoded by *sdiA* mediates a response to *N*-acyl-homoserine-lactone-type autoinducer signals generated by other microbial species (36). The *luxS* system mediates synthesis of autoinducer-2 (AI-2), in response to cell density, and AI-3 that can activate virulence genes in enterohemorrhagic *Escherichia coli* (44). AI-3 has been recently detected also in *Salmonella* (57). AI-2 was shown to be produced from the mid- to the end-exponential growth phase, where maximum levels are reached, followed by a decrease in the stationary growth phase (12, 45). Furthermore AI-2 is released in the exponential growth phase while imported at the transition into the stationary growth phase (47) and environmental regulation of AI-2 has also been reported previously (45). Since AI-2 is produced and detected by a wide variety of bacteria and *luxS* is present in around 50 % of the sequenced bacterial genomes, AI-2 is proposed to enable interspecies communication (59, 64). AI-2 is a by-product of the activated methyl cycle (AMC), which recycles S-adenosyl-L-methionine, the main methyldonor in eubacterial, archaebacterial and eukaryotic cells (55, 61-63). The *lsr* operon genes are the only genes currently known to be regulated by AI-2 in *Salmonella* and these encode for the AI-2 transport and modification system (1, 46, 47). In several other bacterial species roles for AI-2/*luxS* have been observed in biofilm formation, motility, iron

acquisition, and production of virulence factors, but further elucidation of the mechanisms involved is required (55, 64).

Although for *Salmonella* serovar Typhimurium several papers appeared describing transcriptome analysis under various stress conditions compared to the nonstressed condition or of specific genes by using mutant strains (50), no studies have been dedicated to transcriptome analysis of the wild type strain during growth in this nonstressed condition, including transition from the exponential to the stationary phase of growth. Moreover, transcriptome studies with the multiple-antibiotic-resistant emerging *Salmonella* serovar Typhimurium DT104 strain have not been reported (50).

In the present study, the expression of stress response and virulence genes for *Salmonella* serovar Typhimurium DT104 wild type and its *luxS* deletion mutant was assessed over an 3h time course from the exponential to the stationary growth phase by using a thematic microarray. Temporal expression of stress response and virulence genes in the wild type was studied to identify if groups of genes of similar functions reveal the same expression patterns. Furthermore, the temporal expression of these genes in the wild type is compared to that of the *luxS* deletion mutant to elucidate possible roles of LuxS in the expression of stress and virulence genes and to assess its impact on *in vitro* virulence of *Salmonella* serovar Typhimurium DT104.

MATERIAL AND METHODS

Bacterial culturing and sampling.

Salmonella serovar Typhimurium DT104 isolate 7945 (wild type), obtained from the Dutch National Institute of Public Health and the Environment (RIVM) and a *luxS* deletion mutant strain of isolate 7945, which was kindly provided by Jasper Kieboom, were stored at -80 ^{o}C in brain heart broth (Merck, Darmstadt, Germany) plus 50% glycerol (Merck). For the RNA expression experiments, the isolates were grown at 37° C without shaking by inoculating 1/100 brain heart broth (Merck) overnight culture in LBG pH 7.0 medium (20 g Luria Bertani Lennox broth (Difco, Detroit, Mich.), 5 g NaCl , 4.36 g glucose-monohydrate and 100 mM morpholinepropanesulphonic acid (MOPS, Sigma-Aldrich, St. Louis, Mo.) per liter sterile water. The optical density (OD) was measured at 604nm during the growth experiments. At four time points during growth (as indicated in Fig. 1), 40 ml culture samples were taken and added to 10 ml of ice-cold mixture of 95% (v/v) 96% ethanol and 5% (v/v) buffered phenol (Invitrogen, Carlsbad, CA) in 50 ml tubes. The tubes were centrifuged for 5 min at 4.000 rpm at 4 °C.

RNA extraction and labeling.

Total RNA was isolated from the culture pellets by using TRIzol reagent (Invitrogen) and purified as described by the supplier. The purified RNA samples were RQ1 RNase-free DNase (Promega, Madison, Wis.) treated, as described by the supplier. For each RNA sample per hybridization, 20 µg total RNA was converted into cDNA at 37 °C overnight by using 200 Units SuperScript II Reverse Transcriptase (Invitrogen) and adding 4 µl 10x AA-dNTP mixture (5 mM dATP, dCTP, dGTP, 3 mM dTTP (Invitrogen) and 2 mM

amino-allyl-dUTP (Sigma-Aldrich)). The cDNA reactions were stopped by adding 1.5 µl 20 mM pH 8.0 EDTA (Merck) and treated with 15 µl 0.1 M NaOH, heated for 10 min at 70 °C and neutralized with 15 µl 0.1 M HCl to breakdown unconverted RNA and followed by a standard ethanol precipitated. The cDNA pellet was dissolved in 10 µl 0.1 M Na_2CO_3 pH 9.3 buffer and labeled by adding 10 µl Fluorolink Cy monofunctional dye (Amersham Biosciences, Piscataway, NJ). Time samples were Cy5-dye labeled and a mixture of equimolar amounts of all time samples (used as the reference) was Cy3-dye labeled in the dark at room temperature for at least 30 min, ethanol precipitated, dissolved in 100 µl sterile water and purified for removal of uncoupled dye by using a QIAquick PCR purification kit (Qiagen, Valencia, CA), as described by the supplier. Notably, a mixture of all time samples was used as a reference sample for the time-course experiment as reported previously (39). Such a pooled reference sample is more representative than using only one time sample as a common reference, since e.g. some genes are only highly expressed in the exponential growth phase while others are highly expressed in the stationary growth phase. For comparing a diverse set of samples obtained in for example a time-course experiment, this pooled reference approach is preferentially used amongst others in transcriptome analysis of human cell cultures (6, 14, 39, 53). The pellets obtained were dissolved in 17.5 µl hybridization buffer (5xSSC, 0.2% SDS, 5xDenhardt's solution, 50% (v/v) formamide and 0.2 µg/µl denatured herring-sperm DNA), boiled for 5 min and spun down briefly. Finally, the sample Cy5-dye labeled cDNAs and the reference Cy3-dye labeled cDNAs were mixed (1:1).

Microarray procedure and data analysis.

Thematic microarrays were fabricated by spotting, in 5xSSC, 497 oligos designed by using Gene Runner version 3.05 and the first prototype of OligoFaktory (Delphi Genetics S.A., Charleroi-Gosselies, Belgium) (42), representing 426 different genes selected that were mainly involved in stress response and virulence, onto silylated-coated glass slides (Genetix, New Milton, UK) at a 25 mM concentration. The 426 genes selected and their function, and the 497 corresponding oligos and their characteristics for Tm, oligo length and Gene Runner or OligoFaktory designed are thematically shown in Table S1 as supplementary material of Chapter 4. All oligos were synthesized and modified with a 5'-C6-amine linker by Isogen Life Science, Maarssen, The Netherlands.

Two hybridization areas were printed per slide and each oligo was printed twice per hybridization area. After spotting, the silylated slides were treated with sodium borohydride to inactivate free aldehyde groups and to irreversible bind the oligos to the microarray. Furthermore, the silylated slides were blocked by incubating the slides overnight at 42 ^{o}C in hybridization buffer (see above) and washed by dipping five times subsequently in two times sterile water and isopropanol and air-dried. After pre-hybridization, 25 µl Gene Frame windows (Westburg, Leusden, The Netherlands) were fit onto the hybridization areas of the microarrays and covered with cleaned plastic covers (1.5x1.5 cm^2) containing two small pierced holes. The 35 µl mixtures of Cy5/Cy3-dye labeled DNA or cDNA samples were injected into the hybridization areas and the small holes were covered with pieces of plastic cover to prevent evaporation. The slides were incubated for 24 hours at 42 °C in a humid hybridization chamber. After hybridization, the Gene Frame windows were removed and the slides were subsequently first rinsed shortly and second incubated for 5 min in 1x SSC/0.1%

SDS, 0.1x SSC/0.1% SDS and 0.1x SSC. The last incubation in 0.1x SSC was for 1 min. Finally, the slides were dried by centrifugation (440g, 2 min, rT).

Microarrays were scanned with the ScanArray 3000 confocal laser scanner (GSI Lumonics, Kanata, ON, Canada) by using a pixel resolution of 10 μm, a Photo Multiplier Tubes value of 90% and the laserpower was set at a level observing no saturated spots. The fluorescent signals per spot and four background areas around each spot were volume measured (sVOL) by using the software package ArrayVision (Imaging Research, St. Catharines, ON, Canada). From these data the signal-to-noise ratios (S/N) were computed for each spot to discriminate true signal from noise as follows: S/N = (fluorescent spotsignal - average background signal of four areas surrounding the spot)/(standard deviation of the four background area values). A commonly used threshold value to accurately quantify a signal above the noise is an S/N>3 (56). Prior to normalization the obtained Cy5 or Cy 3 values which had an S/N= 3 were discarded. For normalization several parameters are defined: R = Cy5 value of a spot divided by the corresponding reference Cy3 spot value; H = median R value of a hybridization area calculated only from the spots that could be detected in all hybridizations; A = median H value of all hybridization areas; V = median Cy3 hybridization signal per oligo for all hybridization areas. The corrected Cy5 value per spot = R*(A/H)*V. To study patterns of gene expression, the mean corrected Cy5 hybridization signals for different time samples per oligo (duplicate hybridizations and duplicate spots per oligo) that represent the level of expression and the corresponding standard deviation (STDEV) were plotted in the course of time. Note, that the oligo spot names are depicted in Figs 2-5 that are representative for the corresponding genes and that the standard deviations are only depicted in color figures in the supplementary material of Chapter 5. All microarray data can also be obtained from the supplementary material of Chapter 5 presenting in Table S2a wild type only and in Table S2b the wild type and *luxS* deletion mutant comparison. These data are from a single complete experiment. No experimental replicates are used, since it is not necessary to replicate time points when analyzing time series because the noise present in single observations does not contribute significantly when genes are compared across several conditions (15, 58).

Caco-2 cell culturing.

Caco-2 cells of passage 25 to 45, obtained from the American Type Culture Collection were used to study the bacterial adhesion to and invasion into these Caco-2 cells in response to the addition of *Salmonella* serovar Typhimurium DT104 cells from exponential, end-exponential, and stationary growth phase. Therefore, Caco-2 cells were grown confluently at 37 °C and 5% (v/v) CO_2 in air in Dulbecco's modified Eagle's medium (DMEM) with addition of 25 mM Hepes and 4.5 g/l glucose (Invitrogen, Gibco); and per 500 ml DMEM 50 ml heat inactivated (30 min at 60 °C) fetal bovine serum (Integro b.v., Zaandam, The Netherlands), 5 ml MEM non-essential amino acids, 5 ml L-glutamine (final concentration 6 mM), and 0.5 ml gentamicin (final concentration 0.5 μg/ml) (Invitrogen, Gibco), were added. For each experiment, cells cultured for 12 to 19 days (medium was changed three times a week) were seeded at 1.6 x 10^4 cells per well in 12 well tissue culture plates (surface area per well is 401 mm^2) without filter inserts (Corning Costar Europe, Badhoevedorp, The Netherlands). One hour before bacterial infection, medium was

replaced by culture medium without serum and gentamicin, this is called tissue culture medium (TCM).

Bacterial adhesion and invasion assays.

Salmonella serovar Typhimurium DT104 cells from the different growth phases were spun 10 min at 4500*g* and concentrated 10 times in TCM. For each sample 40 µl bacterial suspension containing around 10^9 bacteria were added to a tissue culture plate well and after two hours, the Caco-2 cells were washed three times with TCM. The cells were lysed with 1 ml of 1% (v/v) Triton-X100 (Merck) to study the adhesions or incubated an additional 3 or 24 h with 1 ml 300 µg/ml gentamicin and 300 µg/ml ciprofloxacin in TCM for the invasion assay. For the invasion assay, the cells were washed 3 times with TCM and lysed with 1 ml 1% (v/v) Triton-X100. Adhesion and invasion were determined by counting colony-forming units (cfu), after serial dilution in peptone buffered physiological salt, on trypton soya agar plates. Plates were counted after 18 h incubation at 37 °C. All adhesion and invasion tests were performed three times and plotted relatively (in %) to the total number of bacterial cells added to the Caco-2 cells.

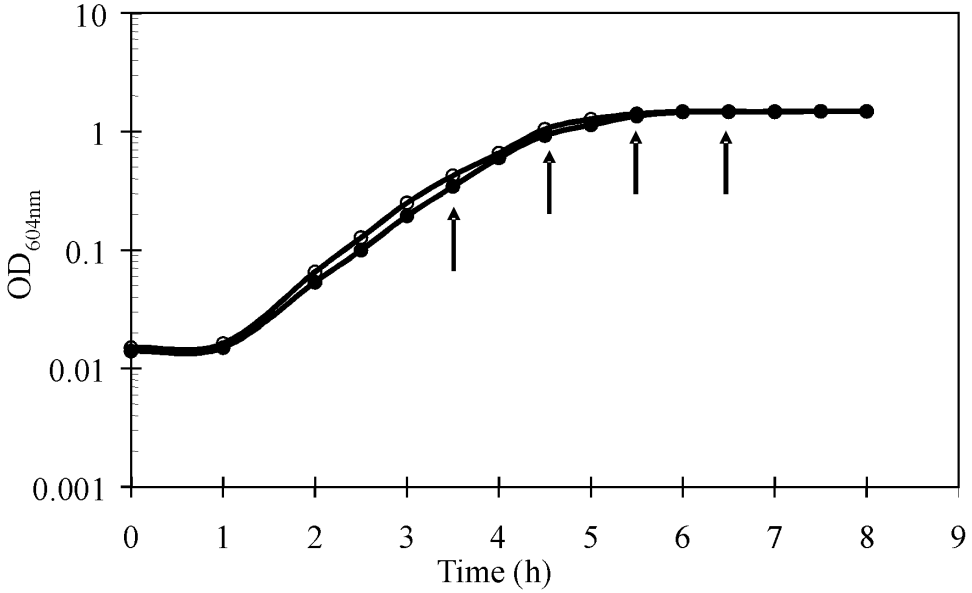

FIG. 1. Growth of *Salmonella* serovar Typhimurium DT104 wild type (open circles) and *luxS* deletion mutant (closed circles) in LBG pH 7.0 medium. Samples for RNA extraction were taken at the time points indicated by the arrows.

RESULTS

Temporal gene expression for *Salmonella* serovar Typhimurium DT104.

Four culture samples were taken (as indicated in Fig. 1) over a 3 h time course during growth of *Salmonella* serovar Typhimurium DT104 from the exponential to the stationary growth phase. RNA was extracted from these samples, fluorescently labeled, and subsequently hybridized to thematic stress response and virulence microarrays. The

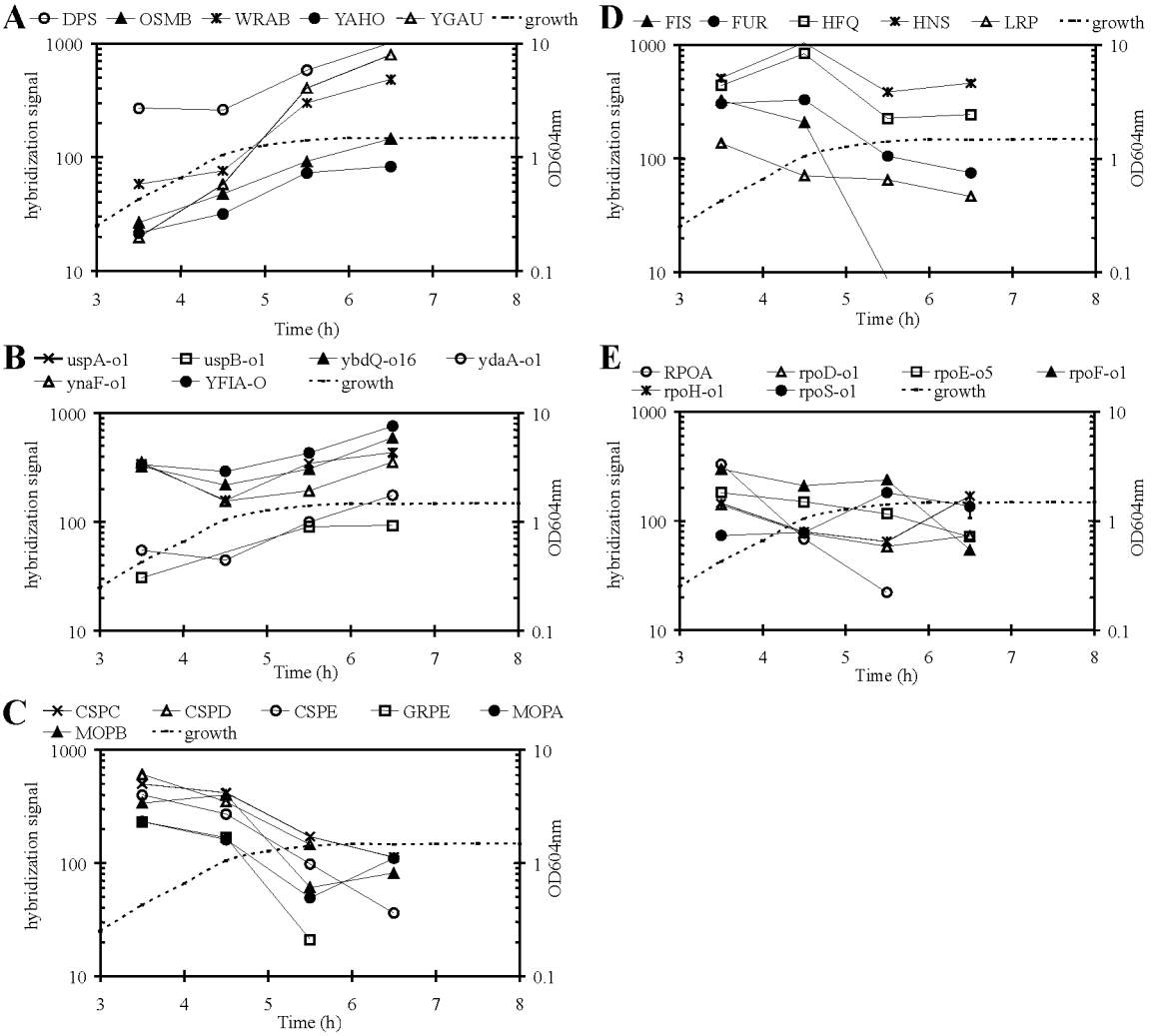

FIG. 2. Expression time patterns for stress response genes (A) RpoS-regulated genes, (B) universal stress proteins encoding genes, (C) heat and cold stress shock induced genes, (D) *fur* and genes involved in repressing RpoS production, and (E) sigma factors in *Salmonella* serovar Typhimurium DT104 in LBG pH 7.0 medium. The dashed line represents the OD_{604nm} during growth. Figure E is additionally presented in color as supplementary material of Chapter 5.

temporal expressions for stress response genes are shown in Fig. 2, and for virulence genes in Fig. 3.

(i) Expression of stress response genes. The response of several RpoS-regulated genes (22, 26, 29) are shown in Fig. 2A. The expression of the RpoS-regulated genes *dps*, *osmB*, *wraB*, *yahO*, and *ygaU* increased at the end-exponential growth phase upon entry into the stationary growth phase. The expression of the universal stress protein (*usp*) and paralogues genes *uspA*, *uspB*, *uspE* (*ydaA*), *uspF* (*ynaF*), and *uspG* (*ybdQ*) (Fig. 2B), that have functions in the protection of DNA (31), and the non-*usp* gene *yfiA* that stabilizes ribosomes against dissociation (33, 60), showed an increase in the stationary growth phase after a decrease in the exponential phase. The chaperones encoding heat and cold shock protein genes *cspC*, *cspD*, *cspE*, *grpE*, *mopA*, *mopB*, (Fig. 2C) decreased in time, although *mopA* and *mopB* showed an increase at the final time point. The genes coding for the nucleoid-associated proteins (Fis, H-NS, and Lrp) and Hfq that are involved in RpoS

regulation, and the major iron regulator Fur (ferric uptake regulator) showed the highest expression in the exponential phase and the expression dropped in the stationary phase (Fig. 2D). The expression of the regular *Salmonella* serovar Typhimurium sigma factor $\sigma^{D/70}$ (encoded by *rpoD*) that initiates transcription of most (housekeeping) genes required for metabolism during exponential growth, decreased in the course of time (Fig. 2E). In addition, *rpoA* that encodes the RNA polymerase alpha subunit decreased around 15 times upon entry into the stationary growth phase and could not be detected in the stationary growth phase because its value was in the noise. It cannot be excluded that *rpoA* is still transcribed at a low level under these conditions. Growth-phase-dependent expression of the four alternative sigma factors, $\sigma^{E/24}$ (*rpoE*), $\sigma^{H/32}$ (*rpoH*), $\sigma^{S/38}$ (*rpoS*), and σ^{28} (*fliA* = *rpoF*), that interact with RNA polymerase core enzyme to initiate transcription under specific conditions is depicted in Fig. 2E. Expression of *rpoN* could not be detected, and is therefore omitted from this figure. The expression of *rpoS* increased upon entry into the stationary growth phase, while *rpoE*, *rpoF*, and *rpoH* decreased, although *rpoH* revealed an upregulation and *rpoF* an additional downregulation in the stationary growth phase.

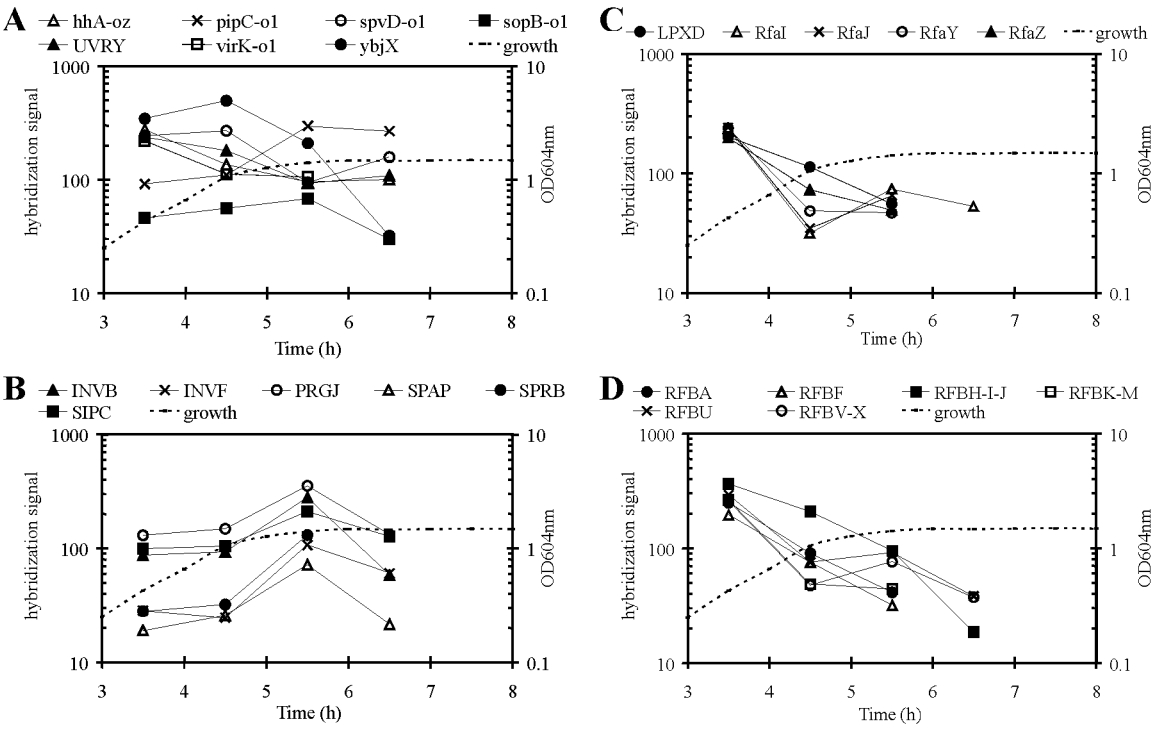

FIG. 3. Expression time patterns for (A) virulence genes, (B) SPI-1 located genes; and lipopolysaccharide synthesis and assembly genes (C) *lpxD* and *rfa* cluster, and (D) *rfb* cluster in *Salmonella* serovar Typhimurium DT104 in LBG pH 7.0 medium. The dashed line represents the OD_{604nm} during growth. Figures A, B and D are additionally presented in color as supplementary material of Chapter 5 showing for figures B and D additional genes with similar expression patterns.

(ii) Expression of virulence genes. Expression of *Salmonella* serovar Typhimurium DT104 virulence genes is presented in Fig. 3A, and displayed large variations in expression patterns. The *Salmonella* pathogenicity island (SPI) 5 encoding *pipC* and *sopB* genes showed a higher expression around the end-exponential/early-stationary growth phase (t = 5.5 h). In addition, the *virK* and the VirK homologue *ybjX*, the virulence plasmid encoded *spvD* gene, *hha* that encodes the hemolysin expression modulating protein, and the gene

encoding the response regulator UvrY (also referred to as SirA in *Salmonella*) decreased in time. The latter two genes are involved in the regulation of the SPI-1 (1). The SPI-1 located genes (Fig. 3B) encoding proteins that mediate the invasion of the gastrointestinal epithelium (25, 34), showed an expression peak at t = 5.5 h. In addition to the six genes depicted in Fig. 3B, 16 SPI-1 located genes showed a similar expression pattern (see supplementary material of Chapter 5). These 16 genes have different functions: the *hilD*, *invF* and *sprB* genes code for transcription regulators, *iacP*, *sipB*, *sipC*, *sipD* code for secreted proteins, *invB, invH, prgI*, *prgJ*, *prgK*, *spaO*, *spaP* and *spaR* code for the secretion apparatus, and *sicA* codes for a specific chaperone involved in activation of SPI-1 genes (25, 34).

Finally, the growth-phase-dependent expression of *lpxD* and the *rfa* and *rfb* genes encoding proteins involved in lipopolysaccharide (LPS) synthesis and assembly was analyzed, since LPS may have different roles in stress survival and in pathogenesis. For example, LPS can mediate resistance to bile (41) and can induce an immune response of the host (20, 40), respectively. Expression of these genes appeared to decrease in the course of time and was the lowest in stationary phase cells (see Fig. 3C and 3D and more LPS genes additionally presented in Fig. 3D as supplementary material of Chapter 5).

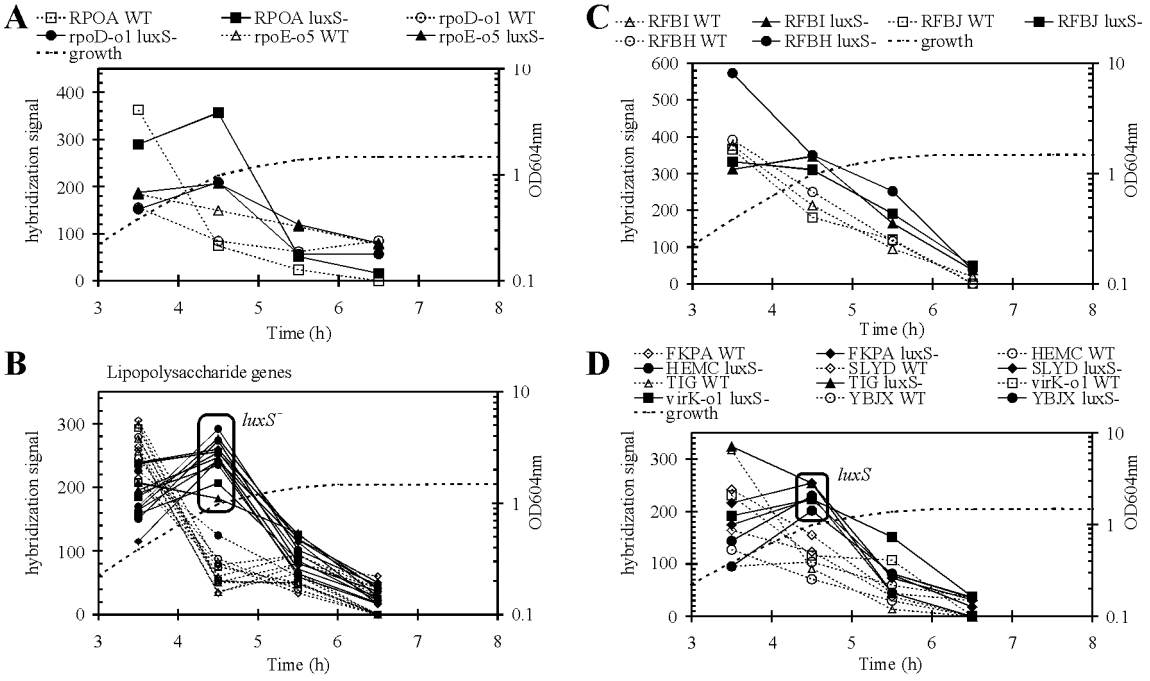

FIG. 4. Expression time patterns for LuxS repressed genes (A) sigma factors, (B, C) lipopolysaccharide encoding genes, and (D) *virK*, *ybjX* and *hemC* genes, and peptidyl-prolyl cis-trans isomerase encoding genes *fkpA*, *slyD*, and *tig* in *Salmonella* serovar Typhimurium DT104 wild type (open symbols, dashed line) and *luxS* deletion mutant (closed symbols) in LBG pH 7.0 medium. The bold dashed line represents the OD_{604nm} during growth. Figures B and D are additionally presented in color as supplementary material of Chapter 5.

LuxS-regulated genes at the various growth phases.

Simultaneously to the wild type *Salmonella* serovar Typhimurium DT104 strain, microarray experiments of four RNA samples over a 3 h time course were performed of a *luxS* deletion mutant. Both strains showed similar growth curves, indicating that deletion of *luxS* did not affect the growth characteristics of *Salmonella* serovar Typhimurium DT104 under the conditions used (Fig. 1). The expression patterns of most of the stress response and virulence genes were similar in both strains; including the RpoS-regulated genes and the SPI-1 located invasion genes (see supplementary material of Chapter 5). The number of genes differentially expressed in the *luxS* deletion mutant was 29, 47, 33, and 20 for the time samples at 3.5 h, 4.5 h, 5.5 h and 6.5 h, respectively. For these four time samples 5, 40, 27 and 11 genes were induced while 24, 7, 6 and 9 genes were repressed. In the exponential growth phase, most of the differentially expressed genes were repressed, while in the three other growth phases these genes were induced. Furthermore, the highest number of genes affected by deleting *luxS* was observed for the end-exponential growth phase. Notably, the downregulation of genes examined in the exponential phase were mainly followed by a higher fold upregulation in the end-exponential phase as also depicted in Fig. 4. The expression time patterns of genes downregulated by LuxS during the transition from the exponential to the stationary growth phase are depicted in Fig. 4 A to 4D and the LuxS-upregulated genes in Fig. 5. The *rpoA* gene, which encodes the RNA polymerase alpha subunit and *rpoD* that initiates transcription of most housekeeping genes required for metabolism during exponential growth were expressed for an extended period of time in the *luxS* deletion mutant compared to wild type. The alternative sigma factor *rpoE* shows similar results, although to a smaller extend than *rpoA* and *rpoD*. The LPS assembly encoding gene clusters *rfa*, *rfb*, and the *lpxD* gene revealed significant different expression patterns for the *luxS* deletion mutant (Fig. 4B and C; see also supplementary material of Chapter 5). The LPS genes were also expressed for an extended period of time in the *luxS* deletion mutant. In addition, the *virK* gene and the VirK homologue *ybjx*, the *fkpA*, *slyD*, *tig* (trigger factor) genes that encode peptidyl-prolyl cis-trans isomerase-type chaperones, and *hemC* show expression patterns similar to that of the LPS genes in the *luxS* deletion mutant. Three genes *ppiB*, *ppiC*, and *gapA* resulted in the second time point sample in lower expression values in the *luxS* deletion mutant (depicted in Fig. 5). The *gapA* gene encodes a metabolic enzyme glyceraldehyde-3-phosphate dehydrogenase A involved in the glycolysis, and *ppiB* and *ppiC* encode peptidyl-prolyl cis-trans isomerase-type chaperones.

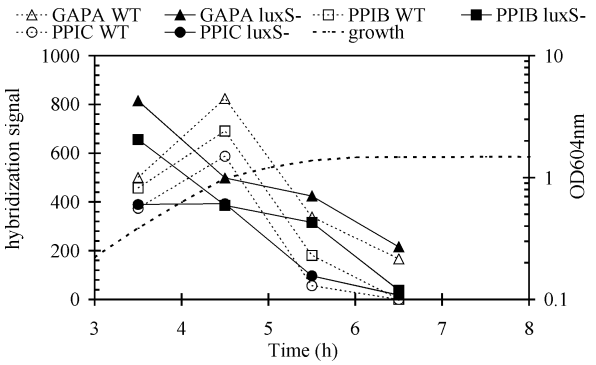

FIG. 5. Expression time patterns for LuxS induced genes in *Salmonella* serovar Typhimurium DT104 wild type (open symbols, dashed line) and *luxS* deletion mutant (closed symbols) in LBG pH 7.0 medium. The dashed line represents the OD_{604nm} during growth.

In vitro virulence assays for the wild type and *luxS* deletion mutant strains.

The wild type and *luxS* deletion mutant were tested for their virulence potency, because differences in gene expression were observed for genes encoding proteins involved in LPS synthesis and assembly, and that of genes encoding chaperone functions in the periplasmic space (see above). Incomplete LPS assembly is known to effect virulence (18, 38) therefore differences in LPS expression might also affects virulence properties such as attachment to cells. Therefore, culture samples of both strains, taken during exponential, end-exponential and stationary phase of growth, were added to Caco-2 cells. The number of bacterial cells that adhered and invaded into the Caco-2 cells was determined (Fig. 6A and 6B). The highest invasion capacity was observed with both wild type and mutant cells from the end-exponential growth phase. The corresponding adhesion capacity was 2.6-fold higher for the *luxS* deletion mutant, whereas the invasion inducing capacity was similar to that of the wild type. The invasion capacity of stationary phase cells from the *luxS* deletion mutant was however significantly higher (2.4-fold) than that of wild type cells, although their adhesion capacity was similar.

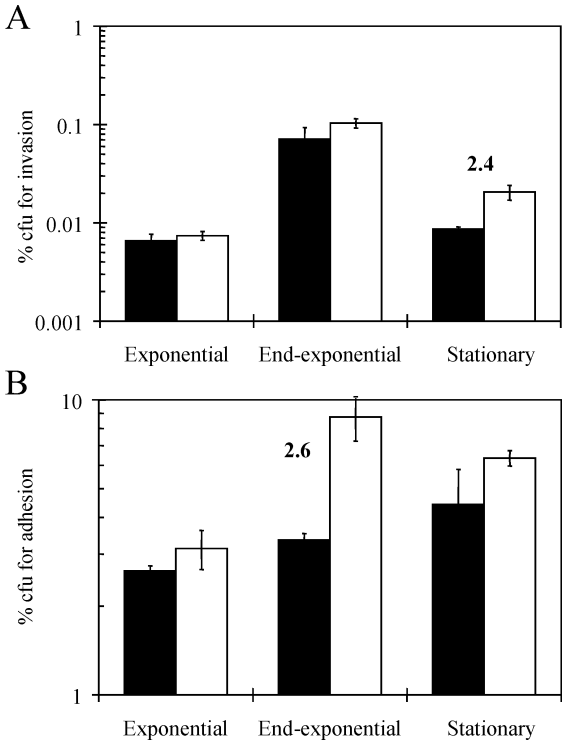

FIG. 6. *In vitro* virulence assays A) invasion and B) adhesion results for the *Salmonella* serovar Typhimurium DT104 wild type (black bars) and *luxS* deletion mutant (white bars) cultured in LBG pH 7.0 medium. Bacterial cells of the exponential, end-exponential, and stationary growth phase were used in these *in vitro* virulence assays. Numbers above the bars indicate the ratios between wild type and *luxS* deletion mutant, only in case of a significant difference.

DISCUSSION

This study describes for the first time gene expression for a *Salmonella* serovar Typhimurium (DT104) wild type strain during growth from the exponential to the stationary growth phase using a transcriptomics approach. Since it is generally accepted that growth phase may have a dramatic impact on cellular characteristics, our transcriptome study of *Salmonella* serovar DT104 wild type and its *luxS* deletion mutant may contribute to the understanding of growth-phase-dependent regulatory mechanisms and their impact on *in vitro* virulence. Several expression patterns for a selection of known stress and virulence factors obtained in our study showed similarities with results of former studies with other *Salmonella* or *Escherichia coli* (*E. coli*) strains, although our results are the first to be reported for *Salmonella* serovar Typhimurium DT104. Similarities include for example the downregulation of genes involved in repressing RpoS production (H-NS, Fis, Lrp, and Hfq) and the upregulation of *rpoS* and the genes under its control upon entry into the stationary

phase of growth (22, 26, 29, 32). Transcription levels of the RNA polymerase alpha subunit encoded by *rpoA* decreased upon entry into the stationary growth phase resulting in values below the detection limit of the microarray. Since transcription of other genes continues in stationary phase, it cannot be excluded that low level transcription of *rpoA* still allows for production of active RpoA, and on the other hand RpoA activity may be maintained in stationary phase. Information about transcription efficiency and activity of the different RNA polymerase subunits is rather limited, but *rpoA* expression patterns in DT104 appeared to be similar to that observed in *Pseudomonas putida* (66). In *E. coli* the levels of the RNA polymerase subunits have been found to decrease as cells enter the stationary growth phase (17, 49). The genes that have chaperone functions revealed the highest expression in the exponential growth phase, such as the heat and cold shock proteins and the peptidyl-prolyl cis-trans isomerases encoding genes. However, the *ppiB* and *ppiC* chaperones showed expression patterns slightly different from that of other genes encoding chaperones. The decreased expression in the stationary growth phase for these chaperones encoding genes may be explained by reduced protein production and turnover rates such that lower amounts of proteins are formed and subsequently fewer chaperones are needed for folding of proteins or re-folding of misfolded proteins (13, 23, 37).

The upregulation observed around the end-exponential growth phase of the SPI-1 located virulence genes might be explained by the decreased expression of *hha* observed in our study. The *hha* gene encodes the hemolysin expression modulating protein that represses *hilA* (19), resulting in increased HilA levels that activate the SPI-1 virulence genes (SPI-1 regulation is reviewed in reference 1). In addition, the SPI-5 encoding *pipC* and *sopB* showed also the highest expression levels in the end-exponential growth phase. Therefore, similarity in regulation might occur for some of the SPI-1 and SPI-5 located genes, which has been suggested previously (58, 67). Finally, the decreased expression in the course of time in the wild type strain of genes coding for the LPS synthesis and assembly machinery is best explained by the large reduction in cellular growth and duplication rates and thus less LPS has to be synthesized and assembled in the outer membrane. Notably, control of LPS synthesis appears to be lost in the *Salmonella* serovar Typhimurium DT104 *luxS* deletion mutant as is discussed in the next section.

In several non *Salmonella* serovar Typhimurium bacterial species, a role for AI-2/*luxS* has been suggested in biofilm production, motility, iron acquisition, and virulence (reviewed in references 55 and 64). Our comparative transcriptome analysis between the wild type and *luxS* deletion mutant upon entry into the stationary growth phase revealed mainly differences in expression of LPS synthesis and assembly genes and for genes having chaperone functions involved in maintenance of protein quality in the cell envelope in response to periplasmic stress. The largest differences in gene expression were observed at the end of the exponential growth phase, at which the highest level for AI-2 is reached (12, 45), indicating that analyzing gene expression in the course of time from the exponential to the stationary growth phase allows for pinpointing genes of which the transcription is affected by LuxS. The chaperones *fkpA*, *ppiB*, *ppiC*, *slyD*, and *tig* are involved in folding or re-folding of newly synthesized proteins such as envelope proteins (13, 27, 37). Notably, deletion of *luxS* also resulted in increased expression of *virK* and the VirK homologue *ybjx*. VirK has been suggested to also contribute to the remodeling of the bacterial outer membrane in response to the host environment (11). A *luxS* mutant strain of

Porphyromonas gingivalis also revealed induced expression of several chaperones and *rpoD* (65), similar as observed in DT104. Information contained in regulatory networks (see below) could offer explanations for changes in gene expression patterns observed in the *luxS* deletion mutant. RpoE, of which the gene expression was also affected in the *luxS* deletion mutant, activates expression of the *fkpA* chaperone gene, several LPS assembly genes and *rpoH* (7). In addition, RpoH has been shown to affect the expression of *rpoD,* (48) *ppiD,* (8) and *gapA* (3) in *E. coli*. Expression of these genes was also altered in our study in the *luxS* deletion mutant. Finally, it is conceivable that changes in the expression of genes encoding chaperones are induced by the high level expression of the LPS synthesis and assembly genes in the *luxS* deletion mutant resulting in high numbers of LPS proteins in the outer membrane and/or periplasmic space. This may have resulted in a high number of misfolded LPS molecules resulting in extra-cytoplasmic stress, which may have triggered in turn expression of *rpoE* and subsequent activation of genes encoding chaperones that are involved in folding or re-folding of newly synthesized proteins in the cell envelope (13, 27, 37). Furthermore, previous work in *E. coli* revealed an induction of *rpoE* in response to overproduction of the outer membrane proteins OmpF, OmpC, OmpT, or OmpX (35).

Although effects of AI-2/*luxS* on e.g. biofilm production, motility, iron acquisition, or virulence have previously been described for other bacteria (reviewed in references 55 and 64) and upregulation (*rfaJ* and *rfaY*) and downregulation (*rfaD* and *rfbC*) of LPS assembly genes was observed in *E. coli* (10), a clear impact of deleting *luxS* on expression of all LPS synthesis and assembly genes and thus a repression of these LPS genes by AI-2 as observed in our study has never been described before. Notably, genes previously reported to be under control of LuxS were found to code often for outer membrane associated proteins involved in biofilm production, iron transport and motility in other bacteria such as the *lsr* operon genes coding for the AI-2 transport and modification system *Salmonella* serovar Typhimurium (1, 46, 47) and TonB involved in iron transport and a putative outer membrane efflux protein in *Porphyromonas gingivalis* (5, 65) (reviewed in references 55 and 64). A linkage between LuxS and LPS production in *Salmonella* serovar Typhimurium DT104 in our work is in line with previous observations that primarily surface characteristics have been affected. A linkage between quorum sensing via LuxS and the formation of surface structures was also proposed by Jeon *et al.* for *Campylobacter jejuni* (30). However, LuxS is involved in both AI-2 and AI-3 synthesis (44, 57) and therefore it remains to be elucidated if the LPS and chaperones genes in the *luxS* deletion mutant were changed by a lack of AI-2 or AI-3 production.

Our *in vitro* virulence assays revealed an increased adhesion of end-exponential *Salmonella* serovar Typhimurium DT104 cells onto Caco-2 cells of the *luxS* deletion mutant when the LPS genes were expressed at the highest level. Furthermore, the higher invasion capacity observed for stationary phase cells might be the result of higher LPS expression observed in the end-exponential sample since the SPI-1 located invasion genes were equally expressed in both strains (see supplementary material of Chapter 5). A *luxS* mutant strain of *Campylobacter jejuni* showed similar invasion capacity into Caco-2 cells as the wild type (16), however this was tested with cells harvested at the mid-exponential growth phase. Notably, the higher invasion capacity of end-exponential *Salmonella* serovar Typhimurium DT104 cells into Caco-2 cells is in agreement with the concomitant upregulation of the SPI-1 located virulence genes, which products mediate the invasion of the gastrointestinal

epithelium (15, 23). Correlations between invasion capacity and expression of SPI-1 proteins have been described previously for other non-DT104 *Salmonella* serovar Typhimurium strains (21, 26). As previously mentioned by Vendeville *et al*. (55), whatever the primary function of LuxS is in a bacterium, it can contribute at several levels to the pathogenesis of infection, and further studies are needed to elucidate these as proposed by Keersmaecker *et al.* (9).

In conclusion, our study reveals growth phase to have a major impact on the expression of stress and virulence genes in *Salmonella* serovar Typhimurium DT104, and correlations with its *in vitro* virulence characteristics were assigned. Furthermore, a role for LuxS in control of LPS synthesis and assembly genes in *Salmonella* serovar Typhimurium DT104 is suggested, since deletion of *luxS* resulted in higher expression of these LPS genes at the end-exponential growth phase, thereby affecting the adhesion and invasion capacities of the *luxS* deletion mutant.

ACKNOWLEDGEMENTS

This study was supported by the Netherlands Organization for Health Research and Development, program “Nutrition: Health, Safety and Sustainability”. We would like to thank Rob de Jonge from the Dutch National Institute of Public Health and the Environment (RIVM) for providing the *Salmonella* serovar Typhimurium DT104 isolate 7945 and Jasper Kieboom from the Wageningen University for the *luxS* deletion mutant.

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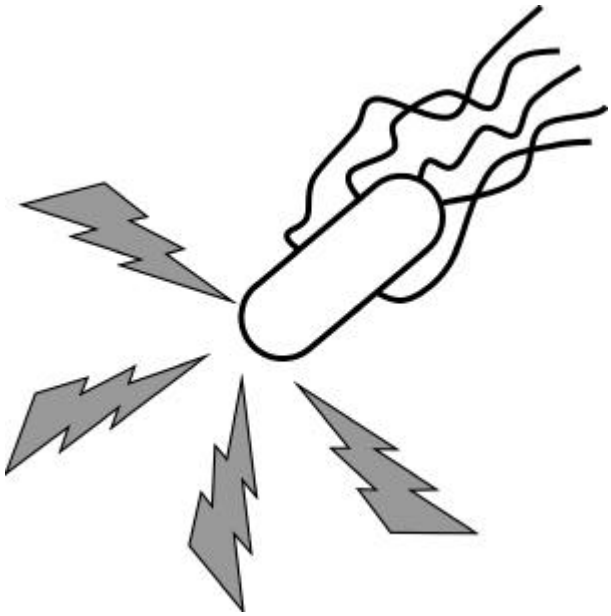
Chapter 6

Comparative transcriptome analysis of *Salmonella enterica* serovar Typhimurium DT104 stress response under aerobic and anaerobic conditions



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Will be submitted

ABSTRACT

A thematic microarray containing stress response and virulence genes was used to assess the *Salmonella* serovar Typhimurium DT104 transcriptomes under aerobic and anaerobic growth conditions and additionally during exposure to heat, hydrogen peroxide or low pH under both growth conditions. Under aerobic growth conditions the genes coding for the stress regulators Fur, OmpR, and RpoS and oxidative stress response genes were higher expressed, whereas under anaerobic growth conditions the genes encoding universal stress proteins and heat shock chaperones were higher expressed. The genes involved in lipopolysaccharide (LPS) synthesis and assembly and the SPI-1 located invasion genes were lower expressed anaerobically.

Exposure of aerobically or anaerobically grown exponential phase cells to the various stresses, revealed stress genes to be mainly induced or repressed in a similar manner under both growth conditions. However, expression of the virulence genes encoding LPS, the PhoPQ regulon, *Salmonella* plasmid virulence (*spv)*, SPI-1, and SPI-2 appeared to be differentially regulated by the selected stresses under aerobic and anaerobic growth conditions. The thematic microarray developed allows for assessment of the impact of stresses and combinations thereof on the expression of stress and virulence genes.

INTRODUCTION

Salmonella enterica subsp. *enterica* serovar Typhimurium (*Salmonella* serovar Typhimurium), like other enteric bacterial pathogens, may be exposed to a range of stresses both in the environment such as in food products or during food processing and during passage and infection in the human host. Stress conditions encountered outside a host may include food-related stresses such as low and high temperatures, acid stress and high osmolarity. Inside the human host for example within macrophages acid stress, iron limitation and oxidative stress may be encountered. Under the various stress conditions encountered, oxygen availability may additionally affect the pathogen responses. For example oxygen availability inside the host varies along the gastro-intestinal tract, together with exposure to low pH, bile salts and low water activity. Many genes and regulators are involved in the control of stress response and virulence factors in *Salmonella* serovar Typhimurium. For example, when *Salmonella* serovar Typhimurium is exposed to acid stress, the genes coding for acid shock proteins (ASPs), the transcriptional regulators RpoS, Fur, PhoPQ and OmpR, and decarboxylases are induced (1, 7, 51). *Salmonella* serovar Typhimurium stress response mechanisms, survival strategies and virulence have recently been reviewed (54) and have been mainly focused on aerobically grown cells. Such stress response mechanisms can enhance survival of pathogens during exposure to stresses encountered outside and inside the host, thereby affecting pathogen numbers and virulence.

Within serovar Typhimurium, *Salmonella* serovar Typhimurium phage type DT104, has been identified as an emerging pathogen (21, 31, 63, 64). *Salmonella* serovar Typhimurium DT104 can adapt to mild acid conditions (pH 5.0) allowing subsequent exposure to extreme acidic conditions (pH 2.5) such as encountered during stomach passage (6, 8, 9, 14). Furthermore, *Salmonella* serovar Typhimurium DT104 is multiple-antibiotic-resistant and additional putative DT104-specific virulence factors have been described recently (29, 55). Differences in virulence between phage type DT104 and other *Salmonella* have not been reported up to now (3, 10, 31). However, it has been suggested that the emergence of DT104 might be explained by its higher stress tolerance (31) and/or its multiple-antibiotic-resistance (10) when compared to other *Salmonella*.

To be able to study simultaneously the expression of the large number of genes involved in the various stress responses and virulence mechanisms, we used the microarray technology. Since the publication of the complete genome sequence of *Salmonella* serovar Typhimurium LT2 (44), most of the *Salmonella* serovar Typhimurium transcriptomics research has been focused on stress adaptation and virulence factors using *Salmonella* serovar Typhimurium strains LT2, ATCC14028(s) or SL1344 (4, 5, 12, 15, 16, 19, 30, 34, 40, 46, 48, 49, 52, 57-60, 66, 68, 69), which are neither emerging nor multiple-antibiotic-resistant. Stresses studied involved exposure to antimicrobial peptides, bile, and hydrogen peroxide, or a temperature upshift from 25 ^{o}C to 37 ^{o}C; and were recently reviewed by Thompson *et al.* (62).

In this study, we investigated the expression of selected stress response and virulence genes in *Salmonella* serovar Typhimurium DT104 both under aerobic and anaerobic conditions by using a thematic microarray. Selected stresses include low pH, hydrogen peroxide and high temperature, which DT104 may encounter in the environment or during passage and infection of the human host.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

Salmonella serovar Typhimurium DT104 isolate 7945, obtained from the Dutch National Institute of Public Health and the Environment (RIVM) was used in all experiments. The strain was stored at -80 ^{o}C in brain heart broth plus 50% glycerol (Merck, Darmstadt, Germany). From an overnight culture of this DT104 isolate grown in brain heart broth (Merck), 0.1% was transferred to LBG pH 7.0 broth that consisted of Luria Bertani broth (Difco, Detroit, Mich.) with the addition of 4 g glucose per liter and 100 mM morpholinepropanesulfonic acid (MOPS, Sigma-Aldrich, St. Louis, Mo.). Cells were cultured in LBG pH 7.0 at 37 ^{o}C (referred to as nonstress condition) in three 2000 ml Erlenmeyers containing 200 ml of culture medium and shaking at 225 rpm for aerobic conditions or in fully filled 500 ml flasks without shaking for anaerobic conditions to an optical density (OD_{600nm}) of around 0.30 (t = 0). Next, the cultures were divided into smaller portions of 40 ml in 50 ml screw cap tubes, and subjected to several stress conditions in triplicate as explained below. Notably, the aerobic cultures were pooled and subsequently divided into smaller portions used in the stress treatments.

Stress treatments and sampling.

Heat stress was applied by adding 4 ml preheated LBG (+/- 82 ^{o}C) to the 40 ml cultures resulting in a final temperature of 44 ^{o}C. Oxidative stress was applied by adding 4 ml LBG supplemented with hydrogen peroxide to a final concentration of 0.1 mM. Acid stress was applied by adding 4 ml LBG acidified with HCl resulting in a final pH of 5.0. As a control, 4 ml of fresh LBG was also added to the non-stressed aerobic and anaerobic cultures. At time zero for the nonstress conditions, and after 10 min of incubation (at 37/44 °C) for all conditions, 40 ml culture samples were taken and added to 10 ml of an ice-cold mixture of 95% (v/v) 96% (v/v) ethanol and 5% (v/v) buffered phenol (Invitrogen, Carlsbad, CA). The tubes were centrifuged for 5 min at 1780g at 4 °C. The remaining 4 ml were used to verify the OD.

RNA extraction and labeling.

Total RNA was isolated from the culture pellets by using TRIzol reagent (Invitrogen) and purified as described by the supplier. Notably, the TRIzol dissolved pellets of the triplicate cultures per condition were mixed. The purified RNA samples were RQ1 RNase-free DNase (Promega) treated, as described by the supplier. For each sample per hybridization, 20 µg total RNA was converted into fluorescent labeled cDNA at 37 °C for two hours by using SuperScript II Reverse Transcriptase (Invitrogen) and 6 µg random hexamers (Invitrogen). Fluorescent label was directly incorporated, by using a mixture of 25 mM dATP, dGTP, dTTP, 10 mM dCTP, and 2 mM Cy3-dCTP or Cy5-dCTP (Amersham Biosciences, Piscataway, NJ). Each specific RNA sample was Cy5-dye labeled, while a mixture of all RNA samples (pooled reference) was Cy3-dye labeled. The cDNA reactions were stopped by adding 1.5 µl 20 mM pH 8.0 EDTA (Merck), subsequently treated with 0.1 M NaOH, heated for 10 min at 70 °C and neutralized with 0.1 M HCl for breakdown of unconverted RNA, followed by an ethanol precipitation and dissolved in 10 µl sterile water.

The sample Cy5-dye labeled cDNAs and the reference Cy3-dye labeled cDNAs were mixed (1:1) and purified for removal of uncoupled dye by using a QIAquick PCR purification kit (Qiagen, Valencia, CA), as described by the supplier. The pellets obtained were dissolved in 35 µl hybridization buffer (5x SSC, 0.2% SDS, 5x Denhardt's solution, 50% (v/v) formamide and 0.2 µg/µl denatured herring-sperm DNA), boiled for 5 min and spun down briefly.

Microarray procedure and data analysis.

Thematic stress response and virulence microarrays were fabricated at Isogen Life Science, Maarssen, The Netherlands, by spotting 507 oligos representing 425 different genes that were predominantly related to stress and virulence (see supplementary material of Chapter 4) onto epoxy coated glass slides (Schott Nexterion Slide E, Jena, Germany).

The oligos designed by using Gene Runner version 3.05 and the first prototype of OligoFaktory (Delphi Genetics S.A., Charleroi-Gosselies, Belgium) (56) were synthesized and modified with a 5'-C6-amine linker by Isogen Life Science, Maarssen, The Netherlands. and spotted at a 30 mM concentration in Nexterion spotting buffer by using four Stealth AMP4 pins (ArrayIt, TeleChem International, Sunnyvale, CA) and the OmniGrid 100 spotter (Genomics Solutions, Ann Arbor, Mi.). Two hybridization areas were printed per slide and each oligo was printed twice per hybridization area. After spotting, the slides were treated for DNA immobilization, washing and blocking as recommended by the manufacturer. Gene frames for 25 µl hybridization samples (Westburg, Leusden, The Netherlands) were fit onto the hybridization areas, and covered with cleaned plastic covers (1.5x1.5 cm^2) containing two small pierced holes and the Cy5/Cy3 labeled cDNA mixture (as mentioned above) was injected into the hybridization area. The slides were incubated for 24 hours at 42 °C in a moisturized hybridization chamber. After hybridization, the Gene Frame windows were removed and the slides were incubated for 5 min in 1x SSC/0.1% SDS, next 5 min in 0.1x SSC/0.1% SDS and finally 1 min in 0.1x SSC and dried by centrifugation (440g, 2 min).

Microarrays were scanned using the ScanArray 3000 confocal laser scanner (GSI Lumonics, Kanata, ON, Canada) by using a pixel resolution of 10 µm, a Photo Multiplier Tubes value of 90% and the laserpower was set at a level observing no saturated spots. The fluorescent signals per spot and four background areas around each spot were volume measured (sVOL) by using the software package ArrayVision (Imaging Research, St. Catharines, ON, Canada). From these data the signal-to-noise ratios (S/N) were computed for each spot to discriminate true signal from noise as follows: S/N = (fluorescent spot signal - average background signal of four areas surrounding the spot)/(standard deviation of the four background area values). A commonly used threshold value to accurately quantify a signal above the noise is an S/N>3 (65). Prior to normalization the obtained Cy5 or Cy 3 values which had an S/N= 3 were discarded. For normalization several parameters are defined: R = Cy5 value of a spot divided by the corresponding reference Cy3 spot value; H = median R value of a hybridization area calculated only from the spots that could be detected in all hybridizations; A = median H value of all hybridization areas; V = median Cy3 hybridization signal per oligo for all hybridization areas. The corrected Cy5 value per spot = R*(A/H)*V.

The fold induction/repression of gene expression under aerobic or anaerobic growth for each stress condition was calculated by dividing the mean corrected Cy5 hybridization signals (duplicate hybridizations and duplicate spots per oligo) from the stress by the nonstress sample. The fold changes of all genes being significantly differentially expressed (i) under nonstress condition in the anaerobically grown cells compared to aerobically grown cells or (ii) in the stress conditions compared to the nonstress conditions for both aerobic and anaerobic grown cells; and including gene function/description can be obtained from Table S1 in the supplementary material of Chapter 6. Notably, the gene function or description used was derived from the *Salmonella* serovar Typhimurium LT2 genome (GenBank accession no. NC_003197) (44). For each gene, significantly differentially expression was tested by comparing the values of a stress condition at t = 10 min with the values of both the nonstress conditions at t = 0 and t = 10 min by using a Student t-test, P-value < 0.05 and all genes of a fold induction/repression of >1.5 were included in our comparative analysis.

RESULTS

Comparative transcriptome analysis between aerobically and anaerobically cultured cells.

Differences in expression for the stress response and virulence genes between aerobically and anaerobically grown *Salmonella* serovar Typhimurium DT104 were assessed. The genes significantly higher expressed in either the anaerobic or aerobic condition are depicted in a Venn diagram (Fig. 1). From the 425 genes present on the microarray, 303 genes were expressed at similar levels in both conditions. Under the anaerobic condition 49 genes were higher expressed, whereas 73 genes were higher expressed under the aerobic condition (see supplementary material of Chapter 6 for functions and descriptions of the genes present on the microarray).

The oxidative stress induced genes (*fhuA, sodA*) and the important stress regulators encoding genes *fur, ompR*, and *rpoS* were higher expressed under aerobic growth conditions. Under anaerobic conditions, other general stress induced genes, including the universal stress proteins (*usp*) and paralogues encoding genes *uspA*, *uspF* (*ynaF*), and *uspG* (*ybdQ*) that have functions in the protection of DNA (39), and *yfiA* that stabilizes ribosomes against dissociation (42, 67), were expressed at higher levels. The stress response involved genes encoding chaperone functions and of the functional group of heat shock proteins (*clpB*, *dnaJ*, *dnaK*, *grpE*, *htpG*, *mopA*, and *mopB*) showed a higher expression in anaerobically grown cells, although other chaperones (*cspE* and *ppiC*) were higher expressed aerobically grown cells.

The virulence associated lipopolysaccharide (LPS) synthesis and assembly encoding genes (18, 53) (*lpxD, oafA, and rfa* and *rfb* genes) were higher expressed under aerobic conditions. Finally, the SPI-1 virulence genes required for type III secretion (TTS)-mediated invasion of epithelial cells (*inv*, *prg*, and *sip* operons) (20, 27, 43) were higher expressed anaerobically and SPI-2 virulence genes involved in survival and replication in host phagocytes (27, 43) were expressed at similar levels under both conditions.

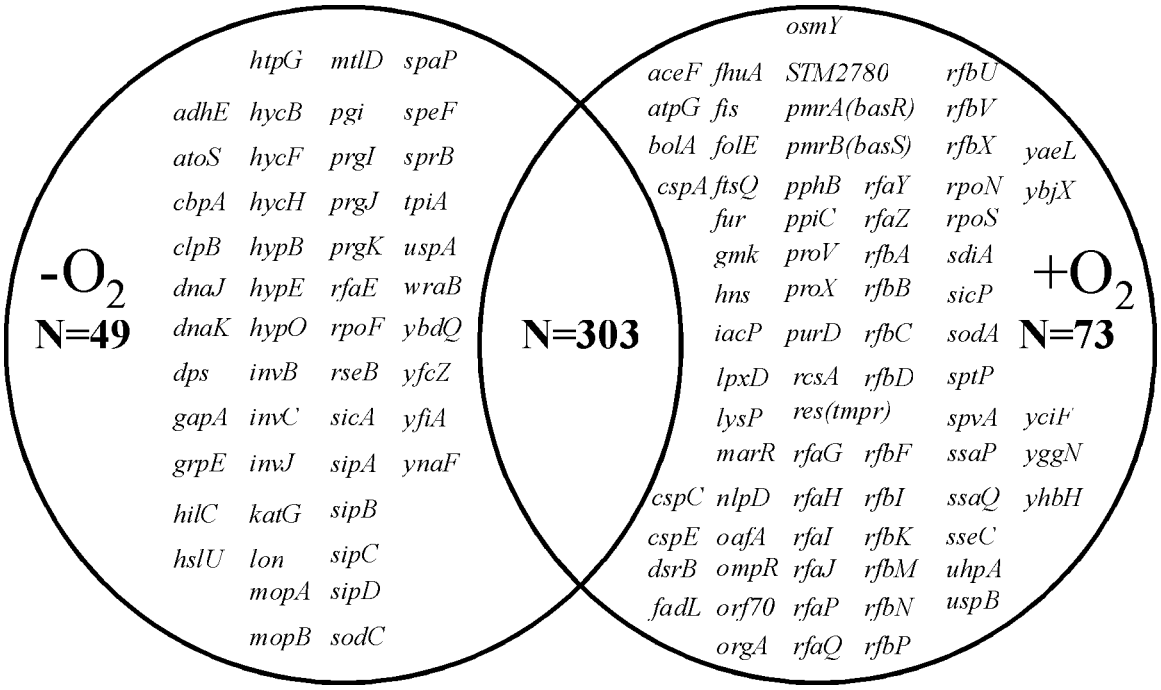

FIG. 1. Venn diagram of 425 stress and virulence genes of *Salmonella* serovar Typhimurium DT104 indicating the genes higher expressed in exponential cells grown under anaerobic (49 genes) or aerobic (73 genes) conditions. Additionally, 303 genes were expressed at similar levels under both conditions. Alternative gene names between parentheses behind the gene name corresponding with.

Comparative transcriptome analysis between cells exposed to heat, oxidative or acid stress under anaerobic and aerobic conditions.

The thematic microarrays were used additionally to determine the gene expression profiles of aerobically and anaerobically grown *Salmonella* serovar Typhimurium DT104 cells exposed for 10 minutes at 44 ^{o}C or with 0.1 mM hydrogen peroxide or at pH 5.0, under aerobic or anaerobic conditions, respectively. The number of genes exhibiting a significant induction or repression under the various stress conditions are depicted in Table 1. Of the 425 genes present on the microarray, 11.3%, 27.1%, and 21.4% was induced in the anaerobic heat, oxidative, and acid stressed cultures, while 8.0%, 20.9%, and 16.9% was repressed, respectively. Under aerobic conditions 22.1%, 19.1%, and 18.6% of the genes were induced and 15.1%, 16.2%, and 18.1% repressed under heat, oxidative, and acid stress conditions.

Table 1. Percentage of stress and virulence genes (100 % = 425 genes)
differentially expressed under specific growth and stress conditions

	Heat (37 → 44 ^{o}C)		H_2O_2 (0.1 mM)		Acid (pH 5.0)	
	-O_2	+O_2	-O_2	+O_2	-O_2	+O_2
Upregulated	11.3	22.1	27.1	19.1	21.4	18.6
Downregulated	8.0	15.1	20.9	16.2	16.9	18.1
Total	19.3	37.2	48.0	35.3	38.4	36.7

(i) Stress genes under anaerobic conditions. An overview of the expression of stress response genes during exposure to heat, oxidative and acid stress under anaerobic conditions is shown in Fig. 2. Specific responses and correlations under these conditions will be highlighted. Acid-induced expression of arginine and lysine decarboxylases (*adiA*, *cadA* and *cadB*) was observed, whereas the corresponding regulator *lysP* (= *cadR*) that represses *cadAB* expression was downregulated as reported previously (41, 50). In addition, for these two decarboxylases only *adiA* upregulation was observed under H_2O_2 stress and the correlation of *lysP* downregulated by H_2O_2 stress resulting in a *cadAB* upregulation under H_2O_2 stress was also not observed. The induction of *adiA* under both acid and H_2O_2 stress might result in a lower arginine concentration explaining the decreased expression of *carA* and *carB* in either one of the stress conditions, since *carAB* is regulated by arginine (36). The *fur* gene encoding the ferric uptake regulator and the Fur-regulated genes (*fhuA*, *sitABCD* and *tonB*) were H_2O_2 induced. Under these anaerobic conditions, the oxidative stress induced genes (*katG*, *oxyR*, *oxyS*, *xthA*, *sodA* and *sodC*) were all H_2O_2 induced, with *sodA* and *sodC* additionally acid-induced. Chaperones of the peptidyl-prolyl cis-trans isomerase-type (*fklB*, *fkpA*, *ppiA*, *ppiC*, *surA* and *tig*) and heat shock chaperones (*clpB*, *dnaK*, *grpE*, *hscA*, *htpG*, *mopA*, *mopB* and *mreB*) were differentially expressed under the various stress conditions. Furthermore, genes of the extracytoplasmic stress RpoE regulon (*rpoE*, *fkpA*, *htrA*, *cpxP* and *rseABC*) (13) were induced by acid stress, with *cpxP*

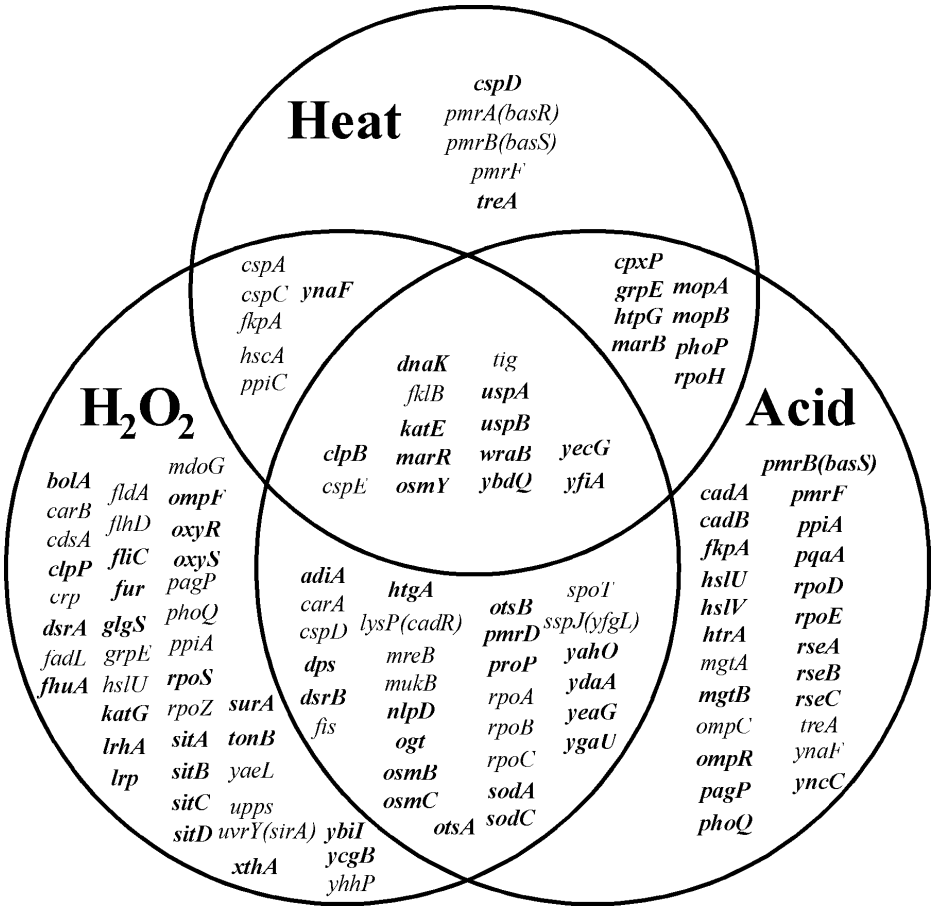

FIG. 2. Venn diagram of stress genes differentially expressed in *Salmonella* serovar Typhimurium DT104 stressed under anaerobic conditions. Alternative gene names between parentheses behind the gene name corresponding with. Genes induced are boldfaced, while repressed genes are not boldface. See text for details.

additionally heat-induced and with *fkpA* heat and H_2O_2-repressed. Our study revealed an induction of the heat stress sigma factor gene *rpoH* by heat and acid stress. In addition, the group of universal stress proteins encoding genes (*uspA*, *uspB*, *uspC* (=*yecG*) and *uspG* (=*ybdQ*)) and *yfiA* were induced by all three stresses under anaerobic conditions. The general stress regulator RpoS encoded by *rpoS* and the RpoS-regulated genes (*bolA*, *dps*, *dsrB*, *glgS*, *katE*, *katG*, *ogt*, *osmB*, *osmC*, *osmY*, *otsA*, *otsB*, *proP*, *rpoS*, *sodC*, *xthA*, *wraB*, *yahO*, *ycgB*, *yeaG*, *ygaU* and *yncC*) (22, 28, 33) were mainly induced by acid and H_2O_2 stress. Finally, most of the genes of the PhoPQ stress regulon (*phoPQ* and PhoPQ-regulated genes *mgtA*, *mgtB*, *pagP*, *pqaA*, and the *pmr* genes (54)) that is amongst others involved in intracellular survival (24, 26) and that has been suggested to affect SPI-1 expression (7, 51), appeared to be acid-induced under anaerobic conditions. However, the *pmr* genes that have been suggested to be under control of PhoPQ (23) were heat repressed in contrast to the other PhoPQ-regulated genes.

(ii) Stress genes under aerobic conditions. Under aerobic stress conditions (Fig. 3), a repression of *lysP* (*cadR*) and induction of decarboxylases and repression of *carAB* was observed as also observed under anaerobic stress conditions, although the genes were more induced or repressed anaerobically (see supplementary material of Chapter 6). For example, the *adiA* gene that encodes the arginine decarboxylase was 10 to 20-fold higher expressed in

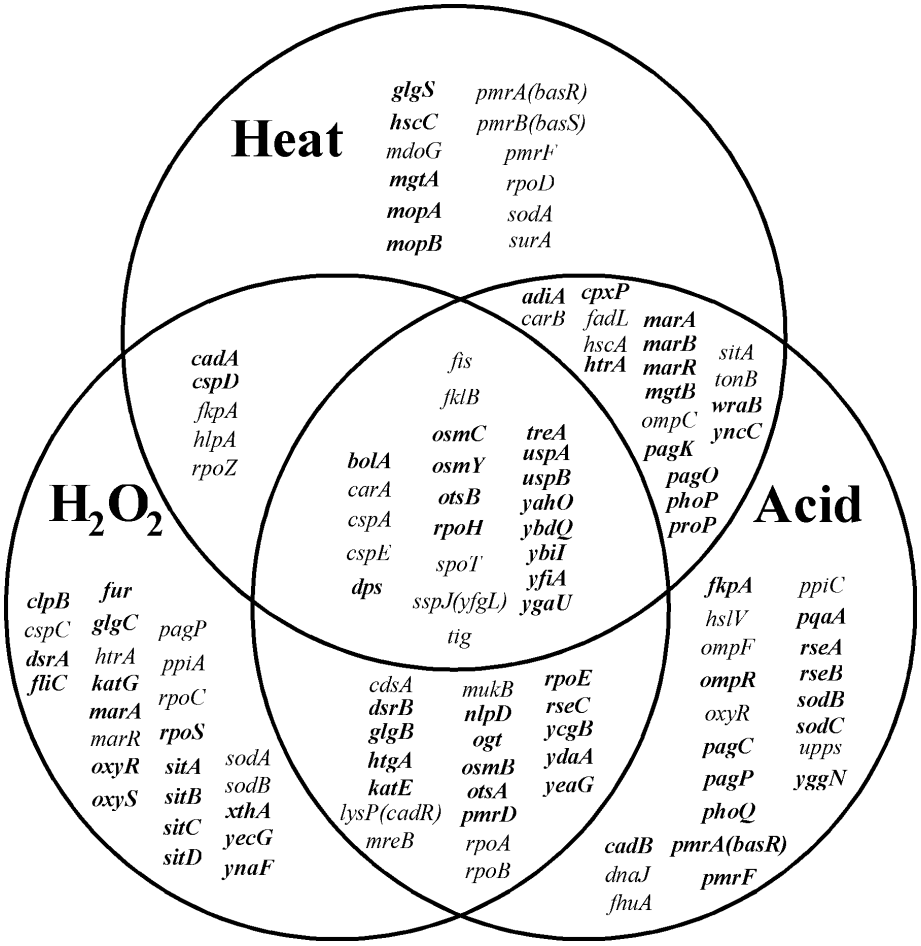

FIG. 3. Venn diagram of stress genes differentially expressed in *Salmonella* serovar Typhimurium DT104 stressed under aerobic conditions. Alternative gene names between parentheses behind the gene name corresponding with. Genes induced are boldfaced, while repressed genes are not boldface. See text for details.

anaerobic cells compared to aerobic cells during H_2O_2 and acid stress exposure. The *fur*, Fur-regulated and oxidative stress induced genes were H_2O_2 induced, as observed under anaerobic conditions. Notably, *fur*, *sitA* and *sodA* were 1.9, 9.9, and 44.2-fold higher induced in the anaerobically H_2O_2 stressed cells than in the aerobically H_2O_2 stressed cells. Furthermore, the RpoE regulon was also induced by acid stress under aerobic growth conditions. Finally, the heat stress sigma factor gene *rpoH* was induced by all three stresses under aerobic conditions. Repression or induction of the expression for the RpoS regulon, chaperones, universal stress proteins and *yfiA* genes and the PhoPQ regulon were fairly similar as was also observed with the anaerobically stressed cells.

(iii) Virulence genes under anaerobic conditions. An overview of the expression of virulence genes during exposure to heat, oxidative and acid stress under anaerobic conditions is shown in Fig. 4. The SPI-1 located genes (20, 27, 43) revealed a varied regulation under the three stress conditions. Most of the genes coding for proteins that form the secretion apparatus (*inv*, and *prg* genes) were repressed by heat. The SPI-1 transcription regulators were either acid (*hilAC* and *sprB*) or H_2O_2 (*hilD* and *invF*) induced and the genes coding for proteins secreted via the secretion apparatus (*sip* and *sop* genes) were H_2O_2 induced, while only the *sip* genes were acid repressed. However, expression of the SPI-1 genes appeared to be mainly induced by H_2O_2 under anaerobic conditions. In addition, the

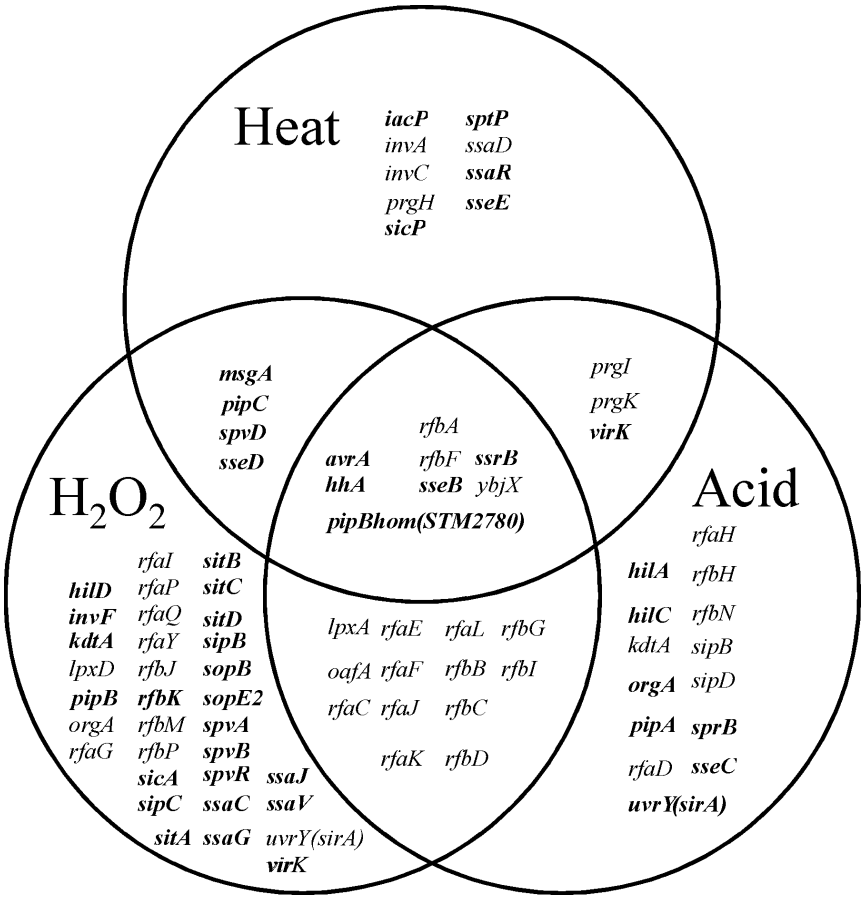

FIG. 4. Venn diagram of virulence genes differentially expressed in *Salmonella* serovar Typhimurium DT104 exposed to the stresses heat (44 ^oC), oxidative (H_2O_2), and acid (pH 5.0) under anaerobic conditions. Alternative gene names between parentheses behind the gene name corresponding with. Genes induced are boldfaced, while repressed genes are not boldface. See text for details.

cluster of the four *sitABCD* genes that is located at the border of SPI-1 and encodes an iron uptake system (70) that is not required for invasion (27), was also induced by H_2O_2, together with the plasmid encoded *Salmonella* plasmid virulence (*spv*) genes. The SPI-2 located genes (*ssa, sse* and *ssrB*) were predominantly heat and H_2O_2-induced. Finally, most of the LPS genes (*lpxA, oafA,* and the *rfa* and *rfb* genes) were repressed under acid and H_2O_2 stress, with the exception of the LPS gene *kdtA* whose expression was repressed by acid, but induced by H_2O_2 under anaerobic conditions.

(iv) Virulence genes under aerobic conditions. Under aerobic stress conditions (Fig. 5), the regulation of the SPI-1 and SPI-2 located genes was more obvious than under anaerobic stress conditions. Various SPI-1 located genes with different functions were aerobically induced by heat and repressed by acid and H_2O_2 and the SPI-2 located genes (*ssa*, *sse*, and *ssr* genes) were predominantly induced by heat. The expression of the *sitABCD* operon revealed to be induced by H_2O_2 as also observed under anaerobic conditions, while the SPI-1 genes were repressed by H_2O_2. Finally, the expression of the LPS genes decreased during exposure to the various aerobic stresses, while these genes were shown to be acid and H_2O_2-induced under anaerobic conditions.

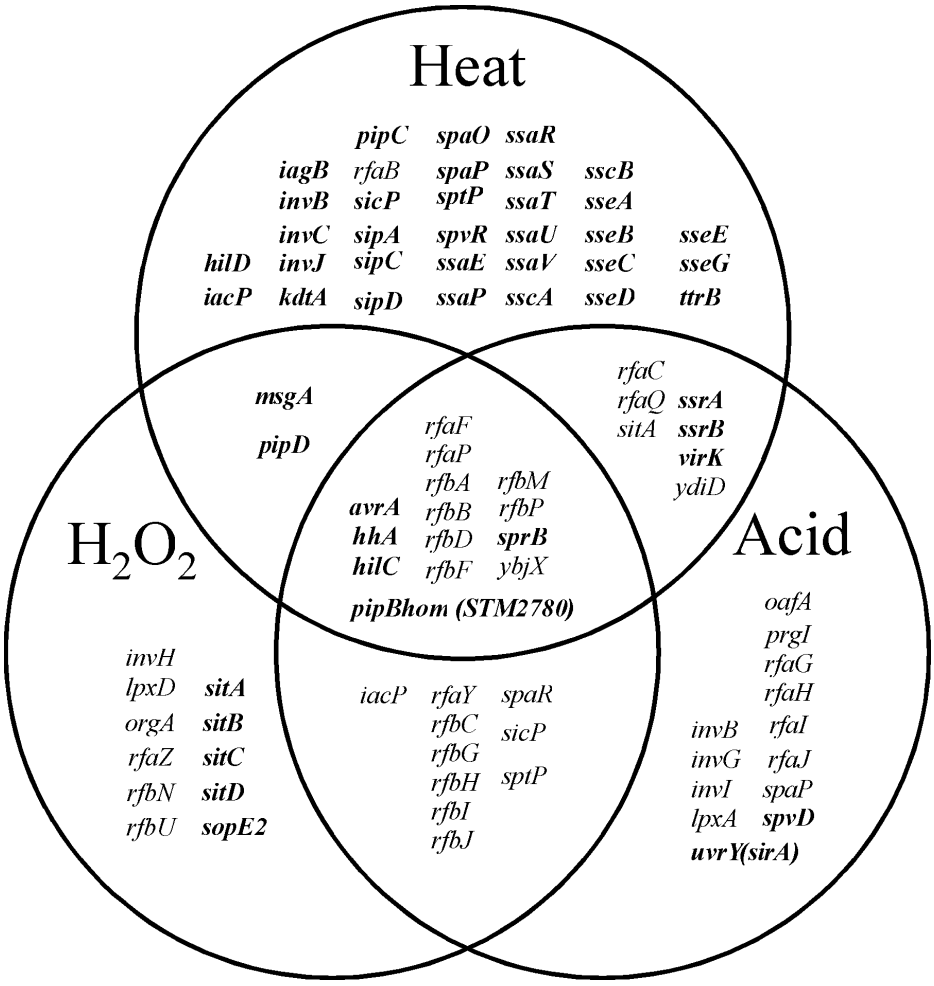

FIG. 5. Venn diagram of virulence genes differentially expressed in *Salmonella* serovar Typhimurium DT104 stressed under aerobic conditions. Alternative gene names between parentheses behind the gene name corresponding with. Genes induced are boldfaced, while repressed genes are not boldface. See text for details.

DISCUSSION

Comparative analysis of transcriptomes from anaerobically and aerobically grown *Salmonella* serovar Typhimurium DT104 cells of the exponential growth phase revealed that 49 and 73 of the 425 predominantly stress and virulence-related genes spotted on the thematic microarray to be significantly higher expressed under anaerobic or aerobic growth conditions, respectively. Apparently, under both growth conditions specific responses were already induced at the transcript level in these exponential cells involving both stress and virulence-related genes, which presets the stage for responses of these cells upon exposure to other stresses.

Subsequently, aerobically or anaerobically cultured *Salmonella* serovar Typhimurium DT104 exponential cells were exposed under aerobic or anaerobic conditions for 10 minutes at 44 ^{o}C or with 0.1 mM hydrogen peroxide or at pH 5.0, and the transcriptomes were compared. Although most of the *Salmonella* serovar Typhimurium transcriptomics research has been focused on stress adaptation and virulence factors (4, 5, 12, 15, 16, 19, 30, 34, 40, 46, 48, 49, 52, 57-60, 66, 68, 69), our present work is the first transcriptomics study of the emerging and multiple-antibiotic-resistant *Salmonella* serovar Typhimurium DT104 under various stresses and, moreover, under both aerobic and anaerobic conditions.

Higher inductions were observed for the *fur*, Fur-regulated and oxidative stress induced genes under H_2O_2 stress in anaerobically grown cells than in aerobically grown cells. Increased expression of the *sitABCD* and *fur* genes under H_2O_2 stress conditions is most likely associated with their function in protecting the cells against oxidative damage (54). Furthermore, the RpoE regulon was induced by acid stress under both anaerobic and aerobic growth conditions. RpoE has been previously reported to be activated in *Salmonella* serovar Typhimurium upon exposure to stresses such as high and low temperatures, antimicrobial peptides, and oxidative conditions, and in the stationary phase of growth (32, 35, 45, 61). However in our study, RpoE induction in DT104 was only observed upon exposure to low pH. The heat stress sigma factor gene *rpoH* was induced under all stresses except upon exposure to H_2O_2 under anaerobic conditions. Increased *rpoH* expression by acid stress may be explained by the acid-induced upregulation of the RpoE-regulon, since RpoE activates the expression of *rpoH* (13).

Several heat shock proteins were induced during incubation of cells at 44 ^{o}C, which is in agreement with the previously observed upregulation of these genes in *Salmonella* serovar Typhimurium at temperatures above 40 ^{o}C (54). However, these heat shock proteins were also induced by acid stress, which is also in line with the observation that heat shock proteins are essential for maintenance of protein quality under a wide range of stress conditions (54).

Exposure to stresses under anaerobic conditions, revealed expression of the various *Salmonella* serovar Typhimurium DT104 SPI-1 located genes (20, 27, 43) to be regulated differently. Most of the genes coding for proteins that form the secretion apparatus were repressed by heat, while different SPI-1 transcription regulators were either acid or H_2O_2 induced, and the genes coding for proteins secreted via the secretion apparatus were H_2O_2 induced or acid repressed. Under aerobic stress conditions, the regulation of these genes was more uniformous, since most of these genes were aerobically induced by heat and repressed by acid and H_2O_2. This suggest additional regulatory mechanisms to affect expression of the

various SPI-1 genes under anaerobic and aerobic stress conditions. Notably, only three virulence genes appeared to be induced under all anaerobic and aerobic stress conditions tested. The SPI-1 located gene *avrA* that encodes an effector protein involved in virulence, the *hha* gene encoding the hemolysin expression modulating protein that can repress SPI-1 gene expression via *hilA* (2, 17), and a homologue of *pipB* (pathogenicity island encoding protein), which mediates the formation of *Salmonella*-induced filaments in mammalian cells as a crucial step in the infection process of this bacterium (37, 38). In addition, expression of the LPS genes *rfbA* and *rfbF* and the gene *ybjX* that may be related to LPS production (47) is repressed under all stress conditions.

Finally, a comparison of our *Salmonella* serovar Typhimurium DT104 stress transcriptome data with transcriptome data of other *Salmonella* serovar Typhimurium strains (see Table 2) revealed that the general stress regulated RpoS regulon was induced under all stresses except in the selected heat stress condition. An induction of the PhoPQ regulon, which is involved in intracellular survival (24, 26), by cationic antimicrobial peptides (CAMP) as present in macrophages, by low pH and/or increased H^+ concentration has been reported (54). However, the low H_2O_2 concentration used in our work had no effect on the PhoPQ regulon expression in *Salmonella* serovar Typhimurium DT104 under both anaerobic and aerobic growth conditions. Although the *Salmonella* plasmid virulence (*spv)* encoding genes can be induced by various stresses (Table 2), we only identified a H_2O_2 induction under anaerobic conditions. This might be explained by the higher *rpoS* induction (4-fold) observed under anaerobic H_2O_2 stress (see supplementary material of Chapter 6) compared to the other five stresses (~1.3-fold induction), since RpoS regulates *spv* expression (11, 25). The LPS genes were mainly downregulated in aerobically grown cells and under the various anaerobic and aerobic stress conditions. Under the various aerobic stress conditions, SPI-1 was induced by an upshift of temperature, while reduced by bile, H_2O_2, CAMP or macrophage conditions. Downregulation of SPI-1 by the various stresses occurs most likely because SPI-1 is mainly involved in invasion and not in survival within the host e.g. in macrophages. Comparative transcriptome analysis of aerobically and anaerobically grown cells revealed SPI-1 located genes to be expressed at higher levels under aerobic conditions. Furthermore, expression of SPI-1 located genes was induced by oxidative stress under anaerobic conditions. Conceivably, the larger impact of the oxidative stress in anaerobic cells triggered SPI-1 expression. In addition, expression of SPI-2 was induced by H_2O_2 and high temperature under anaerobic conditions. Previously, upregulation of SPI-2 was reported to be induced by H_2O_2 under aerobic conditions in *Salmonella* serovar Typhimurium LT2 (19). Notably, in these experiments higher concentrations of H_2O_2 were used which may have induced oxidative stress in the aerobic cells.

In conclusion, transcriptome analysis using the developed thematic microarray has supplied novel information about the performance of aerobically and anaerobically grown *Salmonella* serovar Typhimurium DT104 cells subjected to different stresses and revealed a significant fraction of the selected stress and virulence genes to be differentially regulated under aerobic and anaerobic conditions.

Table 2. Overview of changes in gene expression of selected stress and virulence operons or regulons in response to various stresses and different *Salmonella* serovar Typhimurium strains[a]

Operons or regulons	DT104 (this study)						SL1344	14028(s)		LT2	
	Heat 37→44 °C		0.1 mM H_2O_2		pH 5.0		Within Macrophage (15)	CAMP[b] (4)	3% Bile (50)	Heat 25→37 °C (47)	2 mM H_2O_2 (18)
	$+O_2$	$-O_2$	$+O_2$	$-O_2$	$+O_2$	$-O_2$	$+O_2$	$+O_2$	$+O_2$	$+O_2$	$+O_2$
RpoS	o	o	+	+	+	+	+	+		+	
PhoPQ	+/-[c]	o/-[c]	o	o	+	+	-	+		+	-
Spv	o	o	o	+	o	o	+	+		+	
LPS	-	o	-	-	-	-	-	o		-	+
SPI-1	+	o	-	+	-	o	-	-	-	+	-
SPI-2	+	+	o	+	o	o	+	o		o	+

[a] References between parentheses; +/-, increased/decreased expression compared to nonstress condition; o, no difference compared to nonstress condition.
[b] CAMP, cationic antimicrobial peptides
[c] PhoPQ regulon was induced or equally expressed as depicted, except the *pmr* genes that were repressed, see text for further explanation.

ACKNOWLEDGEMENTS

This study was supported by the Netherlands Organization for Health Research and Development, program “Nutrition: Health, Safety and Sustainability”.

We are grateful to Marco de Boer of Isogen Life Science for his support and advice during microarray construction.

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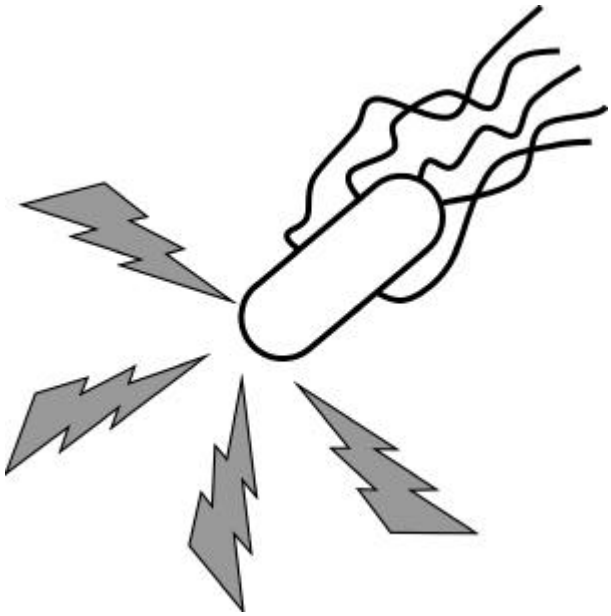
Chapter 7

Summary, concluding remarks and future perspectives



This thesis describes the identification of novel *Salmonella* serovar Typhimurium phage type DT104 sequences and their distribution amongst other phage types. The analysis of expression of stress response and virulence genes in this pathogen under various growth and stress conditions is also part of this thesis. *Salmonella* serovar Typhimurium DT104 was studied because during the past decades the number of infections caused by isolates of serovar Typhimurium has increased in many parts of the world. Additionally phage type DT104 is multiply-antibiotic-resistant and has been generally recognized as an emerging food-borne pathogen (8, 10, 20). In The Netherlands, the percentage of DT104 isolates out of all *Salmonella* serovar Typhimurium isolates from human origin, increased from 7 % in 1990-1995 to 29% in 1996-2001 (23), and to 32 % in 2002-2005 (RIVM, The Netherlands). The molecular approaches to identify novel DT104 sequences and to asses gene expression will be discussed below including the most important results obtained. In addition, we present perspectives for future research and discuss possible applications of the obtained results and insights.

Identification and distribution of novel *Salmonella* serovar Typhimurium DT104-specific sequences

Genomic DNA of the sequenced *Salmonella* serovar Typhimurium LT2 strain (12), was subtracted from genomic DNA of a *Salmonella* serovar Typhimurium DT104 isolate to obtain DT104-specific sequences by using a molecular method called genomic subtractive hybridization (**Chapter 2**). The same principle has been often used to find condition-specific mRNAs in human cell line studies. We applied this method to search for novel *Salmonella* serovar Typhimurium DT104 sequences that could encode genes that may play a role in stress response or virulence and were not present in the genome of *Salmonella* serovar Typhimurium LT2. The subtraction resulted predominantly in the isolation of DNA fragments with sequence similarity to phages ST64B, ST104 and P27. In addition, two fragments obtained were associated with possible virulence factors: one fragment identical to *irsA* of *Salmonella* serovar Typhimurium ATCC 14028, which is suggested to be involved in macrophage survival (2) and another fragment homologous to HldD, an *Escherichia coli* O157:H7 protein involved in a specific lipopolysaccharide (LPS) assembly pathway (11, 22). To find out whether these fragments were DT104-specific, both the *irsA* and HldD homologue fragments and three other fragments with sequence similarity to prophages, were tested for their presence in 17 DT104 and 27 non-DT104 isolates by PCR. Notably, the sequence of the three other prophage fragments were not found in any of the publicly available *Salmonella* genomes. All five DNA fragments selected revealed to be *Salmonella* serovar Typhimurium DT104-specific and the detection of these DNA fragments can be useful for better detection and typing of *Salmonella* serovar Typhimurium DT104.

Based on the genomic subtractive hybridization fragments, new prophage sequences in public databases, and the availability of an unfinished genome sequence of a *Salmonella* serovar Typhimurium DT104 isolate (Sanger Institute) at a later stage in our research, we were able to identify complete DT104-specific prophages instead of only small prophage fragments as mentioned above. The prophages identified were prophages ST104 and ST64B, and a novel prophage remnant that we designated prophage ST104B that harbors the HldD homologue. The distribution of these three DT104 prophages and prophages

Gifsy-1, Gifsy-2, Fels-1, and Fels-2 that were identified in the genome of *Salmonella* serovar Typhimurium LT2, was tested among 19 DT104 and 23 non-DT104 isolates (**Chapter 3**). Notably, additional typing of all isolates tested with the use of the English phage-typing system and the increased insights of us into the Dutch and English phage-typing systems, resulted in the identification of the DT104 subtypes DT104A, DT104B low and DT104L, and the DT104-related phage type U302 within the isolates tested. Furthermore, the presence of the common DT104 antibiotic resistance genes for penta-resistance type ACSSuT, *aadA2*, *floR*, *pse-1*, *sul1*, and *tet(G)* (3, 4, 20), was also studied. Based on differences in prophage presence within their genome, we could divide the isolates in 12 groups. Although no clear relationship was found between different phage types and prophage presence, we could discriminate the different DT104 subtypes based on absence or presence of prophages ST104, ST104B, and ST64B. The novel HldD homologue containing ST104B prophage remnant was identified only in the 14 DT104L isolates and in the DT104-related U302 isolate.

In conclusion, we observed that the presence of the genes for penta-resistance type ACSSuT, the HldD homologue containing ST104B prophage remnant and phage type DT104L revealed to be common features of the emerging subtype of *Salmonella* serovar Typhimurium DT104.

Stress response and virulence transcriptomics for *Salmonella* serovar Typhimurium DT104

To be able to study expression of genes involved in stress response and virulence, a thematic oligo microarray for *Salmonella* serovar Typhimurium DT104 was developed (**Chapter 4**) based on the genome of *Salmonella* serovar Typhimurium LT2 (12) and supplemented with *Salmonella* serovar Typhimurium DT104 genes described, such as the antibiotic resistance genes for penta-resistance type ACSSuT (3, 4, 20). In total, 425 genes were selected of which the majority was involved in stress response and virulence, and oligos were designed by using the software program OligoFaktory. By hybridizing genomic DNA of a LT2 and DT104 isolate to the thematic microarray and comparing these results, we concluded that all oligos designed based on the LT2 genome sequence were also applicable for DT104. In addition, several different microarray parameters were varied, which revealed that using epoxy-coated microarrays and sodium phosphate-based spotting buffer resulted in the most sensitive microarrays allowing the detection of more low-level expressed genes. Furthermore, the expression of the genes encoding six different universal stress proteins and paralogues and the five plasmid-located virulence genes *spvRABCD* was assessed in response to starvation stress during growth at pH 5.0 and pH 7.0 and revealed that the responses of these genes were similar at both pHs. Finally, the results for the development and assessment steps mentioned above showed that our thematic microarray is ready-to-use to study the expression of stress and virulence genes in *Salmonella* serovar Typhimurium DT104.

This stress and virulence thematic microarray developed was now used in two different types of expression studies. Firstly, gene expression was measured in the course of time during growth for a *Salmonella* serovar Typhimurium DT104 wild type strain and a *luxS* deletion mutant strain (**Chapter 5**). Secondly, gene expression was measured for a

Salmonella serovar Typhimurium DT104 wild type strain cultured under aerobic and anaerobic conditions and subjected to heat, oxidative, and acid stress (**Chapter 6**).

The transcriptomes obtained for *Salmonella* serovar Typhimurium DT104 wild type and its *luxS* deletion mutant were compared at various growth stages. LuxS was studied because LuxS might play a role in stress response and virulence in *Salmonella* serovar Typhimurium, since in several other bacterial species *luxS*-mediated regulation has been observed on biofilm production, motility, iron acquisition, or virulence factors (reviewed in reference 24) and this information is rather limited for *Salmonella* serovar Typhimurium (1, 16, 17, 18). When studying the wild type, its expression of stress response and virulence genes in the course of time appeared to be largely growth-phase-dependent. For example, the genes under control of the general stress sigma factor RpoS, the *rpoS* gene itself and the genes encoding universal stress proteins revealed the highest expression levels in the stationary growth phase, while the genes coding for proteins involved in repressing RpoS production (H-NS, Fis, Lrp, and Hfq) were lower expressed at the stationary phase compared to the exponential phase. The invasion genes located at *Salmonella* pathogenicity island-1 (SPI-1) showed the highest expression in the end-exponential growth phase which correlated with the higher Caco-2 invasion capacity of DT104 cells from this growth phase compared to that of cells from other growth phases. The most prominent affect of deleting the *luxS* gene, when comparing the transcriptomes of the wild type and *luxS* deletion mutant strains, was the increased expression of 15 LPS synthesis and assembly genes at the end-exponential growth phase. At this stage of growth a higher expression was observed for the periplasmic stress sigma factor encoding gene *rpoE* and genes under control of RpoE: several of the LPS genes mentioned above, chaperones encoding genes and the heat stress sigma factor encoding gene *rpoH*. Notably, the *luxS* deletion mutant showed higher adhesion and invasion capacity into Caco-2 cells, although the expression levels of the SPI-1 invasion genes appeared to be similar to that in the wild type. We concluded that the loss of *luxS* results in overexpression of LPS genes and most likely also the LPS molecules, thereby affecting *in vitro* virulence characteristics of this DT104 *luxS* deletion mutant.

By using the thematic microarray, transcriptomes of *Salmonella* serovar Typhimurium DT104 grown under aerobic and anaerobic conditions and additionally during exposure to heat, hydrogen peroxide or low pH stress for both growth conditions were assessed. The genes coding for the stress transcriptional regulators Fur, OmpR, and RpoS and oxidative stress response genes were higher expressed under aerobic conditions when not exposed to one of the stresses. In the anaerobic nonstress condition, the genes encoding universal stress proteins and heat shock chaperones were higher expressed. The virulence LPS and SPI-1 genes were higher expressed aerobically. In addition, when the aerobically or anaerobically grown cells were subjected to the different stresses, stress genes such as the RpoS and PhoPQ regulon, chaperones and universal stress proteins were mainly induced or repressed in a similar manner in both types of cells. Furthermore, the virulence(-associated) LPS, PhoPQ, *Salmonella* plasmid virulence, SPI-1, and SPI-2 genes were differentially regulated by the different stresses. The thematic microarray developed allows assessment of the impact of stresses and combinations thereof on the expression of stress and virulence genes for *Salmonella* serovar Typhimurium DT104.

The HldD homologue encoding prophage remnant ST104B

In this paragraph we discuss some additional research results and insights that have not been addressed in the previous chapters, to further characterize the possible function of the HldD homologue. This *Salmonella* serovar Typhimurium DT104L-specific HldD homologue which is located on prophage remnant ST104B might be described as a moron. A moron should be an additional gene located on a prophage which does not have a phage function but may act as fitness factors for the bacterium. We propose that the HldD homologue might be a moron, because changing the LPS of the bacterium, might increase for example survival of stress conditions inside a host and/or its virulence potency. In addition, the HldD homologue as being a moron should be expressed only under very specific conditions to be uniquely beneficial (5). Therefore we initiated additional research to study the role of the HldD homologue.

Firstly, we compared the genome locus of prophage remnant ST104B within the genome of *Salmonella* serovar Typhimurium DT104 with the same genome loci of other *Salmonella* strains (Fig. 1). For this comparison, we used the publicly available sequences of *Salmonella* serovar Typhimurium LT2 (12), *Salmonella* serovar Typhi Ty2 which is a laboratory strain (6) and *Salmonella* serovar Typhi CT18 which is multiply-antibiotic-resistant (13) and the unpublished but finished genome sequences of *Salmonella* serovar Typhimurium DT104 and *Salmonella bongori*, which is a non-*enterica* strain. As mentioned in Chapter 3, the HldD homologue encoded prophage remnant ST104B was found in *Salmonella* serovar Typhimurium DT104 adjacent to the putative integrase encoding gene STM1871, when compared to the *Salmonella* serovar Typhimurium LT2 genome. In

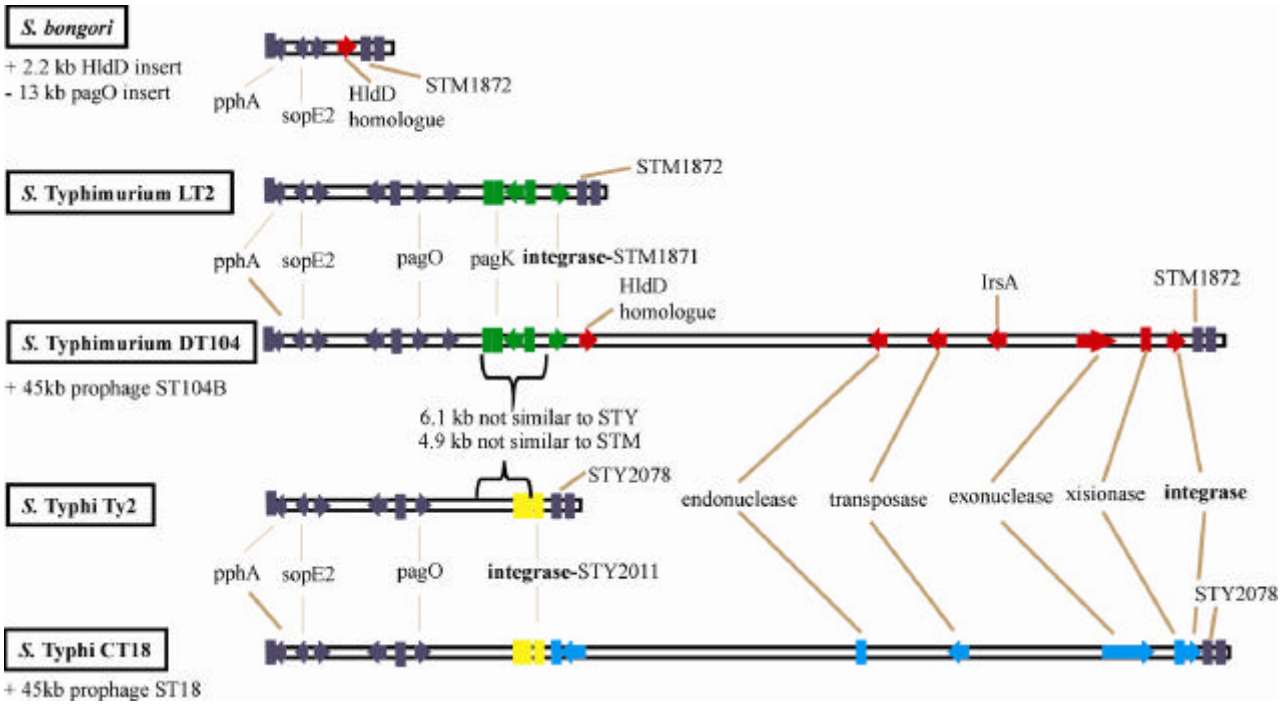


FIG. 1. Genome comparison based on sequence homology of the HldD encoded prophage DT104B for *Salmonella bongori*, *Salmonella* serovar Typhimurium LT2 and DT104 and *Salmonella* serovar Typhi Ty2 and CT18. Notably both LT2 and Ty2 are typical laboratory strains, while both DT104 and CT18 are multiple-antibiotic-resistant and emerged in the past decades. Grey, similar in all strains; green, similar in Typhimurium; yellow, similar in Typhi; red, prophage DT104B-specific; light blue, prophage ST18-specific.

addition to DT104, the HldD homologue sequence was found in the genome of *Salmonella bongori*. The amino acid sequence of the HldD homologue in *S. bongori* was highly similar to the DT104 sequence. The genome loci of these HldD homologues for both strains were also similar (between *sopE2* and STM1872), although many genes surrounding the HldD homologue in DT104 were not found in *Salmonella bongori*. Interestingly, a different prophage called prophage ST18 (19) was present in *Salmonella* serovar Typhi CT18 at the same locus in the genome as prophage remnant ST104B in DT104, when we compared the two *Salmonella* serovar Typhi strains. Both prophage ST18 and prophage remnant ST104B harbor genes of similar functions and which are typical bacteriophage functions such as an endonuclease, a transposase and an integrase, although only little sequence homology occur between both prophages as indicated by the different colors in Fig. 1. Notably, the locus adjacent of prophages ST18 and ST104B also do differ around 5-6 kb between serovar Typhimurium and Typhi as depicted in green and yellow colors in Fig. 1.

Because the total gene homologues to HldD was fully present in *Salmonella* serovar Typhimurium DT104, we were interested if this gene was active and if deleting this gene revealed any effects on typing or virulence. We could not detect expression of the HldD gene in our different transcriptomics experiments performed by using the thematic microarray developed, indicating no functional gene or low expression of this gene under the conditions tested. The HldD homologue may be involved in LPS assembly resulting in modification of the LPS structure and/or architecture. Differences in LPS can affect *Salmonella* sero- and phage-typing (see Chapter 1) and since LPS can induce an immune response of the host (7, 14) a different LPS might result in an altered virulence potency. In addition, changes in expression of the LPS genes already showed an effect on the *in vitro* virulence potency as presented in Chapter 5 of this thesis. Therefore, wild type and HldD deletion mutant cells of different stages of growth in LBG medium were added to Caco-2 cells and the number of DT104 cells that adhered and invaded the Caco-2 cells were determined using the approaches mentioned in Chapter 5.

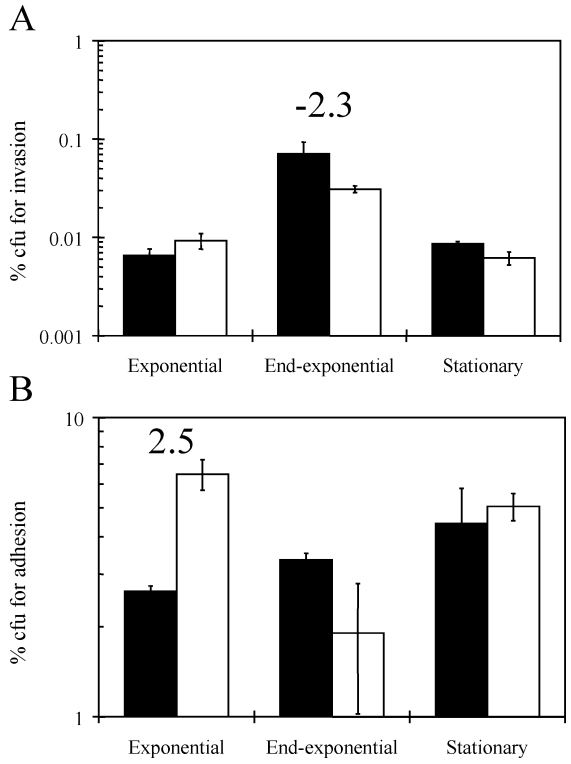


FIG. 2. *In vitro* virulence assays A) invasion, B) adhesion and results for the *Salmonella* serovar Typhimurium DT104 wild type (black bars) and HldD homologue deletion mutant (white bars) cultured in LBG pH 7.0 medium. Bacterial cells of the exponential, end-exponential, and stationary growth phase were used in these *in vitro* virulence assays. Numbers above the bars indicate the ratios between wild type and HldD homologue deletion mutant, only in case of a significant difference.

The *Salmonella* serovar Typhimurium DT104 HldD deletion mutant constructed, remained to be typed as phage type DT104 (data not shown) and revealed a 2.3-fold lower invasion than the wild type for end-exponential cells (Fig. 2), implicating a role for the HldD homologue in virulence.

However, a 2.5-fold higher adhesion was observed for exponential cells of the HldD deletion mutant compared to the wild type. Further experiments are required to assess the possible role of the HldD homologue in *Salmonella* serovar Typhimurium DT104 virulence in more detail. Interestingly, the impact of deleting the HldD homologue on invasion was only observed with cells harvested at the end-exponential growth phase. This points to a moron role of this gene, since a moron should be expressed under very specific conditions to be beneficial (5).

In addition, we studied the expression of the gene encoding the HldD homologue relative to the housekeeping gene *tpiA* by qPCR because qPCR is a more sensitive method to detect gene expression compared to the microarray. The RNA samples of the stress conditions mentioned in Chapter 6, that mimic stress conditions possibly encountered during interaction with the host, were used for this qPCR analysis (Table 1). These preliminary qPCR results indicate that expression of the gene coding for the HldD homologue is detectable under all growth/stress conditions and is specifically induced under oxidative stress, while repressed by acid. These experiments show a specific stress-induced expression of the HldD homologue and thus again underlie the possible moron role for this gene (5). Inside the host *Salmonella* serovar Typhimurium DT104 can be exposed to oxidative stress for example inside the macrophage or in the ileum in the GI-tract. Activation of the HldD homologue might change the LPS structure of *Salmonella* serovar Typhimurium DT104 enhancing its resistance to these specific stresses. Notably previous work of others revealed LPS to mediate resistance to bile (15).

TABLE 1. Relative HldD homologue mRNA levels (*hldD*/*tpiA*) under the various culture or stress conditions

Condition	Aerobically	Anaerobically
Nonstress mid-exp.	0.30 (± 0.06)	0.20 (± 0.04)
Nonstress mid-exp. + 10 min.	0.25 (± 0.04)	0.20 (± 0.01)
Heat stress (44 ^{o}C)	0.36 (± 0.07)	0.58 (± 0.04)
Oxidative stress (0.1 mM H_2O_2)	1.47 (± 0.17)	0.72 (± 0.20)
Acid stress (pH 5.0)	0.12 (± 0.03)	0.16 (± 0.01)

[a] standard deviation between parenthesis

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

When starting with this research, microarrays had to be developed for *Salmonella* serovar Typhimurium DT104. As described in Chapter 4, many different parameters can affect the final results obtained from microarray experiments. We noticed that sensitivity of the microarray is a very important parameter to obtain expression results for most of the genes studied. The design of oligos that are gene specific and do not show a high level of cross-hybridization by *in silico* analysis, is essential for the construction of high quality microarrays. In recent years the number and quality of commercially available microarrays has increased significantly, although the use of these arrays is still costly.

LPS has been recognized to play important roles in pathogen survival and virulence. In chapter 2 and 3 a putative LPS assembly-involved HldD homologue was identified as *Salmonella* serovar Typhimurium DT104-specific that might affect DT104 virulence as

shown in the current chapter. In Chapter 5 large differences were identified in gene expression of almost all LPS genes if *luxS* was deleted, although in a specific phase of growth only. Finally, in Chapter 6 when DT104 cells were subjected to various stresses the expression of the LPS genes was repressed. Furthermore, expression of virulence genes revealed to be regulated or activated under specific conditions. For example expression of the SPI-1 invasion virulence genes had the highest expression during growth at the end-exponential growth phase and was under aerobic conditions induced by heat stress, while repressed by H_2O_2 and acid, and under anaerobic conditions only induced by H_2O_2.

The distribution of prophages in genomes of different *Salmonella* serovar Typhimurium isolates revealed to allow discrimination between phage types. The current phage-typing method performed in *Salmonella* typing laboratories often results in different phage types for the same *Salmonella* serovar Typhimurium isolate or the isolates reveal to be nontypeable. Furthermore, the technicians have to be well trained to interpret the results obtained and the method itself is hard to standardize (9). Therefore, as was stressed before, a molecular method should be developed to replace conventional phage typing (9, 21). A reason that different phage type results can be obtained for the same isolate is the activation of one or more prophages, which may result in an exclusion of this prophage from the bacterial genome. This may also result in loss of prophage-located genes that are not necessary for the bacteriophage machinery and encode for example for bacterial cell wall-related proteins that subsequently may affect the bacteriophage recognition sites on the outside of the bacterial cell. This phenomenon can finally result in a different lysis-pattern in the phage typing method. Therefore, we suggest to include an additional screening of strains by PCR for prophage presence, or to use this as an alternative for phage typing. Molecular prophage profiling of strains can supply an overall picture of prophages and prophage remnants present, and may provide a basis for assessment of correlations between prophages and phage types. Additionally, a PCR-based method for phage typing would be easier to implement and to maintain in laboratories and the results obtained are easier to interpret compared to traditional phage-typing. Moreover, differences in prophage presence can give additional information of the virulence potency of a strain. To make the PCR-based prophage detection approach we presented here more applicable for routine-analysis, allowing larger numbers of samples to be assessed and larger numbers of prophages to be screened, a thematic microarray could be developed containing a large number of prophage sequences. We have obtained reliable results for presence or absence of sequences in a genome (see SGI-1 antibiotic resistance genes in Chapter 4) by using the microarray technology. Other multiplex methods for prophage detection such as the luminex method are also feasible. To our opinion, the use of molecular prophage detection has future potential for a better and faster typing method allowing discrimination between a large variety of different *Salmonella* serovar Typhimurium isolates. If such a method would be available, changes in the genetic make-up of isolates e.g. during an outbreak or as detected in a factory can be easily monitored.

According to our preliminary results for the HldD homologue of *Salmonella* serovar Typhimurium DT104, we recommend future research on the function of the HldD homologue within the LPS assembly and to reveal its role in virulence. Culturing wild type and HldD homologue deletion mutant cells under different conditions, such as the stress conditions mentioned in Table 1, and subsequently assessing the *in vitro* and/or *in vivo*

virulence would increase our knowledge about the regulation and function of this HldD homologue. Moreover, its role in modifying LPS structure remains to be established by structural chemical analysis. The fact that the HldD homologue is detected mainly in the emerging DT104 subtype DT104L, that also harbors the genes for penta-resistance type ACSSuT, would suggest that the HldD homologue may have played an additional role in the emergence of this *Salmonella* serovar Typhimurium variant.

The deletion of *luxS* had effects on the expression of predominantly (outer) membrane protein encoding genes, such as several stress response chaperones encoding genes that function in the folding of new proteins or re-folding of misfolded proteins and virulence genes involved in LPS assembly. Deletion of *luxS* in other bacterial species revealed also effects on expression of (outer) membrane proteins which affected various processes including biofilm formation, motility, and iron acquisition (reviewed in reference 24). Furthermore, the deletion of *luxS* affected *Salmonella* serovar Typhimurium DT104 *in vitro* virulence potency. The gene expression effects suggest that LuxS may have a role in virulence and the expression of chaperones genes directly. However, overexpression of the chaperones encoding genes observed in the *luxS* deletion mutant may also be a secondary effect, since accumulation of high levels of LPS may have resulted in increased levels of misfolded LPS assembly-involved proteins. Therefore, more research has to be performed to assess which genes are under direct control of LuxS in *Salmonella* serovar Typhimurium DT104 and to elucidate the exact role of quorum sensing via AI-2/*luxS* and its impact on cell functioning.

Analysis of gene expression revealed a subset of stress response genes to be induced by the different stresses used in our studies. It remains to be established whether one or more of these genes have an essential role in survival and/or DT104 virulence. If this is the case, inactivation of these specific genes may allow for the construction of an attenuated *Salmonella* serovar Typhimurium DT104 strain to be used for vaccination against salmonellosis, since life vaccines may be more effective than vaccines based on killed virulent strains (www.safe-poultry.com/vaccines.asp). In addition, using the thematic stress response and virulence microarray, transcriptome profiling of *Salmonella* isolates grown under various growth conditions, including those mimicking conditions in the host, may provide information about their virulence potential. Finally, the total overview of which stress response genes are induced under what stress condition also adds information to the current knowledge on stress adaptation capacity and development of resistance to other stresses i.e. cross-protection. For example, cells adapted to acid may be resistant also to heat or oxidative stress, while heat and oxidative stressed cells are not resistant to acid (15). Our transcriptome stress analysis revealed also that aerobic conditions induced transcription of the SPI-1 invasion virulence genes and an additional induction of these genes was observed upon exposure to mild heat shock conditions i.e. exposure of cells grown at 37 ^{o}C to 44 ^{o}C. In conclusion, the results obtained in this study, together with the developed tools, should supply a solid basis for assessment of the impact of industrial (mild) preservation techniques on control of *Salmonella* and for other food safety issues related to this pathogen.

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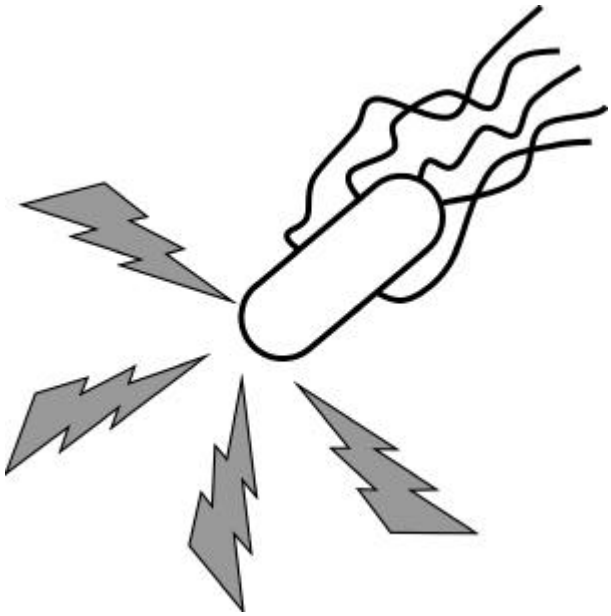
Addendum



Supplemental material of Chapter 4

Table S1. Thematic overview of all genes and their function and corresponding oligos used on the microarray

Gene name	Gene description	Oligo name	Sequence (5' to 3')	Tm (°C)	Length (nt)	Design[a]
1) Stress induced regulators						
adi	*adiA*, arginine decarboxylase, catabolic; inducible by acid	ADI-O	CCTATATGATGCATGCCACCACCTCTCCGTTATATGCCATCTGCGCATCC	78.3	50	OF
		adi-o1	GCGAACGAAGCCATTGACTGTCTGATGTTTAGCTACCAAATGGAACAACCGGATGAGCAC	84.4	60	GR
envZ	osmolarity sensor histidine kinase in two-component regulatory system with OmpR	ENVZ[b]	GCCGAGCCTCCAGCAGTTTAATAAGGTTCTGGCTTACGAAGTCCGTATG	79.6	49	OF
fur	major iron regulator	FUR	GACGCAACAGCATCATCACGACCATCTTATCTGCCTTGATTGCGGAAAA	78.7	49	OF
lysP	cadR; APC family, lysine-specific permease	LYSP	GCGTCAGGTGTTCTGGCGTATTCTGCTGTTTTATGTTTTCGCGATTTTG	79.3	49	OF
ompR	stationary phase transcription response regulator in two-component regulatory system with EnvZ, affecting transcription of ompC and ompF	OPMF	AGGGTAAAAATCAGGACAACCACAGCATTAACTCTCAGAATGGCGATGGCG	78.1	51	OF
phoP	response regulator in two-component regulatory system with PhoQ, transcribes genes expressed under low Mg+	PHOP	GCTGTATCCGGATGCGGAACTGCGGGAAAGTCATACCATTGATGTTCTC	79.0	49	OF
phoQ	sensory kinase protein in two-component regulatory system with PhoP	PHOQ	GGTTCGTATACGTGCTGGCCGCCAATTTACTGTTAGTCATTCCTTTACT	78.1	49	OF
2) Sigma factor						
atoC	putative sigma 54 dependent transcriptional regulator (=STM0652)	ATOC-O	AGCCGCATCTCCTCTCTGTTTTGTCTCTCCTTACCCTCAACTGGCAAAG	78.3	49	OF
		atoC-o1	GAGTCCCGTGGGCACCAGGTACTGATTAGTCGTGGCGGATGTGCGGAAATATTAAAAAAG	84.2	60	GR
		atoC-o4	AACAGGTATTGCACCAGGAGCGGCATACATTACGTGAGGGAAAAACCGTCAGCGCGAAAG	86.2	60	GR
atoS	sensor protein AtoS for response regulator atoC (=STM2361)	ATOS	TCAGGATGTTTTCCCGCTTTTTCGATTTGATTCATCAGCCGTTGGCCGT	79.8	49	OF
cdsA	CDP-diglyceride synthase, sigma E regulon transcribed as a 3 gene operon	CDSA	TTGCTGAAGTATCGCCTGATTTCTGCTTTTGTGTTAATACCCGCGGTCA	78.7	49	OF
flgM	anti-FliA (anti-sigma) factor; also known as RflB protein	FLGM-O	ATGAGCATTGACCGTACCTCACCTTTGAAACCCGTTAGCACTGTCCAGA	78.7	49	OF
		flgM-o3	GCAGCCAGGCGTCAGCGACATTAATATGGAACGCGTCGAAGCATTAAAAACGGCTATCCG	86.5	60	GR
htgA	positive regulator for sigma H (sigma 32) promoters, permitting growth at high temperature	HTGA-O	GCAGATAAAAGCATGTCGACGTTTGAAATAGAACAGCAACTGCTGGAGCA	78.1	50	OF
		htgA-o1	TGGAGCATTTTTTACGTCATACCTGGCAGAAGATGGACGCGGCGCATAAGCAGGAATTTC	85.4	60	GR
rpoA	DNA-directed RNA polymerase subunit alpha	RPOA	GTTGCGCGGTGACCGAGGTTGAGATTGATGGTGTACTACATGAGTACAG	79.2	49	OF
rpoB	RNA polymerase, beta subunit	RPOB	GCGATGGCGTAGAAAAAGACAAACGTGCTCTGGAAATCGAAGAGATGCA	79.8	49	OF
rpoC	RNA polymerase, beta prime subunit	RPOC	GAAACCAACTCCGAAACCAAGCGTAAAAAGCTGACCAAGCGTATCAAAC	78.8	49	OF
rpoD	sigma D (sigma 70) factor of RNA polymerase, major sigma factor during exponential growth	RPOD-O	TCGTAAGGTGCTAAAGATTGCCAAAGAGCCAATCTCCATGGAAACGCCG	79.4	49	OF
		rpoD-o1	GACATCGCTAAACGTATCGAAGACGGGATCAACCAGGTTCAATGCTCCGTTGCCGAATAC	85.0	60	GR
rpoE	sigma E (sigma 24) factor of RNA polymerase, response to periplasmic stress, also important in stationary phase	RPOE-O	GCGCGCTGGATTCTTTCCGGGGGGATAGTGCTTTTTATACCTGGTTGTA	79.8	49	OF
		rpoE-o5	ATTAAGGCCTATCGCGCGCTGGATTCTTTCCGGGGGGATAGTGCTTTTTATACCTGGTTG	84.0	60	GR
rpoF(=fliA)	sigma F (sigma 28) factor of RNA polymerase	RPOF-O	TGTTGCTCGATACCAACAACAGCCAACTTTTCTCTTACGATGAGTGGCG	79.0	49	OF
		rpoF-o1	AATGCGGTCGACCGATATGACGCTTTGCAAGGAACGGCATTTACCACTTACGCAGTGCAG	87.1	60	GR
rpoH	sigma H (sigma 32) factor of RNA polymerase	RPOH-O	CGCTGATCCTGTCTCACCTGCGCTTTGTTGTTCATATTGCTCGTAACTA	78.8	49	OF
		rpoH-o1	CGCTGATCCTGTCTCACCTGCGCTTTGTTGTTCATATTGCTCGTAACTATGCGGGCTATG	84.5	60	GR
rpoN	sigma N (sigma 54) factor of RNA polymerase	RPON-O	AAATAACCCGCTGCTTGAGCAAACCGATCTTCATGACGAAATCGACACC	79.5	49	OF
		rpoN-o1	GATCTTCGCGACTGTCTGCTGATCCAGCTTTCACAATTCGACAAATCCACGCCGTGGCTG	88.6	60	GR
rpoS	sigma S (sigma 38) factor of RNA polymerase, major sigma factor during stationary phase	RPOS-O	AAGAGAACGGTCCGGAAGACACCACGCAAGATGACGATATGAAACAGAG	79.3	49	OF
		rpoS-o1	AACTGGACCACGAACCGAGTGCGGAAGAAATTGCAGAGCAACTGGATAAACCGGTTGATG	86.2	60	GR
rpoZ	RNA polymerase, omega subunit	RPOZ	GCGGAAAGGATCCGCTGGTACCGGAAGAAAACGATAAAACTACCGTTAT	79.7	49	OF
rsD	regulator of sigma D, binding activity to major sigma subunit of RNAP	RSD-O	GCTGGAAGATAATACGCAGCGCATTATGGATTATTACGACACCAGCCTGG	78.5	50	OF

		rsD-o3	GTCAGAGCCTGGTCGATTACCTTTCCGCCGGGCACTTCAGTATTTATGAACGTATTCTCC	83.0	60	GR
rseA	anti sigma E (sigma 24) factor, negative regulator	RSEA-O	CAGCACTATAATGGGCAATCTGAAACATCCCAACAACCTGAAACGCCGG	78.1	49	OF
		rseA-o13	CGTGATGGCCGCTATTGAAAACGAGCCAGTACGTCAGGTGTCGCCATTAATCCCTGAGGC	87.4	60	GR
rseB	anti sigma E (sigma 24) factor, negative regulator	RSEB-O	ATTGTCGATTCTCTGCCTTCCCTGATTTACACCGACTTTAAGCGCCTGG	79.0	49	OF
		rseB-o1	TTGCGTTCACGGTGAGCCAGGATATCGGCAGCAACATGCAGGCGCTGGCGAAGGCGAATC	92.9	60	GR
rseC	regulator of sigma E (sigma 24) factor	RSEC-O	CGGGGCTATTTCTTTGTGCGGCGCTTTTTCAGATGCTGTTTGGATCTGA	79.8	49	OF
		rseC-o1	CGGAGCGCGACGCCTGGCAGCCGGTGATTTTAAACGTTGCCCTCCCGCCTGACCTTGTTC	85.1	60	GR
rtcR	sigma N dependent regulator of rtcBA expression (EBP familiy)	RTCR-O	TGTACGATGCCCGCTCTCGCTCGCTGTTTGAAGGATTAAAGAAGGATAT	79.4	49	OF
		rtcR-o5	CGCTGTTTGAAGGATTAAAGAAGGATATCGCCAGCGTCTCGCCGGAAACGGAAGTCGTCG	87.0	60	GR
yhbH	putative sigma N modulation factor	YHBH-O	GCGGATTAATCAGGTATACGTGGTGTTGAAAGTGGAGAAGGTCACGCAC	78.8	49	OF
		yhbH-oZ	ACGACAAAATTCGCCAAACTTGAACAGTATTTTGAGCGGATTAATCAGGTATACGTGGTG	80.1	60	GR
yhbL	sigma cross-reacting protein 27A to sigma D and sigma H (SCRP-27A)	YHBL-O	AATAGGGACGGATATTGATACCGCTGAAGTACTTGAAGAGATGGGCGCC	78.3	49	OF
		yhbL-o4	CGCTGGGATTCATCTGTATCGCGCCCGCAATGTTGCCGAAAATCTTTGACTTCCCGCTAC	88.3	60	GR
yjfJ	putative Phage shock protein A, IM30, suppresses sigma 54-dependent transcription	YJFJ-O	ATTGGCGCGGGTGAAGCTAAGCCATGACAAGCTGAATGATTTACGTGAG	79.6	49	OF
		yjfJ-o1	GCCGTAACGGCGACAGGCCAGCGGATTGCGCAGTTTGAGCAGCAACTTGAAGTGGTTAAG	89.0	60	GR
		yjfJ-o10	AGCGGATTGCGCAGTTTGAGCAGCAACTTGAAGTGGTTAAGGCGACAGAAGCCATGCAGC	88.1	60	GR
3) RpoS regulators						
barA	sensory histidine kinase, transcription regulator, activator of ompR	BARA	GCCAGCCTAATCGCGGCTCGACCTTCTGGTTTCATATTAATCTTGATCT	78.1	49	OF
clpP	specificity component of ATP-dependent serine protease with clpX, degradates RpoS	CLPP	TATTCTCGTCTACTTAAGGAACGCGTCATATTTCTGACCGGCCAGGTCG	79.9	49	OF
clpX	specificity component of ATP-dependent serine protease with clpP, degradates RpoS	CLPX	AACCGAAGCGGGTTACGTTGGTGAAGACGTCGAGAATATCATTCAGAAA	79.4	49	OF
cspA	major cold shock protein 7.4, transcriptional activator of hns	CSPA	AAATCTCTGGACGAAGGTCAGAAAGTTTCCTTCACCATCGAAAGCGGCG	79.8	49	OF
dksA	dnaK suppressor protein, acts with ppGpp	DKSA	AAGAAAGTGGAAGATGAAGACTTCGGTTATTGCGAGTCCTGCGGGGTGG	79.5	49	OF
dsrA	a small RNA antisilencer of the H-HS-silenced rdsA gene	DSRA	ATTTTCAAGTGCTTCTTGCATAAGCAAGTTTGATCCCGACCCGTAGGGC	78.4	49	OF
fis	site-specific DNA inversion stimulation factor, represses rpoS expression	FIS	CTACCGTTAACTCTCAGGATCAGGTAACCCAAAAACCCCTGCGTGACTC	78.4	49	OF
hfq	host factor I, RNA-binding protein, essential for translation of rpoS	HFQ	GTCAGCCAGATGGTTTATAAGCACGCGATTTCTACTGTTGTCCCGTCTC	78.9	49	OF
hns	DNA-binding protein, binds to HF-1protein, preventing rpoS translation	HNS	GGACTGGCCAGGGTCGTACACCGGCTGTAATCAAAAAAGCAATGGAAGA	79.3	49	OF
lrhA	NADH dehydrogenase transcriptional repressor (LysR family), indirect control on mviA	LRHA	CCATTGGTATTGTGCGGCTGAGTATGTTTTGCAAAGGGGAGAGCCCGTA	79.2	49	OF
relA	ppGpp synthetase I (GTP pyrophosphokinase), ppGpp act as positive signal for *rpoS* transcription	RELA	TGATCAAATTGAAATTATCACTCAGAAGCAGCCGAATCCCAGCCGCGAC	78.5	49	OF
rprA	regulatory RNA	RPRA	TGAAACAACGAATTGCTGTGTGTAGTCTTTGCCCGTCTCCTACGATGGG	79.2	49	OF
spoT	bifunctional : ppGpp synthetase II; also guanosine-3', 5'-bis pyrophosphate 3'-pyrophosphohydrolase	SPOT	TGGTGCTCAAAGAGCAGCGTTTTCACTCGATCATGGACATTTACGCTTT	78.9	49	OF
uvrY	putative response regulator (LuxR/UhpA familiy)	UVRY	TGAAGATATAAAGGGCATTAAAGTTGTCGGTGAAGCGTGCTGCGGAGAG	78.7	49	OF
yhhP	small ubiquitous protein required for normal growth	YHHP	GATATTCCGGGGTTCTGTACCTTTATGGAACACGATTTGCTGGCGCAAG	78.7	49	OF
4) Regulated by Ada or SdiA						
aidB	acyl-CoA dehydrogenase, adaptive response, transcription activated by Ada)	AIDB	CGGCTGGCGGATGATTCATATCGGCTGGTGGGACATAAATGGTTTTTCT	78.5	49	OF
alkA	3-methyl-adenine DNA glycosylase II, inducible, Ada regulated	ALKA	ATACCCACGGCTGGCAACCGTCAATGGATAGCGAAATAGCTGGTATTCA	79.1	49	OF
alkB	DNA repair system specific for alkylated DNA, interaction with ada	ALKB	TGACCGGTGAATTTCGTTACAATCTCACCTTCCGTCAGGCAGCTGAAAA	79.0	49	OF
srgA	sdiA-regulated gene; putative thiol-disulfide isomerase or thioredoxin	SRGA	ATTTTCTTTTTTGTTAGCTGGCTGCGTCTGTCCTGTGGTTGCGCAGGAG	79.7	49	OF
srgB	sdiA-regulated gene; putative outer membrane protein	SRGB	GTCCGGTCTCCCCTGTAACAGAAAACATAACCTTCACCACCACGATGTT	78.0	49	OF

Gene name	Gene description	Oligo name	Sequence (5' to 3')	Tm (°C)	Length (nt)	Design[a]
srgC	sdiA-regulated gene;putative bacterial regulatory helix-turn-helix proteins	SRGC	TCCTGACGTTTGCACTGGTATCGGCCTTTTGTGAGCAGGATATTTTTTTCC	78.1	51	OF
5) Regulated by stress regulator Fur						
cirA	outer membrane porin, requires TonB, putative binding site for fur	CIRA	GTGGAAAACAAAAACCCCGGCAACAGTAGCCCTATCACGTCAGAAAGCA	79.2	49	OF
fhuA	outer membrane protein receptor / transporter for ferrichrome, colicin M, and phages T1, T5, and phi80	FHUA	CCGCCACCACCACCAAAACTGATACACCTATCGAAAAAACGCCACAGTC	79.8	49	OF
fldA	flavodoxin 1, putative binding site for fur	FLDA	TGTGGGTCACTGGCCAACCGCAGGCTATCATTTTGAAGCCTCTAAAGGT	79.0	49	OF
sitA	Salmonella iron transporter: fur regulated	SITA	AAAGAGCTTTATCTCTGGCCAATTAACGCCGATCAACAGGGGACGCCAA	79.6	49	OF
sitB	Salmonella iron transporter: fur regulated	SITB	CCGTCACCGAGTTTTGCGACTATACGGTAATGATTAAAGGAACCGTACT	78.8	49	OF
sitC	Salmonella iron transporter: fur regulated	SITC	ATGGTGTCGCTCAATCCGATGTCGGTGAATATCCAGACGATTATTCTCG	78.4	49	OF
sitD	Salmonella iron transporter: fur regulated	SITD	CGTGTTTTTAGTGTTAAAAGGCTGGGCGTTAATGGGCGATGCAATGAGT	78.7	49	OF
sodA	superoxide dismutase, manganese, putative binding site for fur	SODA	GATTCACCACACCAAACACCATCAAACCTATGTCAACAACGCTAACGCG	78.7	49	OF
tonB	energy transducer; uptake of iron, cyanocobalimin; sensitivity to phages, colicins, putative binding site for fur	TONB	AAACCCAAACCTAAGCCAAAACCCAAGCCAAAACCGAAACCGGTGAAAA	78.8	49	OF
6) Regulated by stress regulator OmpR						
fadL	transport of long-chain fatty acids; putative binding site for ompR	FADL	GCTTTCAGTTAAACGAATTTTCTTCCTCTGGCCTTGGCCGGGCCTATTC	78.7	49	OF
flhD	regulator of flagellar biosynthesis, , putative binding site for ompR	FLHD	TCGCGCGTCGATGACTTACAGCAGATTCACACAGGTATCATGCTTTCAA	79.8	49	OF
lrp	Leucine-responsive regulatory protein, putative binding site for ompR	LRP	CGACACCCGAACTTACGTAGTGATGGAAGAGGTAAAACAGAGTAATCGTC	78.2	50	OF
ompC	outer membrane protein 1b (ib;c), porin	OMPC	GCAGCGCAGTATTCTCAGACCTATAACGCAACCCGTTTTGGTACCTCTA	79.4	49	OF
ompF	outer membrane protein 1a (ia;b;f), porin	OMPR	TACTGGATTTAATGCTGCCAGGTGAAGATGGTCTGTCGATTTGTCGTCG	78.0	49	OF
yfcZ	putative cytoplasmic protein, putative binding site for ompR	YFCZ	GGAGTCTGAACCTTGCCAAATTACGCCAACCTTTACCGAGGAATCCGAA	79.1	49	OF
7) Regulated by stress regulator PhoPQ						
pagK	PhoPQ-activated gene	PAGK	CCAATGCCTCAAAAGTGGTGTAATCTTTGGCCTGCTGGCATACCCTTCCC	78.1	50	OF
pagO	PhoPQ-activated gene; predicted integral membrane protein	PAGO	TGTATCTCGGGGCTTTTGCCGGAGTTTTTGGTATCCTGTGCTACTTTGC	79.0	49	OF
pagP	PhoPQ-activated gene	PAGP	ACTGGAATTATATTCCCATCCCTGTGCTGTTGCCTTTAGCGTCGATAGGCT	78.1	51	OF
pmrA	=*basR*, response regulator in two-component regulatory system with BasS	PMRA	ACCCTCGACCAACACTCTGGAAGTGCATATACATAATTTGCGCGACAAA	78.3	49	OF
pmrB	= *basS*, sensory kinase in two-component regulatory system with BasR	PMRB	GCTGAGTATCGTCAGCCGCATCACCCAACTACATCAGGGACAGTTTTTC	78.8	49	OF
pmrD	polymyxin resistance protein B	PMRD	GGTTAAGAAATCGCATTATGTCAAAAAGAGGGCGTGCCATGTTCTGGTGC	78.1	50	OF
pmrF	putative glycosyl transferase	PMRF	GCGAGAGCCTGGGCAAAGCGTGGGAGATTTTATTGATAGATGATGGTAGC	78.5	50	OF
pqaA	PhoPQ-regulated protein	PQAA	TCAGGATGATAAAAAACCACTGAAAGAGGACGAAAGCGTTTCCCGAAGC	78.2	49	OF
fliC	flagellar biosynthesis, filament structural protein, phoPQ repressed	FLIC	CACTGCAGATGACGGTACATCCAAAACTGCACTAAACAAACTGGGTGGC	78.0	49	OF
8) RpoE regulated						
cutC	copper homeostasis protein	CUTC	TCCAACCATTATGGCTGGTGCGGGGGTTCGTGCAAATAACCTGCAGAAT	79.5	49	OF
dsbC	protein disulfide isomerase II, acting on folding of enveloppe proteins	DSBC	AGACAAAAACAAAGCGTTTGATGACGCCATGGCAGGTAAGGGCGTGAAA	80.0	49	OF
hlpA	histone-like protein, located in outer membrane, acting on folding of enveloppe proteins	HLPA	GGGTGTATCCAATACACTGGAAAACGAATTTAAAGGCCGTGCGGCTGAA	78.9	49	OF
mdoG	periplasmic glucans biosynthesis protein	MDOG	TTGGCAATGTTCAGCACGATAAAGACACGGTAAAAGATTTAGGCTTCGC	78.1	49	OF
mdoH	membrane glycosyltransferase; synthesis of membrane-derived oligosaccharide (MDO)/synthesis of OPGs	MDOH	TCTTTTCGCTGATTCTGTCGCCATTTGTTTCGGTGATCTCCAGTCGTTC	79.3	49	OF
purA	adenylosuccinate synthetase	PURA	GTACTTATCCGTACGTAACTTCTTCTAACACCACTGCAGGTGGCGTGGC	79.9	49	OF
surA	peptidyl-prolyl cis-trans isomerase, acting on folding of enveloppe proteins	SURA	GCCTGAAGCTGGAAGAAATCGCGGCTGACATTAAGAGTGGTAAAACCAC	78.8	49	OF
upps	undecaprenyl pyrophosphate synthetase, sigma E regulon transcribed	UPPS	GGCGCCTGTGGATTTAGTAATTAGGACTGGGGGAGAGCATCGCATTAGT	78.6	49	OF
yaeL	putative membrane-associated Zn-dependent protease, sigma E regulon, down-regulates rpoH and rpoE	YAEL	TCGCATTGGCTCGATTTTGCTGGTGTTGTTAATGGGGCTTGCACTTTTC	79.0	49	OF
yggN	putative periplasmic protein	YGGN	TTTCCAGCAGTTTGGCAAAGATGTCTGTAGCCGCGTAGTGACCTTAGAA	78.8	49	OF

9) RpoS regulated

adhE	alcohol dehydrogenase / acetaldehyde dehydrogenase	ADHE	GTATCGTTCCAACCACTAACCCAACCTCTACCGCGATCTTCAAATCGCT	79.6	49	OF
bolA	involved in modulating cell morphology, putative murein genes regulator	BOLA	AACTTTCCACAACCGTACATGCGCTGGCACTGCATACTTATACCCTCAA	78.6	49	OF
cbpA	curved DNA-binding protein	CBPA	TTTATGGCTCGTTATCCATATTGCCCCGCATCCGCTCTTTGATATCGTC	78.5	49	OF
csgA	curlin major subunit, coiled surface structures, cryptic	CSGA	TTCAGAAACAATGCCACCATCGACCAGTGGAACGCTAAAAACTCCGATA	78.2	49	OF
csgB	minor curlin subunit precursor, nucleator for assembly of adhesive surface organelles	CSGB	ACAAGAAGGAGGAAATAATCGGGCGAAAGTCGACCAGGCAGGGAATTAT	78.2	49	OF
csiE	stationary phase inducible protein	CSIE	ATACTGCCCGTGAGGATATCACTGAGACAAGCCTGGAGATCCAGCGCTA	79.2	49	OF
dpS	stress response DNA-binding protein; starvation induced resistance to H_2O_2	DPS-O	CGTTAGGCACCACGCAAGTTATCAACAGCAAAACTCCACTGAAAAGCTA	78.3	49	OF
		dpS-o8	CGTTCATGAGATGCTGGATGGCTTCCGTACCGCACTGACCGATCATCTGGATACTATGGC	86.5	60	GR
dsrB	regulatory RNA, regulated by DsrA and HNS	DSRB	GACCGGGCGTGGTACTGGCAGTGGAAGAATTTAGTGAAGGCACAATGTA	79.3	49	OF
emrA	multidrug resistance secretion protein, stationary phase	EMRA	GCGTCATATTGAAGAGACAGATGATGCTTACGTGGCAGGGAACCAGGTT	78.2	49	OF
emrB	putative MFS superfamily, multidrug transport protein, stationary phase	EMRB	CCGACGATCACCCTATCGTCGATCTTTCTCTTTTTAAATCGCGTAACTT	78.7	49	OF
gabP	APC family, gamma-aminobutyrate transport protein	GABP	TCTGGCGAATTTCCATCTTTTACCTGTGTTCCATTTTTGTGGTGGTGGCG	78.0	50	OF
galK	galactokinase	GALK	CGTTGCGCAACAGTACGAAGCGAAAACCGGAATCAAAGAAACCTTTTAT	79.7	49	OF
glgB	1,4-alpha-glucan branching enzyme	GLGB	GGAGAAAGTGACGCCAGGCGAAGAACGACAAAAAGCCAATCAGTTTGAT	79.3	49	OF
glgC	glucose-1-phosphate adenylyltransferase	GLGC	TTGTAATATTGATTCGGCAGTGTTGTTACCTGAGGTTTGGGTAGGGCGC	78.0	49	OF
glgS	glycogen biosynthesis, rpoS dependent	GLGS	TTTACTGCCAGCAGGCGACTCAGGCGAAAAGGTTAGAATTAGAACACTA	78.1	49	OF
grxB	glutaredoxin 2	GRXB	CGCGGACTATCGCGATAATATGGCAAAGCAGACGCAAATTAATTTACTC	78.5	49	OF
katE	catalase, hydroperoxidase HPII(III)	KATE	AATTCCGATTAACCGCCCTACCTGCCCCTACCATAACTTCCAGCGTGAC	80.0	49	OF
katG	catalase, hydroperoxidase HPI(I)	KATG	ATGAGCACGACCGACGATACCCATAACACGTTATCCACTGGAAAATGTC	78.5	49	OF
narV	nitrate reductase gamma chain	NARV	CGTCTTTTTTTACGACATCTACCCCTATATTTGCGCCACGGTATTCTTCCT	78.0	51	OF
narW	nitrate reductase delta chain	NARW	CTGTTGATGGAGTATCCGGACGACGAGTTGTGGGAATGTCGGGATGAAG	79.5	49	OF
narY	nitrate reductase beta chain	NARY	GCAGGAGTGGCAAGGCGGTTGGGTTCGTGATGTTAATGGCAAGATAAGG	79.6	49	OF
narZ	nitrate reductase alpha chain	NARZ	ATCTATAATGAACGACCCGCAAAAATGCCAAAGTTACAAACAGGTGCGC	78.1	49	OF
ogt	O-6-alkylguanine-DNA/cysteine-protein methyltransferase, stationary phase	OGT	TATGGAGCAACTGCTAAATATCCACTACCGTCACGAAGGCTATGAACGC	78.4	49	OF
osmB	osmotically inducible lipoprotein	OSMB	AAAAAAATGGCCGCTGCTGTGCTGGCAATCACCGTAGCAATGTCTCTGA	79.9	49	OF
osmC	resistance protein, osmotically inducible	OSMC	TGACAATCCATAAGAAAGGTCAGGCACACTGGGAAGGCGACATCAAACG	78.2	49	OF
osmY	hyperosmotically inducible periplasmic protein, stationary phase	OSMY	CGAAACCGCAGGGCAAAAAGTCGATAGCTCTATGAATAAAGTCGGTAAC	78.4	49	OF
otsA	trehalose-6-phosphate synthase, stationary phase	OTSA	GGAGTGGCGAGACAGGTAACGAGGATGAGCCATTAAAAAAGGTGACAAAAG	78.0	51	OF
otsB	trehalose-6-phosphate phophatase, biosynthetic, stationary phase	OTSB	AACTGGCGTTGCAGCCTGGTAAATGTGTTGTGGAAATCAAACCTAAGGGAA	78.0	51	OF
poxB	pyruvate dehydrogenase/oxidase FAD and thiamine PPi cofactors, cytoplasmic in absence of cofactors	POXB	GCGGCAAAGAGCACGTTGAGTACGATAACCCTTATGATGTGGGAATGAC	78.8	49	OF
proP	MFS family, low-affinity proline transporter (proline permease II)	PROP	TAAACGCGGATTTATGGGAAGCTGGCTGGATTTTGGTTCTATCGCCGGA	80.0	49	OF
proV	ABC superfamily (atp_bind), glycine/betaine/proline transport protein	PROV	AGGCGAAACATCAGCGCACCATTGTCTTTATTTCCCACGATCTTGATGA	78.1	49	OF
proW	ABC superfamily (membrane), glycine/betaine/proline transport protein	PROW	TTTCCGTCCCCTTTTTCAGGGGATTCGTGTGCCGGTGGATTACATCCTC	79.7	49	OF
proX	ABC superfamily (bind_prot), glycine/betaine/proline transport protein	PROX	CGAACAGAAAAATATTGATACTAAACTGCCGAACGGCGCGAACTATGGG	79.0	49	OF
sodC	Gifsy-2 prophage: copper/zinc superoxide dismutase	SODC	ACACCTTACGGTCTGCTTTTCACTCCTCACCTAAATGGTCTTACGCCAG	78.4	49	OF
stiA	putative fimbrial subunit	STIA	AGTCGGGAACGGTCGATGCAACCGCAACATTCTATCTGCAATATAACTG	78.9	49	OF
stiB	putative fimbrial chaparone	STIB	CAATCCGGACAATAAACACAACACACTCATGCTGGCCGTTAAAGCGGAA	79.0	49	OF
stiC	putativie fimbrial usher	STIC	AACAGGCATTATCAGTAAGCATCCCGCAACTCTACATCGCCAACAACGC	79.7	49	OF
treA	trehalase, periplasmic, stationary phase	TREA	ATCGCGAGAAAAAACTGGTCGAAAAAAATGATGTCAGCAGCACCGGAAC	79.4	49	OF
wraB	= *wrbA in E.coli*, tryptophan-repressor binding protein, stationairy phase protein	WRAB	AACCAAGCCAGGAGGAACTCTCTATCGCTCGCTATCAGGGGGAATACGT	79.9	49	OF

Gene name	Gene description	Oligo name	Sequence (5' to 3')	Tm (°C)	Length (nt)	Design[a]
xthA	exonuclease III, may repair singlet oxygen induced lesions, stationary phase oxygen stress resistance	XTHA	CGGAGGAGCGTGAATGGATGTCACGGTTACTGAAGTGGGGATTAGTGGA	79.5	49	OF
yahO	putative periplasmic protein	YAHO	AGAAGATTTAATCAAAAAGGCGGATGAAAAAGGGGCGGATGTGGTGGTGC	78.0	50	OF
ycgB	putative cytoplasmic protein	YCGB	GGCGCACGCCTGTTACGGCCACAACTCCTTTTTTAAAAATAATTATCTCT	78.1	50	OF
yciF	putative cytoplasmic protein	YCIF	GGCCGAGCAGCTCGGCTATAGCAAAGCATTAAAACTGCTCAAAGAAACC	79.5	49	OF
yeaG	putative Ser protein kinase	YEAG	TGACTTTCCGTAACAATAAAAATAACGAAGCGTTCCTCGACCGTGTCTA	78.4	49	OF
yehY	putative ABC-type proline/glycine betaine transport systems, permease component	YEHY[b]	GTGAGTGATGCTGCCAAAAAAATCACGGTTAATCGTGTGCTGTTGCTGT	78.4	49	OF
ygaU	putative LysM domain	YGAU	AAAGCGACAGTGACGGGTGATGGGCTGAGTCAGGAGGCAAAAGAAAAAA	79.4	49	OF
yhjY	putative lipase	YHJY	CAGCGATCACCAACCGAATTACAACGACATACTGGCCGAACGTATTCAG	79.9	49	OF
yjgB	putative alcohol dehydrogenase	YJGB	TCTCATTATTAACACGGTTAACGTCGATCTCGACTGGCAACCCTACTTC	78.1	49	OF
yncC	putative regulatory protein, gntR family	YNCC	GCTGGAAATTAATACGATTCGTACTGCCCTGGAGGAGATGGCGGTGGTC	79.6	49	OF
yohF	putative oxidoreductase	YOHF	GCCCGGCATATGATTAAACAAGGAGAGGGAGGGCGCATTATCAACATCAC	78.2	50	OF
fic	cell filamentation protein, stationary phase induced gene, affects cell division	FIC	TACTGTGAAATTAATGTGCTTCATCCGTTTCGTTTTGGGAGCGGGTTGG	78.1	49	OF
ftsA	ATP-binding cell division protein, septation process, complexes with FtsZ, junctions of inner and outer membranes	FTSA	GGTATGGATAAAGGCGGGGTGAATGACCTTGAGTCAGTGGTGAAATGCG	78.2	49	OF
ftsQ	cell division protein; ingrowth of wall at septum	FTSQ	GGCCTGATGAATTGAAGATTCATCTGGTTGAATATGTGCCGATTGCGCGT	78.3	50	OF
ftsZ	tubulin-like GTP-binding protein/GTPase, forms circumferential ring in cell division	FTSZ	CCGTTGTTGCCACTGGAATTGGTATGGACAAGCGTCCTGAAATCACTCT	78.4	49	OF
10) Heat, cold, acid or osmotic shock induced						
clpB	ATP-dependent protease, Hsp 100, part of novel multi-chaperone system with DnaK, DnaJ, and GrpE	CLPB	ATCGTCTTACTAACAAATTCCAGCTTGCTCTTGCCGATGCCCAGTCGCT	79.9	49	OF
cspC	cold shock protein, multicopy suppresses mukB mutants, putative regulator	CSPC	CGCTATCCAGGGCAATGGTTTCAAAACTCTGGCTGAAGGCCAGAACGTT	79.6	49	OF
cspD	cold shock protein, similar to CspA but not cold shock induced	CSPD	GCGGCGGCGAGGATATTTTCGCCCATTATTCCACCATTCAAATGGATGG	79.6	49	OF
cspE	RNA chaperone, negative regulator of cspA transcription	CSPE	TCCAGACCAATGGTTTTAAAACTCTGGCTGAAGGTCAGCGCGTAGAGTT	78.6	49	OF
dnaJ	chaperone protein DnaJ	DNAJ	GCTAAGTTTAAAGAGATTAAAGAAGCCTACGAAGTGCTGACCGATGCGC	78.4	49	OF
grpE	molecular chaparone, heat shock protein	GRPE	ATGAGTAGTAAAGAACAGAAAACGCCTGAGGGGCAAGCCCCGGAAGAAA	79.0	49	OF
hscA	chaperone, member of Hsp70 protein family, believed to be involved in assembly of Fe-S clusters	HSCA	CGCACAAGGCGATGATGTTGACGCCATAGAACAAGCCATTAAAAACGTA	79.2	49	OF
hscC	putative heat shock protein, homolog of hsp70 in Hsc66 subfamily	HSCC	TTCGTTTTAGCTACGATATTAATGGTCTGCTGGAAGTTGACGTGCTGCT	78.0	49	OF
hslU	ATPase component of the HslUV protease, rpoH controlled heat shock response	HSLU	AAAGACGCGATGAAACTGTTGGTTGAAGAAGAGGCTGCTAAACTGGTCA	78.2	49	OF
hslV	peptidase component of the HslUV protease, rpoH controlled heat shock response	HSLV	GTAACACAGTAATGAAAGGCAACGTGAAGAAAGTCCGCCGCCTCTACAA	79.3	49	OF
htpG	chaperone Hsp90, heat shock protein C	HTPG	CCCGTAACAAGTCGGAAATTAAAGACGACGAGTACAACGAGTTTTACAA	78.2	49	OF
htrA	periplasmic serine protease Do, heat shock protein, transcribed by RpoE	HTRA	AGCGTGATTAAAGTACAGCTTAGCGATGGGCGTAAATTCGATGCTAAAG	78.4	49	OF
mreB	rod shape-determining protein; HSP70 class molecular chaperones involved in cell morphogenesis	MREB	CACTTTATTAAACAAGTGCACAGCAACAGCTTTATGCGCCCAAGCCCGC	79.9	49	OF
ybiI	putative DnaK suppressor protein	YBII	CGGCTGGGCAAATGACGACGCAGTGAATGAACAAATCAACAATACCATT	78.2	49	OF
yegD	putative heat shock protein (Hsp70/DnaK)	YEGD	ATTTTTACAGTAGCGCGAATGGTCGTCTGTTGAACGATTTGGTTCGCAA	79.8	49	OF
dnaK	chaperone Hsp70 in DNA biosynthesis/cell division	DNAK	ATGACTCCGCTTATCACCAAAAACACCACCATCCCGACCAAGCACAGCC	79.9	49	OF

mopA	chaperone Hsp60 with peptide-dependent ATPase activity, affects cell division	MOPA	GTAAACTGATCGCGGAAGCGATGGATAAAGTCGGTAAAGAAGGCGTCAT	79.8	49	OF
mopB	chaperone Hsp10, affects cell division	MOPB	GTAAAGAAGTTGAGTCTAAATCTGCTGGCGGCATCGTACTGACCGGTTC	79.1	49	OF
11) Stress response related						
ada	bifunctional: O6-methylguanine-DNA methyltransferase; transcription activator/repressor (AraC/Xyl family)	ADA	ATGAAAAAAGCGTTACTTATCGATGATGAATGCTGGCTGCGGGTGCAGG	78.9	49	OF
cpxP	periplasmic repressor of cpx regulon by interaction with CpxA, rescue from transitory stresses	CPXP-O	AGCCTCCTGTTAATGTTAGCGAAATGGAGACAATGCATCGGCTTGTCAC	78.1	49	OF
crp	catabolite activator protein (CAP), cyclic AMP receptor protein (CRP family)	CRP	TCGTCGCTTACAAGTCACCTCTGAAAAAGTAGGTAACCTCGCCTTCCTT	79.0	49	OF
nfnB	dihydropteridine reductase/oxygen-insensitive NAD(P)H nitroreductase	NfnB	GTCTCGCTGAAAGATGACCACCAGTGGATGGCGAAGCAGGTTTATCTG	81.2	48	GR
oxyR	regulatory protein sensor for oxidative stress, regulates intracellular hydrogen peroxide (LysR family)	OXYR-O	ACTTTCCCGAAGCTGGAAATGTATCTGCATGAGGCGCAAACGCATCAGT	80.0	49	OF
oxyS	stable RNA induced by oxidative stress	OXYS-O	CGCAAAAGTTCACGTTGGCTTTAGTTATTTCGGGTTCGAGAGATACCCT	78.7	49	OF
		oxyS-oZ	GGTTCGAGAGATACCCTCTGAACGGGCGGTGTCTTCAAGGGTTAAACGAGAAACCGCTCC	85.7	60	GR
pgi	glucosephosphate isomerase, synthesis of organic acids	PGI	ATTTTATCGCCCCGGCTATCACCCATAACCCGCTATCCGATCATCATCA	78.6	49	OF
phoU	regulatory gene for high affinity phosphate uptake	PHOU	TGGCGAAGCGCGTGGTAGAAGGCGATCATCAGGTTAATATGATGGAAGT	79.8	49	OF
purD	phosphoribosylglycinamide synthetase (GAR synthetase)	PURD	GGTCTGATGATCGACAAGCAGGGCAACCCGAAAGTTATCGAGTTCAACT	79.0	49	OF
ramA	putative regulatory protein, resistance against oxidative stress	RamA	GAAGCTAAAACTGGCGGCGCGCGACCTGCTCGACACCGACAGAAGGT	86.3	47	GR
sodB	superoxide dismutase, iron, underexpressed	SODB	ACCAATCTTAACAATTTGATCAAAGGCACGGCGTTTGAAGGCAAATCGC	78.3	49	OF
sspA	stringent starvation protein A, regulator of transcription	SSPA	TCAGGTCCGCATTGTGTTGGCTGAAAAGGGTGTTAGTTTTGAGATCGAAC	78.2	50	OF
sspB	stringent starvation protein B	SSPB	GTAATCTGGAGCTGTCTAATGATGAAGTGCGTTTTAATGCGCGCTTCGG	79.4	49	OF
uspA	universal stress protein A	USPA-O	GCGGCATAAGAAACGCCAGTAGCTCAATGGTCATCGACAACTTATGGAA	78.7	49	OF
		uspA-o1	GACGTCAATCTGGGCGATATGCAGAAACGTATCTCCGAAGAAACCCACCACGCGCTGACC	87.8	60	GR
uspB	universal stress protein B, involved in stationary-phase resistance to ethanol	USPB-O	TCACTATTCTGGGCTTTATGTGTCGTTTGCATTGTTAATATGGCGCGCT	78.1	49	OF
		uspB-o1	CGCATGGTCAGCCCAACAAACAGGTGCGCCTGGTATGGTATATCTACGCTCAGCGCTATC	86.4	60	GR
ybdQ	putative Universal stress protein UspA	YBDQ-O	TCAGTATCGATCCTTCACGAATTAAAACGCATGTTCGCTTTGGTAGCGT	79.2	49	OF
		ybdQ-o16	GCATGAGGCGGAAACGCGTCTGCAAACGATGGTGGGACACTTCAGTATCGATCCTTCACG	88.9	60	GR
ydaA	putative universal stress protein	YDAA-O	TACGCATGTAGAAAAAGGCTTGCCTGAAGAAGTTATCCCGGATCTTGCC	78.0	49	OF
		ydaA-o1	GAAGTTATCCCGGATCTTGCCGAACACCTCCAGGCGGGGATCGTTGTGCTTGGCACCGTT	90.1	60	GR
yecG	putative universal stress protein	YECG-O	TTTACGCGCGGTTATGCATGAAGAGACCGAGAATTTTCTTAAGATGCTG	78.2	49	OF
		yecG-o1	GGTTATGCATGAAGAGACCGAGAATTTTCTTAAGATGCTGGGGGAAAAGGCGGATTATCC	81.9	60	GR
yfiA	ribosome associated factor, stabilizes ribosomes against dissociation	YFIA-O	GATCAACAAGCTGGAACGGCAGCTCAATAAAGTGCAGCACAAAGGCGAA	79.9	49	OF
		yfiA-o1	GACGCCACCATTAATACACCGAACGGACATCTGGTCGCCAGCGCAAAACACGAAGATATG	86.8	60	GR
yicC	putative stress-induced protein	YICC-O	GGAAGCGCACGTCAAAGAGACTTACAACATTCTGAAGAAAAAAGAGGCG	78.4	49	OF
		yicC-o1	TTCAGTTGGAAAACAACCGTCTGGAGCAAGAGCTGGTGATGATGGCGCAACGCATTGATG	88.2	60	GR
ynaF	putative universal stress protein	YNAF-O	CAGAGCTTCCCGCAATGGACGATTTGAAAGCCGAAGCCAAATCTCAACT	79.5	49	OF
		ynaF-o1	GACTGGCTTATTCAGCAGAGCTTCCCGCAATGGACGATTTGAAAGCCGAAGCCAAATCTC	85.7	60	GR
		ynaF-o9	ACGATTTGAAAGCCGAAGCCAAATCTCAACTGGAAGCGATTATCAAGAAATTCAACCTTC	81.4	60	GR
12) Decarboxylase & arginine related						
argI	ornithine carbamoyltransferase 1	ArgI	AGGTTAAGTTCCTGCATTGTCTGCCTGCGTTCCATGATGACGAAACCA	81.3	48	GR
cadA	lysine decarboxylase	CADA	GAGCTGCATGGCGAAACGGAAGAGGTGTATCTTGAAGAGATGGTTGGAC	78.7	49	OF
cadB	lysine/cadaverine transport protein	CADB	ATTGTTAAAAGTATTCTGCTCTGCCTGTGGGCGTTCGTTGGCGTTGAGT	79.7	49	OF
carA	carbamoyl-phosphate synthetase, small subunit, regulated by arginine	CARA	ATTGCCAGCAACTTCCGTAACACCGAAGACCTCTCTTCTTACCTCAAAC	78.1	49	OF
carB	carbamoyl-phosphate synthase, large subunit, regulated by arginine	CARB	CCTTCGAGCCGTCCATCGACTATGTGGTTACTAAAATTCCGCGCTTTAA	79.8	49	OF
oat	putative acetylornithine aminotransferase	OAT	GGGCAGGAGTTTATCGACTGTCTTGGGGGGTTTGGCATCTTTAACGTGG	79.2	49	OF

Gene name	Gene description	Oligo name	Sequence (5' to 3')	Tm (°C)	Length (nt)	Design[a]
potE	APC family, putrescine/ornithine antiporter	POTE	TCACTATCGCTCAGGTCTTTAAATCCTCCGCTGACGAAGGCTATTTCCC	78.7	49	OF
speF	ornithine decarboxylase isozyme, inducible	SPEF	CCCGTAAATTGATCCTCGAAAACTGTCATCACATCCGTCCGTTCGTACC	79.4	49	OF
STM4463	putative arginine repressor	STM4463	AAATCCCTCATTTTGTAAATCGCGGCGAATCTCCTCCTGGGACAGGTAAC	80.5	50	GR
STM4464	putative arginine repressor	STM4464	CTTTTACTCAGGGAATGCTGATGCGAGTACTGCTGTTAATTGTCGGCTAC	76.9	50	GR
STM4465	putative ornithine carbamoyltransferase	STM4465	CCAGCAGGTGGTAAACGCTACCGGTAATCCTGATGTGAAGTTTATGCAC	78.7	49	GR
STM4466	putative carbamate kinase	STM4466	CCCGGCATTCCTCAATCCAACCAAATATATCGGACCGATTTACGACGAAG	81.1	50	GR
STM4467	putative arginine deiminase	STM4467	CAAGGGATAGAAGTGTTGCTGTTAACCGACCTGCTCACCCAAACGCTG	80.7	48	GR
13) Stress & Antibiotic resistance						
marA	transcriptional activator of defense systems (AraC/XylS family), multiple antibiotic resistance protein	MARA	AATTCTGCCGTAGACAAAAAAGAGGTATGACGATGTCCAGACGCAACAC	78.1	49	OF
		MARA-DT104	AAAAAAGAGACCGGTCACTCATTAGGCCAATACATCCGCAGCCGTAAAA	78.4	49	OF
marB	multiple antibiotic resistance protein	MARB	ATTGCAGAACAAACTTTGTTACCTGTGGCGCAAAATAGCCGCGATGTGA	79.5	49	OF
marC	putative MarC Transporter, multiple antibiotic resistance protein	MARC	GGCAGTCCTATATGGCTTCGGTTTATGTCTTCGCTATTATGATGGTGGCG	78.1	50	OF
marR	transcriptional repressor of marRAB operon, multiple antibiotic resistance protein	MARR	GCATCAGGAATTAACAAAAAACTTAACGGCGGACGAAGTGGCAACGCTT	79.9	49	OF
		MARR-DT104	GCATCAGGAATTAACAAAAAACTTAACGGCGGACGAAGTGGCAACGCTT	79.9	49	OF
14) Stress & Cell division						
ftsK	required for cell division and chromosome partitioning, regulates UspA	FTSK	TGGAAGCGTTGTTGATACTTATTGCCCTTTTTGCCGTCTGGTTGATGGC	78.6	49	OF
mukB	kinesin-line cell division protein involved in sister chromosome partitioning	MUKB	GCAAACATCATCCGCAAGACTATCCAGCGTGAACAGAACCGTATCCGTA	79.7	49	OF
tig	peptidyl-prolyl cis/trans isomerase, trigger factor; a molecular chaperone involved in cell division	TIG	GATGGCGTCTGCTTACGAAGATCCGAAAGAAGTGATTGAGTTCTACAGC	78.2	49	OF
15) Stress hyc, hyp operons						
hycA	transcriptional repressor of hyc and hyp operons	HYCA	GATCTTTGCTTTGTCCTGTTTGAGCACTTTCGTATTTACGTCGCGTTGG	79.2	49	OF
hycB	hydrogenase-3, iron-sulfur subunit (part of FHL complex)	HYCB	AATCGTTTTGTAATTGCTGACTCCACTCTCTGTATCGGCTGCCACACCT	78.2	49	OF
hycC	hydrogenase 3, membrane subunit (part of FHL complex)	HYCC	GGTGATTTATCAGTCCTTCTTCAAACTGGGCAACAGCGGTGCGTTTGTT	78.6	49	OF
hycD	hydrogenase 3, membrane subunit (part of FHL complex)	HYCD	GTGATTTAATCACCCTGATTTACCTGTTCGCCATCGCGCGCTTCTTCTT	79.6	49	OF
hycE	hydrogenase 3, large subunit (part of FHL complex)	HYCE	GGAAGTGGTGGAGTTTCTTTACTACCAGCAGGGTGGGTGGCTGTCGGTG	80.0	49	OF
hycF	hydrogenase 3, putative quinone oxidoreductase	HYCF	TAAACTGTCGCAAGAGTACGAACTGGCGGTGTGGAAGAAAGAAGATTTC	78.3	49	OF
hycG	hydrogenase activity	HYCG	ACGATCCGCGTTTAACCGAAATTGTCACCCATCTCAATCATGTTGTTGA	78.0	49	OF
hycH	processing of HycE (part of the FHL complex)	HYCH	GGTTATCGATTGTCTGGAGGCCGCACTCACCTGCCCATGGGATGAGTAT	79.4	49	OF
hycI	protease involved in processing C-terminal end of HycE	HYCI	AGCTGAAAGAGGATGTCGGGGAGGTGATTTTTGTTGGTATCCAGCCGGA	78.8	49	OF
hypA	guanine-nucleotide binding protein in formate-hydrogenlyase system, nickel donor for HycE of hydrogenlyase 3	HYPA	CACGATAGCAGAAGGTTGTAAACTGCACCTCGAAGAACAGGAAGCCGAA	79.5	49	OF
hypB	hydrogenase-3 accessory protein, assembly of metallocenter	HYPB	TGGTATTCTGTTTATCGAAAACGTCGGTAATCTTGTCTGCCCGGCCAGC	79.9	49	OF
hypC	putative hydrogenase expression/formation protein	HYPC	CTACAAAATATGTTTGATGTTGAGCCGGACGTCGGCGCACTGCTGTATG	79.8	49	OF
hypD	putative hydrogenase expression/formation protein	HYPD	TGAGCAGAAAATAGCGGCCCTAAGCCAGGTTGAAAATCAATACCGTCGC	79.7	49	OF
hypE	putative hydrogenase expression/formation protein	HYPE	ATCCTTGAAGAAGGGCTGCCGATGGAGACGTTAAAAAGCGTGGTCAATA	78.9	49	OF
hypO	putative Ni/Fe hydrogenases, small subunit	HYPO	ACCCAACCGTTGAAAACCTCGTTCTGGAGACCATCTCTCTGGAATACCAC	78.3	50	OF
16) Two-component system, LuxR related						
orf7	putative bacterial regulatory proteins, luxR family	ORF7	GATTTCAGCCGGATATGGTACGACCGGCTTTGTATCGAATTTAACAGCG	79.3	49	OF
rcsA	positive transcriptional regulator of capsular/exo- polysaccharide synthesis	RCSA	GGGTATCTGGTCAGCAGAGGGGTGAAAAAAAGGGAAATCAACGACATCGAAAC	78.2	53	OF
rcsB	response regulator (positive) in two-component regulatory system with RcsC	RCSB	AAGAAGCTCAACCGCAGCATTAAGACCATCAGCAGCCAGAAGAAATCGG	78.9	49	OF
uhpA	response regulator (repressor) in two-component system with UhpB, regulates uhpT operon (LuxR/UhpA family)	UHPA	GATATTTCCGGACTGGAGCTGCTAAGCCAACTGCCAAAAGGGATGGCGA	79.9	49	OF

uhpB	sensory histidine kinase in two-component regulatory sytem with UhpA	UHPB	CAGCCAGACCTGGCACGATCATCCCGTCGATTTATTACTTTCGCTGTTG	79.8	49	OF
uhpC	membrane protein, regulator of uhpT expression	UHPC	CCTATATCTGGCTGCTTTCGTTGTGTTATGTCTTAGTGTACGTGGTGCG	78.1	49	OF
uhpT	MFS family, hexose phosphate transport protein	UHPT	GGCGTAAAATGTGGTTCAAGCCGTTCATGCAGTCCTATCTGGTGGTTTT	78.3	49	OF
yhjB	putative transcriptional regulator (LuxR/UhpA familiy)	YHJB	GCTGTCGCTCGCGGGATGATGTTTTTACCGGGGGATTGGTTAAATTCAA	79.9	49	OF
17) Cell structure & Lipopolysaccharide						
aas	bifunctional: 2-acylglycerophospho-ethanolamine acyl transferase; acyl-acyl carrier protein synthetase	AAS	ATTTTGTGCCGCTCGACCCCACTAAACCCATGTCCATTAAACACCTGGT	78.8	49	OF
nlpB	lipoprotein-34	NLPB	ATTGGGTGAACTGGAATCGCCTGGATGAAGACGAACAGTATCGTGGACG	79.7	49	OF
nlpD	lipoprotein (upsteam of rpoS)	NLPD	TGAACAAAGTGCTAACAAAATGTTGCCAAACAACAAGCCTGCTGGGACG	78.0	49	OF
rcsC	sensory histidine kinase in two-component regulatory system with RcsB, regulates colanic capsule biosynthesis	RCSC	GCGTTACTCATTTGGCTCTTAATCGCCTTTGTTTCGGTGTTTTACATCG	78.3	49	OF
ftsN	essential cell division protein	FTSN	ATAAGAAAGAAGAGTCCGAAACGCTACAAAACCAGAAAGTCACCGGCAA	78.1	49	OF
kdtA	3-deoxy-D-manno-octulosonic-acid transferase (KDO transferase)	KdtA	GATCCTAAGCTGGTATTGATCATGGAGACTGAGCTCTGGCCAAATCTG	77.5	48	GR
kdtB	phosphopantetheine adenylyltransferase	KdtB	CCTGGGTAACGTAGAGGTGGTAGGCTTTAGCGACCTGATGGCCAATTTC	79.7	49	GR
lpxA	UDP-N-acetylglucosamine acetyltransferase, lipid biogenesis	LPXA	TCGAAATTGGTGAGGGAACCGTACTGAAGTCTCATGTGGTCGTGAATGG	78.4	49	OF
lpxD	UDP-3-O-(3-hydroxymyristoyl)-glucosamine n-acyltransferase, lipid biogenesis	LPXD	TTGCGTCCATGCAATCTGCAACAACAGGCCACATTACGTTTATGGTGAA	78.3	49	OF
oafA	O-antigen five: acetylation of the O-antigen (LPS)	OAFA	AAATGACTGAAAAGTCTTTTGTTGTATGGGGTGACTCGCATGCCGCACA	78.1	49	OF
rfaB	UDP-D-galactose:(glucosyl)lipopolysaccharide-1,6-D-galactosyltransferase	RfaB	TGCAGAATGTATAACGTACGCGGATTATCATCTGGCCATCAGTAGTGG	77.3	48	GR
rfaC	heptosyl transferase I	RfaC	GCGAACCGCTGGCCAGCCTGTTCTATAACCGTAAACACCATATCGCAAAG	81.8	50	GR
rfaD	ADP-L-glycero-D-mannoheptose-6-epimerase, lipid biogenesis	RFAD	AATACATTCCGTTCCCGGATAAGCTGAAAGGTCGCTATCAGGCGTTTAC	79.0	49	OF
rfaE	bifunctional; putative sugar nucleotide transferase domain of ADP-L-glycero-D-manno-heptose synthase	RfaE	GGTTGTTCCACGACCAATATCATCAAAAAGATCCAGACCGAGAGCGAG	79.4	48	GR
rfaF	ADP-heptose; LPS heptosyltransferase 1	RfaF	TATCACCAGAGCCTGATCGATATCACGCCGCAGCGGGTTCTGGAAGAG	83.8	48	GR
rfaG	glucosyltransferase I	RfaG	GCTAAATGAGGTTTTACTCAAAGCGCTGACACAGCCTTCCTTACGCAAC	77.9	49	GR
rfaH	transcriptional activator affecting biosynthesis of lipopolysaccharide core, F pilin, and haemolysin	RfaH	CGCATTTGAAGGGCTGAAAGCGATTTTTACCGAACCGGATGGCGAAAC	83.2	48	GR
rfaI	UDP-D-galactose:(glucosyl)lipopolysaccharide-alpha-1,3-D-galactosyltransferase	RfaI	GCTGAGACTGTATTTGTTCATTATATCGGACCAACGAAGCCCTGGCATAG	77.6	50	GR
rfaJ	UDP-D-glucose:(galactosyl)lipopolysaccharide glucosyltransferase	RfaJ	ATTCATTATACGGGCGCTACAAAACCATGGCATGCCTGGGCAAATTATCC	80.9	50	GR
rfaK	putative hexose transferase, lipopolysaccharide core biosynthesis	RfaK	GTTTATAAGCGTCTTTTTCAGAAATGGACTCGTCTCGACCCACTACCC	75.8	48	GR
rfaL	O-antigen ligase	RfaL	CAAGTTTAGGACTTCGCTGCCTTGCAGAGAGTATTCTGTATATCGAGG	74.7	48	GR
rfaP	lipopolysaccharide core biosynthesis; phosphorylation of core heptose	RfaP	CCCACCATTAGCCTTGAAGACTACTGTGCTGACTGGGCTGTTAATCCAC	78.8	49	GR
rfaQ	lipopolysaccharide core biosynthesis; modification of heptose region of core	RfaQ	ATGCGTTTTCATGGAGACATGTTACTTACCACCCCTGTCATCAGCACG	79.7	48	GR
rfaY	lipopolysaccharide core biosynthesis; modification of heptose region of the core	RfaY	TGATATGCCAGAAATTTCTGACGAGGTGAGAGGGAAAATCAAACAGTCG	78.1	49	GR
rfaZ	lipopolysaccharide core biosynthesis	RfaZ	GTCAAGCCATTTTTGTACCTTCTGACGGATATTCGCTTCCTCCATCGTC	78.8	49	GR
rfbA	dTDP-glucose pyrophosphorylase	RFBA	TATCTATATGGAGCAGGGAAGATTGTCTGTCGCTATGATGGGGCGCGGT	79.0	49	OF
rfbB	dTDP-glucose 4,6 dehydratase	RFBB	GCGCGCTTCATATGGTAGTGACTGAAGGCAAGGCAGGGGAGACTTATAA	79.1	49	OF
rfbC	dTDP-4,deoxyrhamnose 3,5 epimerase	RFBC	CAGGGGAAGTTAGTTCGTTGTGCTGTCGGTGAGGTTTTTGATGTTGCGG	79.9	49	OF
rfbD	TDP-rhamnose synthetase	RFBD	CCTGCAGGATAACTGCCCTAAACACCTTATCTTCCGCACCAGTTGGGTT	78.3	49	OF
rfbF	LPS side chain defect: glucose-1-phosphate cytidylyltransferase	RFBF	TTGGTTGATACGGGTGATTCTTCAATGACTGGTGGTCGTCTGAAACGTG	78.0	49	OF
rfbG	LPS side chain defect: CDP glucose 4,6-dehydratase	RFBG	TGTGGTGGCGCAACGCTTATATACAGAAGGTGCTAAGTTTTCTGAAGGA	78.0	49	OF
rfbH	LPS side chain defect: CDP-6deoxy-D-xylo-4-hexulose-3-dehydrase	RFBH	TCCGGGAAGGTTATTGGTGCCAAAGAGTTACAATTGATGGTTGAGGCGT	78.1	49	OF

Gene name	Gene description	Oligo name	Sequence (5' to 3')	Tm (°C)	Length (nt)	Design[a]
rfbI	LPS side chain defect: CDP-6-deoxy-delta3,4-glucoseen reductase	RFBI	GGGTGATTGTGGTATCTGTGAGTCCGATTTGTTGGCGGGAGAAGTTGTT	78.2	49	OF
rfbJ	LPS side chain defect: CDP-abequose synthase	RFBJ	TGTAATTGTCAGTGGGGCTTCCGGCTTTATTGGTAAGCATTTACTCGAAGC	78.1	51	OF
rfbK	LPS side chain defect: phosphomannomutase	RFBK	TGTTATTTATTCATCAGGTATTGTGTTTGGAACGAGTGGGGCTCGCGGT	78.0	49	OF
rfbM	LPS side chain defect: mannose-1-phosphate guanylyltransferase	RFBM	TCGTCGTGGTGAGTTGATAGGAAATGACGCTTATGCAGTGGCTGAATTT	78.3	49	OF
rfbN	LPS side chain defect: rhamnosyl transferase	RFBN	GTCGCCTACTGCGCTGAAGCGGTGGTAAGACACTCCCATAATTATACCC	80.0	49	OF
rfbP	LPS side chain defect: undecaprenol-phosphate galactosephosphotransferase/O-antigen transfer	RFBP	CGCATTTTATTTTGTCAGTAGTATGTGTCGGTTGGTTTTGGATTCGTTTGCGA	78.1	53	OF
rfbU	LPS side chain defect: mannosyl transferase	RFBU	GCAGTTGCCTGATGCAATTTCCCGATTTAACAACTATGTCGCACGGTAT	78.3	49	OF
rfbV	LPS side chain defect: abequosyltransferase	RFBV	TCGGCGTAATAGCGTTAACCTTGGGCCAGATAGGAATTTTCTTGCTTCA	78.4	49	OF
rfbX	LPS side chain defect: putative O-antigen transferase	RFBX	GCACAATGGTATTTTTCTAGTACGCTTGGAATCAGTGGAGTGCTGCTTGGC	78.1	51	OF
waaD	homologue of WaaD (=HldD) in DT104: putative lipopolysaccharide |core biosynthesis enzyme	WaaDhom	ATTAGCTCCTGCAAATGGAAGCCGGTATCCCCCTCTTTTACGAACTCAG	79.0	49	GR
18) Pathogenicity						
pipA	Pathogenicity island encoded protein: SPI3	PIPA-O	TGCTACGATGAACTATCCTCCGTACTTCAAAATGCGTATACCCAAAGCG	78.3	49	OF
		pipA-o1	AATTCCGTCTGGAGCCAGAGTGTGTGGTGGAGTACCTTATCTCAGGCGCGGGTGGAATAG	85.7	60	GR
STM2780	homologue of *pipB*, putative pentapeptide repeats (8 copies)	PIPBHOM-O	GGGCGCAAATCTATCGGGAACATCACTAGGCGACAGTAATTTCAAGAAC	78.1	49	OF
		pipBhom-o1	GGTGCAAATATGGATCACACTAATCTTTCAGGCGCAACCCTTATACGTGCTGATATGAGC	81.3	60	GR
pipB	Pathogenicity island encoded protein: SPI3	PIPB-O	TAGCGAAAGCATTAACAGGAGCCGATCTGACAGGTAGTCAACATACCCC	78.1	49	OF
		pipB-o1	ACTTTAGCGAAAGCATTAACAGGAGCCGATCTGACAGGTAGTCAACATACCCCTACTCCA	80.5	60	GR
pipC	Pathogenicity island encoded protein: homologous to *ipgE* of *Shigella*	PIPC-O	CCCTTTATGCCATTGCCTGACGACATCCTGACTTTGCAGCATTTTTTACGT	78.2	51	OF
		pipC-o1	GACGACATCCTGACTTTGCAGCATTTTTTACGTCTGAACTACACCAGCGCCGTCACTATC	83.2	60	GR
pipD	Pathogenicity island encoded protein: SPI3	PIPD-O	AAAAACGATACCACCTATAATTATCCGCGCGTCTGGACGCTACAACACC	79.9	49	OF
		pipD-o1	GCGGTCATCAATGGCTGGCAGTACGACTTCCGGCAGATAGCTATTTCGTTTCCGCCAATC	86.8	60	GR
ydiD	homologue of a plant pathogenicity factor	YDID-O	GCGAGAAGCAGAGCTGGTTTGGGTGCTGAACAAATGTCAGGCTAAAATA	78.0	49	OF
		ydiD-o1	GCTGAACAAATGTCAGGCTAAAATATTCTTCGCCCCCACCGTGTTCAAACAGAATCGTCC	83.8	60	GR
19) Virulence associated						
bigA	putative surface-exposed virulence protein	BIGA-O	AGAAAAACGCGCAAAACTCTCACCGACCTGCCTTGCTACACCTGAAAAT	80.0	49	OF
		bigA-o1	ACTCTGTTATTACCTTCAGCAACGGCGTCACCATCGATAAAGGCAAAGACACCCTGACCT	83.1	60	GR
		bigA-o2	GGCGATATTAACGTTGTCTCAGTCCAGGACAGCGAAGGCGTGTTTAGCTCAGCGACAGGG	86.1	60	GR
cobB	putative nicotinate-nucleotide dimethylbenzimidazolephosphoribosl-transferase, homologue of virulence factor	COBB-O[b]	GGCCCGGCAAGCCAGGTTGTGCCGGAATTTGTTGATAAATTCCTGAAAG	79.3	49	OF
		cobB-o1	GGCGGATGGCCTTTGGGAAGAGCATCGGGTTGAAGACGTGGCAACGCCGGAAGGATTCGC	92.6	60	GR
hhA	hemolysin expression modulating protein, involved in environmental regulation of virulence factors	HHA-O	CAATGAGCTGGCTGTATTTTACTCAGCTGCGGATCACCGTCTTGCAGAA	78.9	49	OF
		hhA-oz	ATTGACACTCTGGAGCGCGTCATTGAGAAAAATAAATATGAATTGTCCGACAATGAGCTG	81.2	60	GR
hnR	Response regulator in protein turnover: mouse virulence	HNR-O	TCTCAAACTGGTAGAAAACTTACGTAATCGCGGAGACCAGACGCCTATT	78.8	49	OF
		hnR-o5	ACTCGCGGGTTGAAGAAGAAGAACGCCTGTTTCGTGACTGGGATGCAATGGTGAGTAATC	85.4	60	GR
lon	DNA-binding, ATP-dependent protease la; cleaves RcsA and SulA, heat shock k-protein (DNA binding activity)	Lon	GTGTAAACGATCTTTTCACCGTCGGGACCGTGGCCTCTATTTTGCAGATG	81.5	50	GR
msgA	Macrophage survival gene; reduced mouse virulence	MSGA-O	AACGCATTAAACAGTGACTGTACAAAAACCGAGAAAGAACGGCTGCACC	79.1	49	OF
		msgA-oz	GCAAGTGAAAGTTAAGCCAATGCAGGCGAACGCATTAAACAGTGACTGTACAAAAACCGA	82.6	60	GR
mviM	putative virulence factor	MVIM-O	ATCATTTTTCCGCCGACAAACTACAAATTACCACCAGTATGCACCGGCG	79.5	49	OF
		mviM-o1	GCTTGAACAGCGTGGTTTTGTCGGATGCGCGCGGCATTTCATTGACTGCGTACAAAATCA	89.0	60	GR
mviN	putative virulence factor	MVIN-O	TTACATTTCCCTATATTCTGCTGATCTCGCTGGCTTCACTGGTTGGCGC	78.6	49	OF
		mviN-o2	TGGATGTGGTTCCTGATGCGTCTGATCATTTCCGTACTGGTGATGGCCGCCGTATTGTTC	87.6	60	GR

pagC	putative outer membrane protein, virulence gene	PAGC	AAGGAAGCAACATCTCCTCTACAAAAATAAACGGCTTCAACGTCGGGGT	78.4	49	OF
pagD	PhoP regulated, virulence gene	PAGD	GTCCGCACCAGTCAATGACGCCAGGAAAGGGAATACTTTCTCCAGAACA	79.0	49	OF
sicP	chaparone, related to virulence on SPI, acts as a specific chaperone for SptP	SICP-O	GGTGAACTGGCTGCGAATAATGAAGGTACGTTAGCGTATATTGATGCCG	78.8	49	OF
		sicP-o1	GTGGCGATTCTATCTGGCGGCAGATTATGGTGATTAATGGTGAACTGGCTGCGAATAATG	83.7	60	GR
spvA	Salmonella plasmid virulence: outer membrane protein	SPVA-O	TGCCATTAACCAGCAAATACCGACCCTGCTGCCGTATCATTTTCCACAC	78.8	49	OF
		spvA-o6	ACCAGAGCAGCAGACACTGCCGACCGAGCCGGACAACAGTACCGCCACTGACCTGACCTC	90.8	60	GR
spvB	Salmonella plasmid virulence: hydrophilic protein	SPVB-O	TGGTTTTTCATCTGCCACTTTAGCGCTGATCACTCCCCCTTTCCTGCCA	79.0	49	OF
		spvB-o1	TCCTGATGAACTGGGTGAAGCCGATACGCTGGTTTCCCGTCTGCTGCTGGAGTATGACGA	88.1	60	GR
spvC	Salmonella plasmid virulence: hydrophilic protein	SPVC-O	GAGGACAGTCCGGTAGATAAGTGGAAAGTGACCGATATGGAGAAGGTCG	78.0	49	OF
		spvC-o1	CTGACTATTCAGGGATGCGCCAGAGTGGTTTCTTTGCTATGAGCCAAGGTTTTCAGCTGA	84.0	60	GR
spvD	Salmonella plasmid virulence: hydrophilic protein	SPVD-O	ATTAAGTTCGAAGAACACTGCCTCTCCTACAGTGGGATTAGACAGCGCC	78.4	49	OF
		spvD-o1	GCTTCTGGCTGTAGCGAAAAAATTCCGTAAATTCCGTAAATACGCATGGCTAAAGGAATA	79.6	60	GR
spvR	Salmonella plasmid virulence: regulation of spv operon, lysR family	SPVR-O	GTTCCTTCAGTATCGCAACATCAGTACTGTATATCACCCGAACCCCGCT	78.5	49	OF
		spvR-o4	TGGGGAGAGCCGTTAACAGCCAAATAATAGAAGAACTGTGTCAGACAAACAACTGCATTG	81.3	60	GR
		spvR-o6	CGCACTGCACATCAAAGGCGTAGCGCTCTGTACCTCCTTGTATTACCCGACCAAGAAACG	85.4	60	GR
virK	virulence gene; homologous sequence to virK in Shigella	VIRK-O	TTAACGCTGTGCTTCAATGATACGCCGTTGGCACGTTTATCATTTTCCT	79.0	49	OF
		virK-o1	GCTAAACGGCGTTAAGAAAGGTGCCTTATATCATCTGCCATCACAGGTGATGCGTAAAGC	82.1	60	GR
ybjX	virulence gene virK homologue	YBJX-O	TTACCCTGTGTCGCTTTAACCAACAACGCACACTATTTATCGGCGGATT	79.3	49	OF
		ybjX-o1	CATGCCGCGTGCGACCAGCCAATTACTGACCAATCTCACCCAATGGCCGGAGTTAAATAC	87.5	60	GR
20) Virulence & Stress and/or LuxR related						
sdiA	transcriptional regulator of ftsQAZ gene cluster (LuxR/UhpA family), regulator of rck operon on virulence plasmid	SDIA	CCCGTCCCCTTTACCCGGCCTAAAATATCGCTTCGTACCACTTATCCTC	79.8	49	OF
luxS	quorum sensing protein, produces autoinducer signaling molecules	LUXS	ATGCCATTATTAGATAGCTTCGCAGTCGATCATACCCGGATGCAAGCGC	79.6	49	OF
mgtA	P-type ATPase, Mg2+ ATPase transporter	MGTA	GAGGTTGGGCTGGATGCGGGCGATGTCATTATTGGCAGTGATATTGAAG	78.3	49	OF
mgtB	Mg2+ transport protein	MGTB	AGAAAAATGGTCATACCGTTGGTTTTCTTGGGGATGGGATCAACGACGC	78.3	49	OF
sspJ	= *yfgL*, putative serine/threonine protein kinase, resistance against superoxide and replication within macrophages	SspJ	TTTGCTGACCTCCCCGGTGCTGTATAATGGTGATTTAGTCGTCGGCGATA	81.7	50	GR
21) Virulence others						
rcK	resistance to complement killing	RCK	GTCATCGATCTGGGCTATGAGGGAAGTAAAGTTGGCGCAGCGAAACTGA	79.8	49	OF
recD	exonuclease V, alpha chain	RECD	GGTCGGCAATCCAGGCTGTTTTTCAGCAGGGGTTTAGCGATATAGAGAA	78.5	49	OF
sopB	Salmonella outer protein: homologous to ipgD of Shigella	SOPB-O	AAAAAATACACTCACGCATAACGGGCATCACTATACCAACACGCAGCTC	78.4	49	OF
		sopB-o1	CGCGGCAAAAGCATTGAAGAAAAATCTTATCGAACTTATTGCAGCACGCACTCAGCAGCA	85.0	60	GR
vacB	putative exoribonuclease	VACB	TGGATAACGATTACTATCGCTTCGATCAGGTAGGGCAACGTCTGATTGG	78.2	49	OF
22) Virulence SPI1 & Invasion						
avrA	putative inner membrane protein	AVRA	TCCGGCGGCGGGAAGCAGTATAGTAAACAAAAAGAATGAAACGCTTTAT	78.9	49	OF
hilA	invasion genes transcription activator	HILA[b]	GTGGCAGGAAAGCTGTTTATGAGCGTATCAAGTCTGAAGCGAAAACTGG	78.2	49	OF
hilC	bacterial regulatory helix-turn-helix proteins, araC family	HILC	GTGGCAGGAAAGCTGTTTATGAGCGTATCAAGTCTGAAGCGAAAACTGG	78.2	49	OF
hilD	regulatory helix-turn-helix proteins, araC family	HILD	GTTAATTCCTTATTTTCTGCTGTTCCTGCTTACTGCTTTTCTCCGACTCCCGG	78.0	53	OF
iacP	putative acyl carrier protein, invasion	IACP	TGATGACCTTTGCGGATATATGCCGTGTTGTTAAAAAAAGTCTTGAGTCCAGGGTGT	78.1	57	OF
iagB	cell invasion protein	IAGB	ATCAGATATGATGAAAATCTACGGTTATAGCTGGGAGGCCGTTGGCGCT	78.1	49	OF
invA	invasion protein	INVA	TTTTTCAACGTTTCCTGCGGTACTGTTAATTACCACGCTCTTTCGTCTG	78.7	49	OF
invB	surface presentation of antigens; secretory proteins	INVB	GTATGGATCTGGGCGCAATTGGGTGCTGACAGCATGGTGGTATTACAAC	78.1	49	OF
invC	surface presentation of antigens; secretory proteins	INVC	TGACGCGTTTGGAAGAGCTCCAGCTTTTCATTGACTTGGGAGAATATCG	78.5	49	OF
invE	invasion protein	INVE	TGTTGCGCTCCGCTGACCTACTGTTTGTGAGTACATTGTTGTCGTATTC	79.2	49	OF

Gene name	Gene description	Oligo name	Sequence (5' to 3')	Tm (°C)	Length (nt)	Design[a]
invF	invasion protein	INVF	TGGTTTTTGCAGCAAATTATTACGCCTTCTCCGGCCTTCAATAAGGTACTGG	78.1	52	OF
invG	invasion protein; outer membrane	INVG	GCGGTGCCTGGAGTGGGGACGATAAGTTACAGAAATGGGTTCGTGTTTA	79.7	49	OF
invH	invasion protein	INVH	CTGTCTTCCTGTCTTTTTACTGATCGGCTGTGCTCAGGTGCCCCTCCCT	79.4	49	OF
invI	surface presentation of antigens; secretory proteins	INVI	GCTGCGTGCAGAAAACAGACAGCTCAGTCGTGAGGAAATTTATACGTTA	78.7	49	OF
invJ	surface presentation of antigens; secretory proteins	INVJ	TGCCGCAGCAGGATGAGGTTGGCGGTTTATCAGAAGCATTAAAAAAAGC	79.0	49	OF
mutS	methyl-directed mismatch repair, recognize exocyclic adducts of guanosine	MUTS	TGTTGTATGCCGAAGATTTTGCTGAAATGGCGTTAATAGAGGGACGCCG	79.4	49	OF
orgA	putative flagellar biosynthesis/type III secretory pathway protein	ORGA	TAACGCCATTAACGCACTTATTGATGAATGGAAGACAAAAACGCAAGCG	78.2	49	OF
pphB	serine/threonine specific protein phosphatase 2	PPHB	AGGATGGCAATTTCTGGTATGTAAACGGTGGTTACTGGTACGACTCGGT	78.4	49	OF
prgH	cell invasion protein	PR	GTACCGTAACGCGCAATCGAACACGGTAAAAGTCTTTAAGGATATTGAT	78.1	49	OF
prgI	cell invasion protein; cytoplasmic	PRGH	ATGAAAGAGATACGTTGTGGGCTCGTCAGGTTTTAGCGAGGGGCGATTA	79.9	49	OF
prgJ	cell invasion protein; cytoplasmic	PRGJ	CCGTTATAGGGCAGGCGGTCAATATCAGGTCTATGGAAACGGACATTGT	78.2	49	OF
prgK	cell invasion protein; lipoprotein, may link inner and outer membranes	PRGK	ATTCGGCTATTGAACAGCGACTGGAACAGTCATTACAGACGATGGAGGG	78.5	49	OF
sicA	surface presentation of antigens; secretory proteins	SICA	ACAGTGTTTTGAACTTGTCAATGAACGTACTGAAGATGAGTCTCTGCGGGC	78.0	51	OF
sipA	cell invasion protein	SIPA	AAAGTAAAGATGGAAAGGTGGTCACGTCAGAAAAGGGCACTACGGGTGA	78.1	49	OF
sipB	cell invasion protein	SIPB	TGTGGGTAGCAAGATGGGCCTGCAAACGAATGCCTTAAGTAAAGAGCTG	78.4	49	OF
sipC	cell invasion protein	SIPC	CGGAACCAGCGCCACGGAAAGTCTGGGTATTAAAGACAGTAATAAACAA	78.0	49	OF
sipD	cell invasion protein	SIPD	TCAAATAAACAGTAATACCGTTTTATTTCCAGCGCAGTCAGGCAGCGGC	78.8	49	OF
spaO	surface presentation of antigens; secretory proteins	SPAO	AACTGCAGAAACTCTGCCTGGCTTGAATCAATTGCCCGTCAAACTGGAA	78.3	49	OF
spaP	surface presentation of antigens; secretory proteins	SPAP	CGCGTTTAAAATTGGTTTTTATCTTTATTTGCCCTTTGTCGTCGTCGACC	78.4	50	OF
spaQ	surface presentation of antigens; secretory proteins	SPAQ	TGCAGGTAATAAGGCGCTCTATCTTGTTTTGATCCTGTCAGGGTGGCCG	79.0	49	OF
spaR	surface presentation of antigens; secretory proteins	SPAR	GTAGAAAACGCGGGCAAAGACGTTATTCAGCCACAAGAAAACGAGGTAC	79.9	49	OF
spaS	surface presentation of antigens; secretory proteins	SPAS	CTGCTGTCATGGCCTTTTTGGGTTATGCATGCGCTGGGTTGTATTATCG	78.4	49	OF
sprB	transcriptional regulator	SPRB	ATTATGTTTTAAAAGACGAACTATCCTGCGCTATCCGCTCGGAGCGAGA	79.5	49	OF
sptP	protein tyrosine phosphate	SPTP	ACAATGGGGAACCTGTGGAGGAGAGGTTGAGAGGTGGGTTGATAAAGCCTCT	78.1	52	OF
sopE2	TypeIII-secreted protein effector: invasion-associated protein, not on SPI-1	SOPE2	GCGCAGAATGCGGGGCTACCTGGGGAGATAAAAAATGGCGTATTTACTC	79.6	49	OF
23) Virulence SPI2						
ssaB	Secretion system apparatus	SSAB	TACCTAAGCCTTGTCTTGCCTATGTGACACTACTGCTTGCAGCACACCC	78.4	49	OF
ssaC	Secretion system apparatus	SSAC	TCCGTCGTGGTTCCAGGGGTCGTTAGTGTATTGCGTGAGATGAGTAAAA	79.8	49	OF
ssaD	Secretion system apparatus	SSAD	CCAGCGAAGGGCCGGGGAGCGTGTTAATTCATGATGATATACAAATGGA	78.3	49	OF
ssaE	Secretion system effector	SSAE	AGGATCCGCAGCAATATCAGCAAAACACCTTATTGCTTGAAGCGATCGA	78.5	49	OF
ssaG	Secretion system apparatus	SSAG	TGGCGCACCAGGCAGGCCAGGCCATTAATGACAAAATGAATGGTAATGA	78.3	49	OF
ssaH	Secretion system apparatus	SSAH	TTGCGGGCGTTAACCATAGCCTGATTTCCCAGGTACATGCGATGTTACC	79.8	49	OF
ssaI	Secretion system apparatus	SSAI	CTGTAAGCACTCAATCTTATGTAAAGTCCTCTGCAGAACCGAGCCAGGAGCA	78.1	52	OF
ssaJ	Secretion system apparatus: homology with the yscJ/mxiJ/prgK family of lipoproteins	SSAI	CAGCATTAGTACGTTTGCGTCGTTTGCTGTTATTCCTTGGCCTTGAAAA	78.4	49	OF
ssaK	Secretion system apparatus	SSAJ	GAATGCTGAGTCAGATGGAGGGCGTGATTAATGCAAAAGTGACCATTGCG	78.2	50	OF
ssaL	Secretion system apparatus	SSAK	AACTTTTGGCAAGCGGTTTACGTTGATTATCGAGCCTGGTTTCTCTCCC	79.0	49	OF
ssaM	Secretion system apparatus	SSAM	CCAGCTCCGCCGAGCTCTGGTTACGATTACATCATCGACAAATAAAATT	78.2	49	OF
ssaN	Secretion system apparatus: homology with the YscN family of proteins	SSAN	ACGACTTTTAGAACGTACGGGAATGGGAGAAAAAGGCAGTATTACCGCA	79.0	49	OF
ssaO	Secretion system apparatus	SSAO	GTTGGATAAGAAACAACAAATGGCCGGGTTATTCACTCAGGCGCAGAGC	78.7	49	OF
ssaP	Secretion system apparatus	SSAP	GTGGCGGTTATCTTGACGGTGTAGAGTGTGAAGTATGTGAATCAGGGGG	78.0	49	OF
ssaQ	Secretion system apparatus	SSAQ	CGTTCGGATTCATTGCTTCGGCGACATCAGACTCGGTTTTTTTGCTATT	79.6	49	OF

ssaR	Secretion system apparatus: homology with YscR of the secretion system of Yersinia	SSAR	GTCCCCCCAAATATCGCACTGTATGGCCTTGCGCTTGTACTTTCCTTAT	78.4	49	OF
ssaS	Secretion system apparatus: homology with YscS of the secretion system of Yersinia	SSAS	ACTTTTATGGATCGTCCTTTTTACGTCTATGCCGGTAGTGTTGGTGGCA	78.1	49	OF
ssaT	Secretion system apparatus: homology with YscT of the secretion system of Yersinia	SSAT	CGGTTCCCTTTTGGGCCGTTGATATGGCGGGGTTTCTGCTTGATACTTT	79.7	49	OF
ssaU	Secretion system apparatus: homology with YscU of the secretion system of Yersinia	SSAU	CCGTACTGTGGGTTAGCCTGTGGCGTGCTTGTGGTTTCTTCTTTAATAA	78.4	49	OF
ssaV	Secretion system apparatus: homology with the LcrD family of proteins	SSAV	TTATTGTCATTACAAAAGGTATCGAGAGGGTGGCGGAAGTTAGCGCACG	79.2	49	OF
sscA	Secretion system chaparone	SSCA	ATGCTGCCAGGCTCAAAAACATTGGGGGGAAGCGATATACGCTTATGGA	78.7	49	OF
sscB	Secretion system chaparone	SSCB	GGGTTGGCGAGAGAGGCTTTTCAAACCGCAATCAAGATGAGTTATGCGG	79.8	49	OF
sseA	Secretion system effector	SSEA	GCTTGCTGAAAGGGCAGAGAGCCCCAAAAATAGCAGAGAGACAGAGAGT	78.1	49	OF
sseB	Secretion system effector, enhances serine sensitivity	SSEB	GACGCCAACCGGAATACCGATCTTATGAGTCAGGGGCAGATAACAATTC	78.0	49	OF
sseC	Secretion system effector	SSEC	GCGTATGGTAGGTGCAGGGGAAGCAGAAATAGAGGAGTTGGCTGAAAAG	78.2	49	OF
sseD	Secretion system effector	SSED	TATGGAGAAAGCAACTGAAATTATGCAGCAAATCATCGGCGTGGGGTCG	78.5	49	OF
sseE	Secretion system effector	SSEE	TTGTTTCACTTACTGGGACTTTTACGCGGGATATTTGTGCATCACCCGC	78.9	49	OF
sseF	Secretion system effector	SSEF	GCTATTTCTATGTTGGTTTTACCCCTACAGTTTCCACTGCCCGCGGCTG	79.1	49	OF
sseG	Secretion system effector	SSEG	AGAGTGGTAGAATAGGACCTCAACAAGGAAAAGAGCGGGTATTGGCCGG	78.5	49	OF
sspH2	Leucine-rich repeat protein, induced by the SPI-2 regulator ssrA/B	SSPH2	TGGACAGACTGAGTGAGACGGAAAACTTCATAAAGGACGCGGGGTTTAA	78.7	49	OF
ssrA	secretion system regulator,sensor component	SSRA	GCAAACCATCCAGGGGCCAGCGCAAAGCAAAAAACTGTCATTACGTACT	79.7	49	OF
ssrB	Secretion system regulator: transcriptonal activator, homologous with degU/uvrY/bvgA	SSRB	TGAATTAAACGCTGACACGACCAATCATCAACTGCTTACTTTGCGCGAG	79.4	49	OF
ttrA	Tetrathionate reductase complex, subunit A	TTRA	CAAACACACTGGCAGCAAAATCCACAACAAACCATCGCCATGACGCAAT	78.8	49	OF
ttrB	Tetrathionate reductase complex, subunit B	TTRB	GTGAACCAATACCAGGTCCAGCGTGAAGGTAGTCAGGAAGTCACGAATG	78.3	49	OF
ttrC	Tetrathionate reductase complex, subunit C	TTRC	TTCAATAAAAGTTACAACGTCACTAAATGGTTGGCGTTAGCCAGCGCGC	80.0	49	OF
ttrR	Tetrathionate reductase complex: response regulator	TTRR	CGAAAAAATGGCCTCGCCAGAGACCAGAATACGAACAACGTATGAGCCA	79.9	49	OF
ttrS	Tetrathionate reductase complex: sensory transduction histidine kinase	TTRS	TTCTGGCGTTCTTGTTGCTCACGCTCAATTATATTTGGGTCATGCTGCT	78.1	49	OF
24) DT104 SGI-1 located (including 5 antibiotic resistance genes)						
groEL/intI1	GroEL/integrase fusion protein	GROEL/INTI1	TACGCTACTTGCATTACAGCTTACCAACCGAACAGGCTTATGTCCACTG	78.2	49	OF
rep	replication protein	REP	TCGTTCAAGAGTAAATGGATCTGAGTTTGAGAGGCGTTGTGGTGCTGCT	78.4	49	OF
res	= *tmpr*, resolvase	RES	TCTTAGACCTGGGGATACGTTGGTCGTTTGGAGGCTGGATAGGTTAGGC	79.0	49	OF
rt	reverse transcriptase	RT	GCGCATTTGCATATAAAAAGGGCAGTAGCATTAAGACCAACGCTCAAGTTCA	78.1	52	OF
tnpa	transposase	TNPA	ATGACGGATTTCAAGTGGCGCCATTTCCAGGGTGATGTGATCCTGTGGG	79.0	49	OF
urt	hypothetical protein	URT	GCAAAGTCGATGTACGTGCACCAATTCCCCCCAGTTTTAGGGATAGACT	78.2	49	OF
yidY	putative drug translocase	YIDY[b]	CGATGTCCGCGATGGGCATTATTGTGGGCCTGTTATCACTGTTTATCCT	78.4	49	OF
aada2	streptomycin/spectinomycin resistance protein	aadA2[c]	CTTTGACCCGGTTCCTGAACAGGATCTATTCGAGGCGCTGAGGGAAACCTTGAAGCTATG	84.7	60	GR
		AADA2[b]	AGATCACTTGGCCTCACGCGCAGATCACTTGGAAGAATTTATTCGCTTT	78.5	49	OF
dhfrX	trimethoprim resistance protein, dihydrofolate reductase, found in *Salmonella* serovar Agona (accession number in Genbank AY049746)	DHFRX	GCTTGTCTATTGATAAGTTTGTTAGACCAGCTTCGCTGGTTGGGGTGCC	78.0	49	OF
flo	chloramphenicol and florfenicol resistance protein	flo[c]	GCGTGGGATGGCGTTGCTTGTTTGCGGAGCGGTCCTGTTGGGGATCGGCGAACTTTACGG	92.5	60	GR
		FLO[b]	GCTGATGGCTCCTTTCGACATCCTCGCTTCACTGGCGATGGATATTTAT	78.5	49	OF
pse-1	beta-lactamase Pse-1 precursor	pse1	CACTGGTAATTTACTACGTTCAGTATTGCCGGCGGGATGGAACATTGCGGATCGCTCAGG	85.7	60	GR
		PSE-1	TGGCGGATTTGGTGCTCGGAGTATTACAGCAGTTGTGTGGAGTGAGCAT	79.6	49	OF
sul1	sulfonamide resistance protein	sul1[c]	GATTTTTCTTGAGCCCCGCACCGGAAACATCGCTGCACGTGCTGTCGAACCTTCAAAAGC	88.8	60	GR
		SUL1	GCTACCTGAACGATATCCAAGGATTTCCTGACCCTGCGCTCTATCCCGA	79.4	49	OF
		sul1 NEW[c]	TTTCTTGAGCCCCGCACCGGAAACATCGCTGCACGTGCTGTCGAACCTTCAAAAGCTGAA	89.9	60	GR

Gene name	Gene description	Oligo name	Sequence (5' to 3')	Tm (°C)	Length (nt)	Design[a]
		tet(G)[c]	ATCTAAGCTCTATCGCAGGACCGCTTGGCTTCACAGCACTCTATTCTGCCACCGCCGGGG	88.0	60	GR
tet(G)	tetracycline resistance protein	tet(G) NEW[c]	ATCTAAGCTCTATCGCAGGACCGCTTGGCTTCACAGCACTCTATTCTGCCACCGCCGGGG	88.0	60	GR
		TETA(G)	TTCTTCTGGCTTTTGCCACGCAGGGATGGATGGTGTTCCCGATTCTGTT	79.4	49	OF
tetR	tetracycline resistance regulator protein	TETR	GCTACCCGAAGAGAATGAGGACTGGCGGGTGTTCCTGAAAGAGAATGCC	79.8	49	OF
25) Housekeeping						
aceF	pyruvate dehydrogenase, dihydrolipoyltransacetylase component	ACEF	GCGTCTGATGATGCCTATCTCTCTTTCCTTCGACCACCGTGTGATCGAT	79.5	49	OF
aceK	isocitrate dehydrogenase kinase/phosphatase, also has ATPase activity	ACEK	AGATTGCGGAGAGCTTTTTCAACTCCGTTTATTGCCGGTTATTTGACCA	78.0	49	OF
fklB	FKBP-type 22KD peptidyl-prolyl cis-trans isomerase (rotamase)	FKLB	TGCATTACACCGGTAAGCTTATTGATGGCACCGTATTTGACAGCTCCGT	79.0	49	OF
fkpA	FKBP-type peptidyl-prolyl cis-trans isomerase (rotamase), acting on folding of enveloppe proteins	FKPA	AGGCGAAGATGGAAAAAGACGCTGCCGATAACGAAGCAAAAGGTAAAAC	79.6	49	OF
folA	dihydrofolate reductase type I, trimethoprim resistance	FOLA	GAGTCCATCGGACGCCCCTTACCGGGACGTAAAAATATTGTTATCAGCA	79.3	49	OF
gapA	glyceraldehyde-3-phosphate dehydrogenase A	GAPA	TGAAATGGGACGAAGTTGGTGTTGACGTAGTGGCTGAAGCTACCGGTAT	79.5	49	OF
gmk	guanylate kinase	GMK	AAGTCATTGCCAAACGAATGGCGCAAGCGGTTGCAGAAATGAGCCATTA	80.0	49	OF
mdh	malate dehydrogenase	MDH	TGATTTGAGCCACATCCCCACCGCTGTAAAAATCAAAGGTTTCTCCGGT	78.2	49	OF
mtlD	mannitol-1-phosphate dehydrogenase	MTLD	GAAAGCATTACATTTTGGCGCAGGTAATATCGGTCGTGGCTTTATCGGC	79.4	49	OF
polA	DNA polymerase I	POLA	TATTGATGTGCCGTTGCTGGTGGAAGTCGGTAGCGGGGAAAATTGGGAT	79.8	49	OF
ppiA	peptidyl-prolyl cis-trans isomerase A (rotamase A)	PPIA	TTGGCTATGCGGTATTTGGTAAAGTGGTAAAAGGTATGGATGTCGCCGA	78.1	49	OF
ppiB	peptidyl-prolyl cis-trans isomerase B (rotamase B)	PPIB	TTTTCCACCGTGTGATTAACGGTTTTATGATTCAGGGCGGCGGCTTTGA	79.9	49	OF
ppiC	peptidyl-prolyl cis-trans isomerase C (rotamase C)	PPIC	GAAGAGAAACTGGCTTTAGATCTTCTGGAGCAAATTAAAAACGGCGGCG	78.3	49	OF
slyD	FKBP-type peptidyl prolyl cis-trans isomerase (rotamase)	SLYD	CCTGGCCTATCAGGTACGTACAGAAGACGGTGTGTTGGTTGATGAGTCT	78.4	49	OF
tpiA	triosephosphate isomerase	TPIA	ACATCCTTTAGTGATGGGTAACTGGAAACTGAACGGCAGCCGCCACATG	79.1	49	OF
26) Rich/Minimal medium dependent expression						
atpG	membrane-bound ATP synthase, F1 sector, gamma-subunit	ATPG	TAAGATCGCAAGCGTCCAGAACACGCAAAAGATCACTAAAGCGATGGAG	79.6	49	OF
cheM	methyl accepting chemotaxis protein II, aspartate sensor-receptor	CHEM	TGCTGGTGCTGATTTTGATGGTGGTTTGGTTCGGCATTCGTCATGCCCT	79.8	49	OF
entD	enterochelin synthetase, component D	EntD	TGGCTGAACGTACCGTCCGTACAGAATGGTTTCAACGTGACAATAGCG	80.8	48	GR
entF	enterobactin synthetase, component F (nonribosomal peptide synthetase)	ENTF	CCGTGCAGTTTTGACGTCTCAGTATGGGAGTTTTTCTGGCCGTTTATCG	79.7	49	OF
fabD	malonyl-CoA-[acyl-carrier-protein] transacylase	FABD[b]	GCCGAGATGGCGGCAAATTACCCTATCGTAGAAGAAACGTTTGCTGAAG	79.8	49	OF
fadA	3-ketoacyl-CoA thiolase; (thiolase I, acetyl-CoA transferase), in complex with FadB catalyzes	FADA	CGGTCATGATGCAGACGGCGTGTTGAAGCAGTTTAACTACGATGAAGTG	79.8	49	OF
fadB	3-hydroxyacyl-coA dehydrogenase	FADB	GGCTCGTGCGCTGGTCGGTATCTTCCTCAACGATCAATATGTAAAAGGT	79.6	49	OF
fadR	negative regulator for fad regulon and positive activator of fabA (GntR family)	FADR	AGAGCCCGGCGGGTTTCGCGGAAGAGTATATTATTGAAAGTATCTGGAA	79.0	49	OF
folE	GTP cyclohydrolase I	FOLE	TATATCCCCAAAGATTCCGTGATCGGCCTGTCGAAAATTAACCGCATCG	79.0	49	OF
gst	glutathionine S-transferase	GST	TGTTCTACAAACCGGGTGCCTGCTCTCTTGCTTCCCATATTACCCTGCG	79.8	49	OF
hemC	Hydroxymethylbilane synthase	HEMC	CTTTGGCAGGCACATTATGTCAAAGACGCATTGATGGCAACCCATCCGG	78.8	49	OF
metE	5-methyltetrahydropteroyltriglutamate- homocysteine S-methyltransferase	METE	CACCCACATGTGTTACTGCGAGTTCAACGACATTATGGATTCGATCGCC	79.2	49	OF
modE	transcriptional repressor of modABCD operon (molybdate uptake)	MODE	GAGGGAAAAGAAGTGCTGATCCTGCTAAAAGCGCCGTGGGTTGGCATTA	79.7	49	OF
27) Putative function						
orf242	putative regulatory proteins, merR family	ORF242	GCTATGGTTTATTGAAACCGCAGCGTACTGATGGCGGACATCGCTTATA	79.4	49	OF
orf245	putative cytoplasmic protein	ORF245	ATAAAGGGAAAGTATGTTGTTTTGCCAGTGATTGTTCGCCGCACTGGGG	78.2	49	OF
orf319	putative inner membrane protein	ORF319	CTATCCTGCGCCACCCCGCTTCACGTAAAAATCACACCAATGTGCTGAT	80.0	49	OF
orf32	putative hydrolase or acyltransferase	ORF32	ACAGAAAATCATTACTCACGCCGCTCATTATCCTTCATGGCGGCCCTTC	79.0	49	OF
orf408	putative regulatory protein, deoR family	ORF408	CATCACCTCATACAGCGCGTATCAAACAGATAAAGCACCCACCGGTAGC	79.4	49	OF
orf48	putative amino acid permease	ORF48	TATTACTGCTGGATTATCTTTTGTTCCCTACGCTCGTGGCAGTGCTGGG	78.4	49	OF

orf70	putative cytoplasmic protein	ORF70	TGGAAATGCGCGATCTGGGGCAGGAGCCTAAATATATTGTTATTGCGGG	78.8	49	OF
yhjC	putative transcriptional regulator, LysR family	YHJC	AGGACGGAATGGTTTACTACGAACGGGCAAAAGATCTGTTGAGTAACCT	78.0	49	OF
yhjD	putative tRNA-processing ribonuclease	YHJD	GCTTAAAAATACGATTAATACGGCGGTTCAACAGCGTACCACGGTAGGG	79.8	49	OF
yhjE	putative MFS family transport protein	YHJE	TCATCATTACCACGCTCATCATTCTCTTCGCGTTGTTCGCCTTTACCCC	79.7	49	OF
yhjG	putative inner membrane protein	YHJG[b]	ACTGGAACCGCCTCAAACCGACCATCAACCAGAAAGTCTCTACCGAATT	79.2	49	OF
yhjH	putative diguanylate cyclase/phosphodiesterase domain 3, flagellar regulon related	YHJH	CTTCTCACTGTCGTCACCCATCCTGATAACCCTTCCAGACGTATTGCCC	78.4	49	OF
yibR	putative inner membrane protein	YibR	ACTACGCCTTAAAGCAAAAAGAGAGATGTCAGGTAAAAATTACTCTATAG	68.7	50	GR
yodD	putative cytoplasmic protein	YODD	GAGCGGGTGAGCGTAGACGGACGGGAATATCATACATGGCATGAATTAG	79.1	49	OF
ytfJ	putative transcriptional regulator	YTFJ	GCGATCCCAGGCTCCGGTATGTTCGTGCGTAGCAGTATAGAAAGTAATA	79.5	49	OF
28) Control spike gene						
luc	The firefly *Photinus pyralis* luciferase gene (accesion no. M15077)	LUC1	GACGCAAGAAAAATCAGAGAGATCCTCATAAAGGCCAAGAAGGGCGGAAAGT	78.2	52	OF
		LUC2	GCGGTCGGTAAAGTTGTTCCATTTTTTGAAGCGAAGGTTGTGGATCTGG	78.7	49	OF
		Luc50-o15m	GCACTGATAATGAATTCCTCTGGATCTACTGGGTTACCTAAGGGTGTGGC	76.9	50	GR
		Luc50-o18e	TTCCATCTTCCAGGGATACGACAAGGATATGGGCTCACTGAGACTACATC	78.1	50	GR
		Luc50-o4b	GGTTGGCAGAAGCTATGAAACGATATGGGCTGAATACAAATCACAGAATC	76.8	50	GR
		Luc60-o2b	TGTCCGTTCGGTTGGCAGAAGCTATGAAACGATATGGGCTGAATACAAATCACAGAATCG	83.5	60	GR

[a] GR indicates oligo designed by using Gene Runner and OF indicates oligo designed by using OligoFaktory

[b] oligo is incorrect: resulted continously in no signal for DNA hybridization

[c] indicates that the oligo was adapted from Angela van Hoek

Supplementary material of Chapter 5

Table S2A. Signal intensities of wild type (WT) only using all data timepoints of this strain for data normalization.

Spot labels[a]	Signal intensities				Standard deviations[b]				No. of datapoints[c]			
Time	**3.5**	**4.5**	**5.5**	**6.5**	**3.5**	**4.5**	**5.5**	**6.5**	**3.5**	**4.5**	**5.5**	**6.5**
adi-o1	ND	49.9	90.9	32.6	NS	5.6	2.1	6.1	0	3	4	2
CSPC	499.3	417.9	170.8	112.3	82.8	6.0	3.3	43.1	4	2	4	4
CSPD	605.4	347.6	145.2	ND	160.0	28.4	3.5	NS	4	3	4	0
CSPE	397.7	269.7	97.7	36.1	85.6	33.3	3.6	1.1	4	4	4	2
DPS	268.8	262.0	581.9	1008.5	18.6	25.6	4.0	47.5	4	4	4	4
FIS	326.1	208.5	8.7	ND	37.3	16.2	NS	NS	4	4	1	0
FKPA	156.1	118.6	37.2	10.0	9.9	2.4	1.7	NS	4	2	4	0
flgM-o3	232.6	213.8	362.9	323.3	13.9	10.5	20.5	69.6	4	4	4	4
FLHD	85.1	42.3	66.0	ND	5.5	NS	2.8	NS	3	1	4	0
FLIC	336.4	980.1	555.7	822.7	23.5	39.4	12.7	110.4	4	4	4	4
FUR	304.6	329.1	105.0	74.9	18.7	39.6	2.8	0.4	4	4	4	2[c]
GAPA	534.0	888.3	369.8	169.7	70.5	40.6	7.9	11.1	4	4	4	3
GRPE	230.9	167.8	21.0	ND	46.3	9.1	1.8	NS	4	3	4	0
GRXB	47.7	53.0	75.7	21.2	4.6	NS	3.0	NS	4	1	4	1
HEMC	90.8	98.0	57.0	32.7	19.8	12.9	1.8	NS	3	2[c]	3	1
HFQ	439.2	841.7	224.7	243.5	19.1	67.2	6.9	NS	4	4	4	1
hhA-oz	276.4	135.4	94.9	100.3	21.1	3.0	5.8	23.5	4	4	3	4
HILC	152.8	623.7	430.7	160.6	4.9	22.7	29.0	36.7	4	4	4	4
HILD	160.3	148.0	321.8	29.6	9.2	9.3	19.2	NS	4	4	4	1
HLPA	147.3	184.9	49.6	10.0	11.4	1.2	2.4	NS	3	2	4	0
HNS	508.3	1037.9	386.2	458.5	32.3	153.6	18.1	103.5	4	4	4	4
HYCA	40.1	137.6	80.6	43.7	5.5	6.6	2.0	2.3	4	3	4	2
HYCI	22.1	43.2	61.0	84.2	1.9	NS	2.4	1.0	3	1	4	2
IACP	122.4	141.5	227.1	58.9	8.5	10.6	3.5	NS	4	3	4	1
INVB	87.3	93.2	281.3	58.1	4.2	0.1	7.0	5.6	4	2	4	3
INVF	28.3	24.6	107.1	60.4	0.9	NS	6.7	2.6	4	1	4	3
INVH	128.5	99.5	266.6	ND	5.4	10.2	14.6	NS	4	4	4	0
LPXD	202.5	113.2	55.9	ND	19.6	3.2	4.7	NS	4	2	4	0
LRP	136.1	70.8	64.9	46.6	9.8	6.0	3.3	NS	4	4	4	1
MOPA	232.2	160.8	49.1	110.2	8.7	27.7	3.9	25.4	4	2[c]	4	2[c]
MOPB	338.9	400.4	60.6	81.2	14.1	31.3	1.4	14.8	4	4	4	3
NLPD	104.7	62.0	171.9	173.2	13.0	5.0	9.4	32.4	4	2	4	3
OMPC	339.7	342.8	205.2	81.5	32.4	27.2	5.3	5.6	4	4	4	4
ORGA	132.1	52.9	116.2	ND	10.4	1.9	3.8	NS	4	2	4	0
OSMB	26.6	47.8	91.8	145.4	2.0	2.9	3.1	6.8	4	3	4	3
PAGK	13.2	31.9	194.0	228.5	0.4	1.6	6.9	28.0	3	2	4	3
PAGP	370.0	261.7	23.8	ND	17.8	19.0	0.2	NS	3	2	2[c]	0
pipC-o1	91.7	110.4	296.3	266.2	8.8	5.9	6.7	60.1	4	4	4	4
PPIB	400.3	612.7	162.3	ND	32.0	29.8	1.3	NS	4	4	4	0
PPIC	313.9	501.1	48.5	ND	14.8	14.0	1.9	NS	4	4	4	0
PRGI	71.9	91.2	238.2	70.0	3.6	4.1	11.1	20.7	4	3	4	4
PRGJ	130.2	148.7	354.0	131.8	10.3	6.9	13.1	19.0	4	3	4	3
PRGK	55.7	59.6	138.8	25.8	4.5	2.8	10.1	NS	3	2	4	1
RES	137.1	97.7	91.7	118.1	7.5	12.4	1.4	5.1	3	4	3	3
RfaI	239.4	31.7	74.2	53.2	6.1	6.3	4.4	10.4	4	2	4	2[c]
RfaJ	243.1	34.7	65.1	ND	13.3	8.5	3.1	NS	3	2	4	0
RfaY	239.8	48.6	46.8	ND	9.3	4.9	1.3	NS	4	2[c]	4	0
RfaZ	198.6	73.1	49.4	ND	22.9	0.5	2.8	NS	4	2	4	0
RFBA	248.8	90.3	41.0	ND	16.9	8.4	0.8	NS	4	3	4	0
RFBF	194.7	75.1	31.7	ND	26.7	6.9	2.0	NS	2	3	4	0
RFBH	340.5	219.4	103.6	ND	36.1	19.3	3.1	NS	4	4	4	0
RFBI	364.0	209.0	94.7	18.5	9.5	13.6	5.4	6.2	4	3	4	2
RFBJ	328.3	164.3	110.7	ND	24.8	13.2	3.6	NS	4	4	4	0

Spot labels[a]	Signal intensities				Standard deviations[b]				No. of datapoints[c]			
Time	3.5	4.5	5.5	6.5	3.5	4.5	5.5	6.5	3.5	4.5	5.5	6.5
RFBK	262.9	48.6	44.2	ND	11.2	1.2	1.3	NS	4	3	4	0
RFBM	272.8	46.8	47.3	ND	9.8	2.2	1.5	NS	4	2	4	0
RFBU	291.3	75.5	91.7	38.2	12.9	4.1	6.1	0.2	4	3	4	2
RFBV	229.3	50.2	80.7	23.1	17.6	1.7	1.8	NS	4	2	4	1
RFBX	256.9	47.2	76.1	37.3	12.1	5.1	2.7	1.7	4	3	4	2
RPOA	329.7	68.1	22.2	ND	34.4	3.6	1.5	NS	2[c]	3	4	0
rpoD-o1	140.3	77.7	58.1	73.2	13.5	8.8	4.7	16.5	4	3	4	2[c]
rpoE-o5	181.0	148.8	116.5	71.9	20.9	8.3	6.8	17.7	4	4	4	3
rpoF-o1	296.6	208.3	237.2	54.1	16.1	15.1	12.9	12.3	4	4	4	2[c]
rpoH-o1	144.9	79.4	64.7	168.8	13.4	2.1	2.0	43.0	4	2	4	3
rpoS-o1	73.5	78.0	181.2	135.6	5.5	2.6	6.5	29.5	4	3	4	4
rseA-o13	71.2	52.7	73.2	30.6	3.9	2.1	0.4	0.7	3	2	4	2
RT	236.2	309.4	35.6	ND	23.4	9.6	0.7	NS	4	2	4	0
SICA	85.2	70.5	110.0	66.5	5.0	5.6	5.5	4.5	4	3	4	2
sicP-o1	314.6	327.9	349.6	161.9	20.6	29.4	8.1	40.5	4	4	4	4
SIPB	20.9	38.8	54.7	38.4	3.3	NS	3.3	NS	2[c]	1	4	1
SIPC	99.6	104.7	210.4	128.0	11.7	1.7	2.7	14.7	4	4	4	4
SIPD	36.5	53.0	66.0	24.2	4.1	NS	5.5	NS	3	1	4	1
SLYD	229.3	150.3	42.9	27.9	12.6	2.3	4.1	NS	4	3	4	1
SODB	346.8	561.5	383.5	499.6	29.7	34.8	7.3	27.3	4	4	4	4
SODC	126.3	120.3	183.1	144.6	6.3	8.0	3.1	3.7	4	4	4	4
sopB-o1	46.1	56.1	68.0	30.0	3.8	2.3	1.7	NS	4	2	4	1
SPAO	25.3	35.8	109.0	50.5	2.0	0.6	3.9	NS	3	2	4	1
SPAP	19.1	25.9	71.9	21.5	1.1	NS	3.0	0.1	3	1	4	2[c]
SPAR	24.0	33.6	76.3	ND	1.2	0.2	2.1	NS	4	2	4	0
SPRB	28.1	32.1	130.8	ND	2.0	2.3	6.9	NS	4	4	3	0
SPTP	224.5	197.3	197.7	54.4	11.1	1.3	7.8	2.9	4	2	4	2
spvD-o1	242.6	269.3	94.3	157.7	7.3	19.4	3.2	4.8	4	4	4	4
SUL1	221.5	161.4	127.9	58.2	18.4	11.7	4.7	2.3	4	4	4	4
TIG	289.5	83.2	12.5	ND	16.7	5.2	0.2	NS	2[c]	2	2[c]	0
TPIA	273.5	276.0	145.9	61.5	15.1	19.9	1.0	0.6	4	4	4	3
uspA-o1	339.5	157.7	345.8	433.1	16.2	8.3	7.6	37.3	4	4	4	4
uspB-o1	30.8	ND	90.3	92.8	3.2	NS	5.5	1.3	3	0	4	2
UVRY	236.5	180.8	93.4	108.7	11.0	8.3	2.5	22.8	4	4	4	4
virK-o1	220.0	111.7	105.6	ND	16.4	6.3	5.2	NS	4	2	4	0
WRAB	58.1	76.0	299.8	482.2	3.5	6.0	22.5	33.1	4	3	4	3
YAHO	21.6	31.7	72.6	82.6	1.0	NS	4.1	15.7	3	1	4	3
ybdQ-o16	322.8	219.8	304.1	592.4	16.8	8.8	10.8	37.0	4	4	4	4
ybjX	344.4	494.8	209.8	32.1	24.0	40.3	5.1	NS	4	4	4	1
ydaA-o1	55.0	44.7	99.8	175.7	5.2	2.9	1.9	8.0	4	2[c]	4	4
yecG-o1	33.2	30.8	44.0	50.7	1.1	4.3	3.2	3.1	3	2	4	2
YFCZ	279.8	208.7	124.1	168.9	15.5	12.2	3.5	9.8	4	4	4	4
yfiA-o1	366.4	1072.6	595.1	897.4	21.5	41.1	18.7	92.0	4	4	4	4
YGAU	19.8	57.7	405.4	796.2	0.2	0.3	23.0	34.4	3	2	4	4
YHBH-O	151.4	202.5	56.4	5.0	8.5	22.0	1.2	NS	4	4	4	0
yhbL-o4	88.1	102.6	83.6	42.2	11.3	8.5	1.7	1.3	4	3	4	3
YHJH	199.0	206.6	248.4	115.2	9.3	11.6	11.2	23.4	4	4	4	4
yicC-o1	176.9	149.0	54.2	ND	19.8	11.4	1.5	NS	4	2	4	0
ynaF-o1	350.7	154.8	192.8	354.2	37.1	12.5	2.3	9.7	4	4	4	4

[a] grey color indicates less reliable results due to fewer datapoints; ND, not detectable

[b] NS, no standard deviation because of only one signal intensity value obtained

[c] only one value for each duplicate hybridization

Table S2B. Signal intensities of wild type (WT) and *luxS* deletion mutant (*luxS*$^-$) using all data timepoints of both strains for data normalization

Spot labels[a]		Signal intensities				Standard deviations[b]				No. of datapoints			
	Time	3.5	4.5	5.5	6.5	3.5	4.5	5.5	6.5	3.5	4.5	5.5	6.5
adi-o1	WT	ND	56.1	101.2	40.0	NS	12.3	4.6	14.9	0	3	4	2
adi-o1	luxS-	ND	10.3	210.7	71.2	NS	1.3	37.2	7.9	0	3	2	2
CSPC	WT	502.0	412.4	168.1	117.7	146.2	11.9	6.6	88.5	4	2	4	4
CSPC	luxS-	426.4	618.1	315.9	101.1	135.8	3.9	15.0	34.9	4	4	4	4
CSPD	WT	565.7	326.2	133.6	ND	279.1	56.7	6.4	NS	4	3	4	0
CSPD	luxS-	232.1	420.0	187.9	69.1	40.9	19.2	40.0	25.1	4	4	3	3
CSPE	WT	595.8	390.4	139.7	57.1	278.7	91.9	10.3	3.4	4	4	4	2
CSPE	luxS-	400.1	346.6	111.1	64.5	166.5	47.1	44.1	11.9	4	4	3	4
DPS	WT	71.8	60.9	224.8	1029.1	1.9	3.2	19.2	412.7	2	2	4	4
DPS	luxS-	36.8	118.1	168.8	444.8	0.1	14.1	13.7	207.8	2	3	4	4
FIS	WT	355.6	225.2	9.3	ND	66.4	37.6	NS	NS	4	4	1	0
FIS	luxS-	427.5	310.1	22.8	ND	116.6	14.4	3.4	NS	3	4	4	0
FKPA	WT	164.3	123.3	37.8	ND	27.6	5.0	3.5	NS	4	2	4	0
FKPA	luxS-	174.5	224.0	79.5	17.5	23.6	39.4	6.9	3.1	4	4	4	2
flgM-o3	WT	249.9	226.4	378.6	365.6	23.3	25.0	42.9	163.5	4	4	4	4
flgM-o3	luxS-	222.3	285.4	325.6	325.1	8.3	51.5	14.7	56.2	4	4	4	3
FLHD	WT	94.9	46.1	70.2	ND	15.9	NS	5.9	NS	3	1	4	0
FLHD	luxS-	64.9	105.8	95.6	36.6	10.8	4.7	13.6	NS	4	4	4	1
FLIC	WT	364.8	1047.5	585.9	942.5	35.1	76.9	26.6	275.7	4	4	4	4
FLIC	luxS-	798.4	829.7	399.2	363.3	93.5	153.0	36.0	38.9	4	4	4	4
FUR	WT	337.2	359.1	112.8	86.9	36.3	90.7	6.1	1.8	4	4	4	2
FUR	luxS-	345.4	322.3	145.1	70.2	30.4	32.8	19.0	11.8	4	4	4	3
GAPA	WT	500.8	824.6	338.3	166.2	113.0	83.5	14.4	17.9	4	4	4	3
GAPA	luxS-	815.2	498.1	424.5	216.1	130.7	14.0	18.3	22.7	4	4	4	4
GRPE	WT	253.1	179.2	22.0	ND	110.5	21.5	3.7	NS	4	3	4	0
GRPE	luxS-	239.7	225.0	31.6	25.4	22.0	10.7	4.3	NS	4	4	3	1
GRXB	WT	49.5	54.5	76.0	22.5	9.5	NS	6.1	NS	4	1	4	1
GRXB	luxS-	25.3	46.0	86.9	32.6	NS	3.7	3.1	1.8	1	4	3	2
HEMC	WT	95.2	103.6	59.3	36.0	38.8	28.8	3.8	NS	3	2	3	1
HEMC	luxS-	95.1	201.5	81.2	35.6	31.1	9.2	13.4	NS	3	4	4	1
HFQ	WT	466.4	876.5	231.1	276.8	44.7	129.5	14.3	NS	4	4	4	1
HFQ	luxS-	567.4	617.7	315.6	168.5	167.7	222.9	37.9	23.8	4	4	4	4
hhA-oz	WT	330.0	159.2	110.0	125.8	43.7	8.8	13.5	60.6	4	4	3	4
hhA-oz	luxS-	334.2	180.2	172.0	105.6	26.5	9.0	8.1	1.8	4	4	4	3
HILC	WT	164.2	657.8	447.7	181.1	14.1	52.9	60.3	86.1	4	4	4	4
HILC	luxS-	133.5	273.8	533.9	243.8	10.8	37.9	94.8	12.3	3	4	4	4
HILD	WT	172.2	155.7	333.4	33.9	27.1	21.4	39.9	NS	4	4	4	1
HILD	luxS-	129.3	155.6	384.7	103.4	8.3	16.4	62.8	8.5	4	4	4	3
HLPA	WT	150.9	185.5	48.5	ND	25.8	2.4	4.7	NS	3	2	4	0
HLPA	luxS-	112.0	223.1	81.1	20.1	13.7	22.8	10.3	2.7	4	4	3	3
HNS	WT	514.4	1032.2	379.8	488.7	45.0	293.5	35.6	228.5	4	4	4	4
HNS	luxS-	866.6	620.1	440.4	319.2	335.0	144.5	81.1	68.4	4	4	4	4
HYCA	WT	41.5	138.9	80.1	45.9	13.0	11.5	3.9	4.8	4	3	4	2
HYCA	luxS-	13.9	112.9	67.4	55.6	NS	8.0	5.7	2.1	1	4	4	2
HYCI	WT	24.8	48.6	66.9	97.7	5.4	NS	5.2	2.4	3	1	4	2
HYCI	luxS-	ND	63.9	50.1	67.8	NS	8.8	8.6	NS	0	4	3	1
IACP	WT	129.5	147.1	231.7	63.5	23.1	23.5	7.1	NS	4	3	4	1
IACP	luxS-	125.7	200.8	151.1	120.2	6.2	13.6	20.1	5.0	4	4	4	2
INVB	WT	82.0	86.8	255.5	56.4	9.7	NS	12.7	9.7	4	2	4	3
INVB	luxS-	85.3	123.9	276.2	93.4	7.5	11.7	22.9	8.0	3	4	4	3
INVF	WT	31.2	26.9	114.1	69.0	3.2	NS	14.2	6.7	4	1	4	3
INVF	luxS-	34.2	45.5	103.5	68.5	2.9	4.3	8.3	10.3	4	4	4	4
INVH	WT	138.2	105.1	277.2	ND	16.3	22.7	30.5	NS	4	4	4	0
INVH	luxS-	119.3	170.1	345.1	42.4	5.2	37.4	34.0	3.6	4	4	4	4
LPXD	WT	225.7	124.5	60.0	ND	53.2	7.0	10.1	NS	4	2	4	0
LPXD	luxS-	150.1	273.1	80.8	36.5	6.7	16.0	13.0	5.8	3	4	3	3

Spot labels[a]		Signal intensities				Standard deviations[b]				No. of datapoints			
	Time	3.5	4.5	5.5	6.5	3.5	4.5	5.5	6.5	3.5	4.5	5.5	6.5
LRP	WT	153.8	78.3	70.6	56.1	28.6	14.2	7.2	NS	4	4	4	1
LRP	luxS-	100.0	138.1	108.8	41.6	8.6	5.3	4.5	8.1	4	4	3	2
MOPA	WT	251.3	171.3	51.5	124.0	21.0	61.5	8.3	53.5	4	2	4	2
MOPA	luxS-	218.8	269.1	61.3	61.2	27.1	14.5	9.0	6.1	4	4	4	2
MOPB	WT	346.2	402.2	59.9	85.6	30.3	67.7	2.8	29.7	4	4	4	3
MOPB	luxS-	208.9	291.8	99.0	78.1	13.5	23.6	5.7	3.1	4	4	4	3
NLPD	WT	113.1	66.0	178.7	196.3	32.2	10.7	19.6	75.8	4	2	4	3
NLPD	luxS-	72.2	127.4	149.2	184.9	3.5	3.2	18.3	14.1	4	4	3	4
OMPC	WT	388.1	387.4	228.3	98.0	58.2	66.2	11.8	13.5	4	4	4	4
OMPC	luxS-	625.6	284.0	322.9	151.1	109.3	22.2	31.9	13.9	4	4	4	4
ORGA	WT	143.7	56.8	121.8	ND	27.9	4.2	8.0	NS	4	2	4	0
ORGA	luxS-	117.3	206.5	130.0	37.1	6.2	7.0	7.2	12.2	4	4	4	3
OSMB	WT	30.6	54.1	101.9	175.7	5.8	7.1	6.9	15.2	4	3	4	3
OSMB	luxS-	40.9	51.1	60.3	124.1	3.9	5.5	1.9	18.2	4	4	3	3
PAGK	WT	13.6	32.9	195.2	245.6	1.5	3.3	13.9	55.0	3	2	4	3
PAGK	luxS-	20.4	30.7	62.5	321.2	NS	4.4	6.0	34.9	1	4	4	4
PAGP	WT	396.5	275.6	24.5	ND	22.4	40.0	0.4	NS	3	2	2	0
PAGP	luxS-	454.3	280.5	52.5	ND	36.1	21.0	7.8	NS	3	4	4	0
pipC-o1	WT	101.6	120.6	319.0	310.4	17.7	13.5	14.4	144.9	4	4	4	4
pipC-o1	luxS-	109.9	173.8	206.0	301.7	3.1	30.0	12.5	46.6	4	4	4	4
PPIB	WT	457.1	691.1	180.4	ND	53.7	75.7	3.0	NS	4	4	4	0
PPIB	luxS-	655.6	385.9	316.4	37.9	71.7	56.8	26.6	3.2	4	4	3	3
PPIC	WT	373.7	587.3	56.0	ND	24.8	37.9	4.3	NS	4	4	4	0
PPIC	luxS-	389.7	392.6	96.8	17.3	20.5	21.2	4.4	NS	4	4	4	1
PRGI	WT	78.2	97.4	250.2	79.9	11.1	8.4	23.5	49.0	4	3	4	4
PRGI	luxS-	96.0	139.1	192.4	123.5	3.2	22.2	12.6	7.4	4	4	4	3
PRGJ	WT	132.0	148.2	346.4	139.0	24.9	15.2	25.7	44.0	4	3	4	3
PRGJ	luxS-	154.3	226.6	343.5	187.6	18.0	43.1	5.7	16.9	4	4	3	4
PRGK	WT	57.8	61.9	140.6	27.6	11.9	5.9	20.5	NS	3	2	4	1
PRGK	luxS-	72.9	86.9	131.3	44.7	7.2	7.5	18.1	3.9	4	4	4	3
RES	WT	150.5	103.9	96.0	134.4	22.4	27.7	2.9	10.7	3	4	3	3
RES	luxS-	103.5	183.5	80.5	82.7	4.2	2.6	6.0	6.0	4	2	4	4
RfaI	WT	258.9	34.1	77.8	60.7	4.8	13.6	9.2	25.4	4	2	4	2
RfaI	luxS-	184.8	254.3	125.9	40.7	16.9	15.5	15.9	3.3	2	4	4	3
RfaJ	WT	255.7	35.7	65.2	ND	37.6	17.5	6.3	NS	3	2	4	0
RfaJ	luxS-	207.0	182.5	127.7	28.2	21.2	24.5	6.6	4.4	4	4	4	2
RfaY	WT	264.1	52.5	49.7	ND	31.1	11.4	2.8	NS	4	2	4	0
RfaY	luxS-	164.5	241.9	113.6	16.3	23.1	36.3	3.2	1.2	4	4	4	3
RfaZ	WT	208.0	75.4	49.7	ND	56.5	1.0	5.7	NS	4	2	4	0
RfaZ	luxS-	157.8	206.3	85.0	22.0	20.7	12.2	3.3	1.5	3	4	3	2
RFBA	WT	245.9	87.7	39.1	ND	43.8	17.3	1.5	NS	4	3	4	0
RFBA	luxS-	190.4	235.0	64.7	18.4	16.1	14.4	9.2	2.2	4	4	4	2
RFBF	WT	214.7	81.1	33.5	ND	69.9	15.8	4.3	NS	2	3	4	0
RFBF	luxS-	114.8	242.4	59.9	17.1	14.9	47.4	8.5	9.0	4	4	2	2
RFBH	WT	391.6	249.9	116.2	ND	66.5	46.5	7.1	NS	4	4	4	0
RFBH	luxS-	573.5	349.7	251.9	37.8	33.1	54.7	20.3	3.2	4	4	4	4
RFBI	WT	375.9	213.1	94.7	19.6	18.1	30.1	10.7	13.2	4	3	4	2
RFBI	luxS-	311.2	346.8	165.2	36.5	18.8	88.8	23.1	8.9	4	3	4	3
RFBJ	WT	366.0	180.8	119.9	ND	42.9	31.3	7.7	NS	4	4	4	0
RFBJ	luxS-	331.8	310.4	190.6	48.5	15.9	31.3	9.0	7.6	4	2	4	3
RFBK	WT	292.9	53.2	47.5	ND	34.2	2.8	2.8	NS	4	3	4	0
RFBK	luxS-	237.1	255.8	95.2	24.5	5.0	13.4	6.5	4.9	3	4	4	2
RFBM	WT	298.1	50.7	50.0	ND	29.6	4.8	3.2	NS	4	2	4	0
RFBM	luxS-	195.3	246.5	71.8	28.3	37.2	4.9	2.0	7.3	4	2	4	2
RFBU	WT	305.0	77.8	93.0	40.9	32.0	8.2	12.3	0.4	4	3	4	2
RFBU	luxS-	238.6	259.8	118.8	50.4	22.2	30.9	7.0	10.0	4	4	4	4
RFBV	WT	276.4	59.8	93.7	28.3	54.0	4.0	4.2	NS	4	2	4	1
RFBV	luxS-	166.1	276.8	117.7	42.3	6.1	21.1	27.2	1.2	4	4	4	2

Addendum

Spot labels[a]		Signal intensities				Standard deviations[b]				No. of datapoints			
	Time	3.5	4.5	5.5	6.5	3.5	4.5	5.5	6.5	3.5	4.5	5.5	6.5
RFBX	WT	278.8	50.6	80.1	41.5	18.0	10.4	5.7	3.7	4	3	4	2
RFBX	luxS-	169.6	291.5	101.9	46.5	21.7	15.7	9.3	5.9	4	4	3	3
RPOA	WT	362.6	74.3	23.7	ND	56.5	8.7	3.2	NS	2	3	4	0
RPOA	luxS-	289.5	356.7	51.6	16.1	21.6	24.1	8.8	0.6	3	4	3	2
rpoD-o1	WT	155.3	84.5	62.0	85.0	35.1	19.9	10.1	40.8	4	3	4	2
rpoD-o1	luxS-	151.8	209.2	56.7	57.0	17.2	11.8	9.2	2.8	3	4	3	2
rpoE-o5	WT	183.9	149.2	115.1	77.3	37.4	18.1	13.5	38.6	4	4	4	3
rpoE-o5	luxS-	186.8	206.7	118.9	80.0	9.7	8.9	13.6	16.6	3	3	4	4
rpoF-o1	WT	311.1	215.5	241.7	60.0	21.2	33.9	26.2	29.0	4	4	4	2
rpoF-o1	luxS-	271.7	198.9	272.1	66.6	15.0	10.1	23.9	2.8	4	4	4	3
RPOH-O	WT	64.1	45.8	26.7	61.1	8.8	NS	6.9	1.2	3	1	4	2
RPOH-O	luxS-	35.4	59.2	23.5	35.6	1.3	3.2	4.7	5.6	3	3	3	3
rpoS-o1	WT	71.3	74.5	169.8	138.0	12.3	5.6	12.2	62.7	4	3	4	4
rpoS-o1	luxS-	71.9	103.3	184.9	179.4	4.3	10.7	25.1	33.2	4	4	4	4
rseA-o13	WT	80.2	58.2	78.8	34.8	10.6	4.6	0.7	1.7	3	2	4	2
rseA-o13	luxS-	86.3	69.8	72.7	51.5	7.6	5.0	3.4	9.4	4	4	2	2
RT	WT	243.2	314.3	35.3	ND	57.5	19.5	1.3	NS	4	2	4	0
RT	luxS-	154.7	289.7	80.7	10.3	14.0	11.1	7.4	NS	4	4	3	1
SICA	WT	84.0	68.6	105.1	67.1	9.6	11.5	10.4	9.1	4	3	4	2
SICA	luxS-	91.6	104.0	111.6	80.7	2.7	8.3	8.7	9.1	4	4	3	4
sicP-o1	WT	326.1	335.4	352.1	176.7	30.9	63.8	16.3	91.3	4	4	4	4
sicP-o1	luxS-	392.5	246.8	356.6	245.7	21.4	14.9	69.0	35.8	4	4	4	4
SIPC	WT	109.7	112.4	222.7	145.9	30.1	5.0	5.7	29.9	4	4	4	4
SIPC	luxS-	135.3	204.2	170.7	198.6	19.3	40.2	12.6	27.4	4	4	4	4
SIPD	WT	39.8	57.8	70.2	27.2	10.7	NS	11.6	NS	3	1	4	1
SIPD	luxS-	39.1	64.6	56.2	35.9	6.3	4.3	5.2	2.6	4	4	4	3
SLYD	WT	241.5	155.4	43.6	30.0	35.9	5.3	8.3	NS	4	3	4	1
SLYD	luxS-	216.6	254.4	72.6	30.9	26.1	6.0	8.8	4.1	4	3	4	4
SODB	WT	378.9	605.6	407.5	572.7	52.6	82.4	15.4	49.6	4	4	4	4
SODB	luxS-	546.5	401.0	491.3	467.1	15.9	32.2	27.0	76.1	4	4	4	4
SODC	WT	145.2	135.6	203.2	173.4	20.1	19.7	6.9	5.3	4	4	4	4
SODC	luxS-	114.3	162.3	192.7	182.7	9.5	25.3	11.8	11.7	4	4	4	3
sopB-o1	WT	52.2	62.7	74.2	34.6	10.4	5.0	3.7	NS	4	2	4	1
sopB-o1	luxS-	49.3	71.5	71.9	36.7	5.2	0.7	6.9	6.4	3	4	3	2
SPAO	WT	28.6	39.5	117.3	57.5	5.0	1.4	8.5	NS	3	2	4	1
SPAO	luxS-	42.4	66.3	109.5	71.8	1.4	6.6	5.5	4.7	3	4	4	2
SPAP	WT	21.0	27.9	77.4	25.1	3.4	NS	6.4	0.5	3	1	4	2
SPAP	luxS-	21.2	35.7	55.8	36.9	NS	3.1	5.7	5.4	1	4	4	3
SPAR	WT	26.5	36.7	81.5	ND	6.1	0.9	9.3	NS	4	2	4	0
SPAR	luxS-	27.4	47.7	57.2	31.2	NS	5.9	12.0	NS	1	3	4	1
SPRB	WT	27.0	30.2	121.1	ND	5.1	4.4	12.8	NS	4	4	3	0
SPRB	luxS-	29.4	34.7	177.8	24.6	2.9	2.6	19.3	4.0	4	4	4	3
SPTP	WT	216.5	188.3	184.1	53.5	30.2	2.5	14.7	5.6	4	2	4	2
SPTP	luxS-	252.0	224.2	184.7	69.2	4.4	47.8	11.8	2.7	4	4	4	4
spvD-o1	WT	270.0	294.4	101.5	183.6	22.4	45.9	6.8	11.5	4	4	4	4
spvD-o1	luxS-	294.6	307.3	141.4	132.0	15.7	62.0	9.2	4.6	4	4	4	3
SUL1	WT	245.6	174.9	136.5	67.1	51.2	27.5	10.0	4.8	4	4	4	4
SUL1	luxS-	355.0	344.6	312.9	138.7	19.7	39.5	22.5	12.1	4	4	4	3
TIG	WT	317.2	90.8	13.3	ND	19.7	11.3	0.5	NS	2	2	2	0
TIG	luxS-	324.0	254.0	44.6	ND	13.9	58.6	15.9	NS	3	3	4	0
TPIA	WT	313.2	309.7	161.2	72.9	46.8	48.3	2.3	2.5	4	4	4	3
TPIA	luxS-	212.1	230.9	218.2	86.6	5.1	35.9	19.6	9.7	4	4	4	4
USPA-O	WT	155.0	81.9	192.9	330.9	23.5	10.3	3.0	111.2	4	3	4	4
USPA-O	luxS-	78.8	152.4	223.5	237.3	4.3	24.8	15.5	10.6	4	4	4	4
USPB-O	WT	26.4	ND	88.4	87.6	5.0	NS	14.6	8.3	4	0	4	2
USPB-O	luxS-	16.6	35.9	83.2	107.4	NS	4.7	12.3	4.9	1	4	4	2
uspB-o1	WT	30.0	ND	85.6	92.9	7.4	NS	10.5	2.5	3	0	4	2
uspB-o1	luxS-	16.7	32.7	63.1	103.6	NS	1.5	10.7	4.7	1	4	4	3

Spot labels[a]		Signal intensities				Standard deviations[b]				No. of datapoints			
	Time	3.5	4.5	5.5	6.5	3.5	4.5	5.5	6.5	3.5	4.5	5.5	6.5
UVRY	WT	262.1	196.8	100.2	126.5	28.4	20.4	5.4	55.4	4	4	4	4
UVRY	luxS-	193.0	263.8	109.3	112.0	12.0	26.5	12.0	8.5	4	4	4	4
virK-o1	WT	230.8	115.9	107.0	ND	40.4	13.1	10.6	NS	4	2	4	0
virK-o1	luxS-	191.7	223.5	151.0	37.2	75.4	23.1	6.7	4.0	4	4	4	4
WRAB	WT	64.7	83.1	322.1	555.2	9.8	13.4	48.3	70.4	4	3	4	3
WRAB	luxS-	48.9	90.0	246.9	411.8	6.0	4.2	10.2	99.4	4	4	4	4
YAHO	WT	24.3	35.0	78.0	95.9	3.2	NS	8.8	39.3	3	1	4	3
YAHO	luxS-	1.0	53.6	50.8	69.5	NS	6.0	8.0	NS	0	4	4	1
ybdQ-o16	WT	354.1	237.6	324.0	681.4	22.9	21.0	23.0	76.3	4	4	4	4
ybdQ-o16	luxS-	239.8	250.9	343.6	471.3	12.0	36.4	20.9	97.2	4	4	4	4
YBJX	WT	127.3	70.7	29.7	ND	39.5	3.8	7.1	NS	3	2	4	0
YBJX	luxS-	143.6	230.1	45.2	ND	18.6	9.0	9.2	NS	4	2	3	0
ydaA-o1	WT	56.1	44.4	98.0	186.2	13.1	5.1	3.8	13.3	4	2	4	4
ydaA-o1	luxS-	36.1	76.0	75.0	168.6	7.4	7.7	5.5	3.9	4	4	4	4
yecG-o1	WT	34.1	30.9	43.1	52.5	3.1	8.5	6.3	6.5	3	2	4	2
yecG-o1	luxS-	19.9	31.1	49.0	58.8	NS	2.9	28.4	6.8	1	3	3	2
YFCZ	WT	303.4	221.7	129.9	190.8	44.5	28.4	7.4	17.8	4	4	4	4
YFCZ	luxS-	382.3	341.3	154.2	239.3	21.4	70.5	10.5	12.5	4	4	4	4
YFIA-O	WT	373.9	319.1	464.3	884.5	54.6	53.9	10.5	93.8	4	4	4	4
YFIA-O	luxS-	152.9	418.5	479.7	613.4	4.8	57.3	23.4	152.1	4	4	3	4
YGAU	WT	21.3	60.8	416.8	886.4	NS	NS	47.3	99.2	3	2	4	4
YGAU	luxS-	20.8	44.2	269.5	481.5	2.3	4.9	9.5	36.1	2	2	4	4
YHBH-O	WT	173.5	227.1	62.4	5.0	25.2	49.2	2.7	NS	4	4	4	0
YHBH-O	luxS-	146.1	264.7	71.0	22.1	18.1	8.3	5.2	9.6	4	4	4	2
yhbL-o4	WT	90.6	103.4	82.6	44.7	26.5	18.2	3.3	1.6	4	3	4	3
yhbL-o4	luxS-	71.2	127.3	86.0	62.0	13.4	7.6	6.9	10.0	3	4	4	4
YHJH	WT	204.3	208.2	246.8	124.2	21.9	24.7	22.3	52.6	4	4	4	4
YHJH	luxS-	112.9	254.6	168.0	230.8	9.6	60.5	8.9	20.8	4	4	4	4
yicC-o1	WT	190.1	157.8	56.0	ND	50.4	24.1	3.1	NS	4	2	4	0
yicC-o1	luxS-	163.1	198.6	63.2	28.6	15.5	19.5	2.3	7.2	4	4	3	2
ynaF-o1	WT	391.0	170.8	209.5	415.6	67.1	29.7	5.1	15.4	4	4	4	4
ynaF-o1	luxS-	234.5	205.8	178.4	264.7	8.7	7.7	18.5	6.3	4	4	4	4

[a] grey color indicates less reliable results due to fewer datapoints; ND, not not detectable

[b] NS, no standard deviation because of only one signal intensity value obtained

FIG. 2E in color and including standard deviations

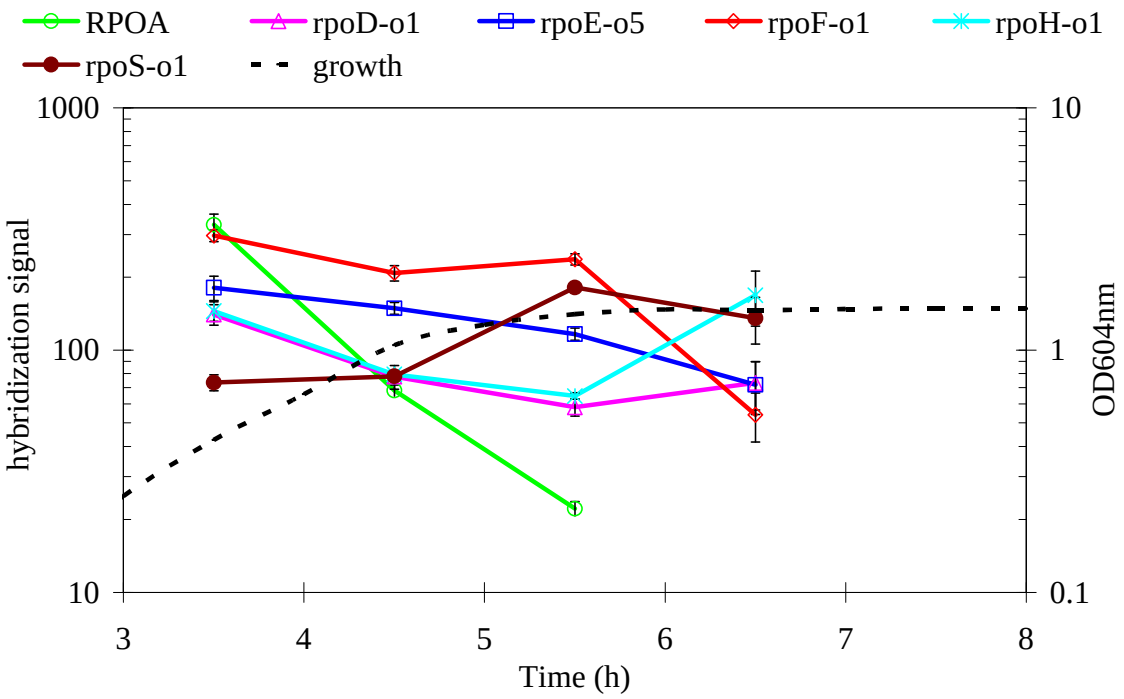


FIG. 3A in color and including standard deviations

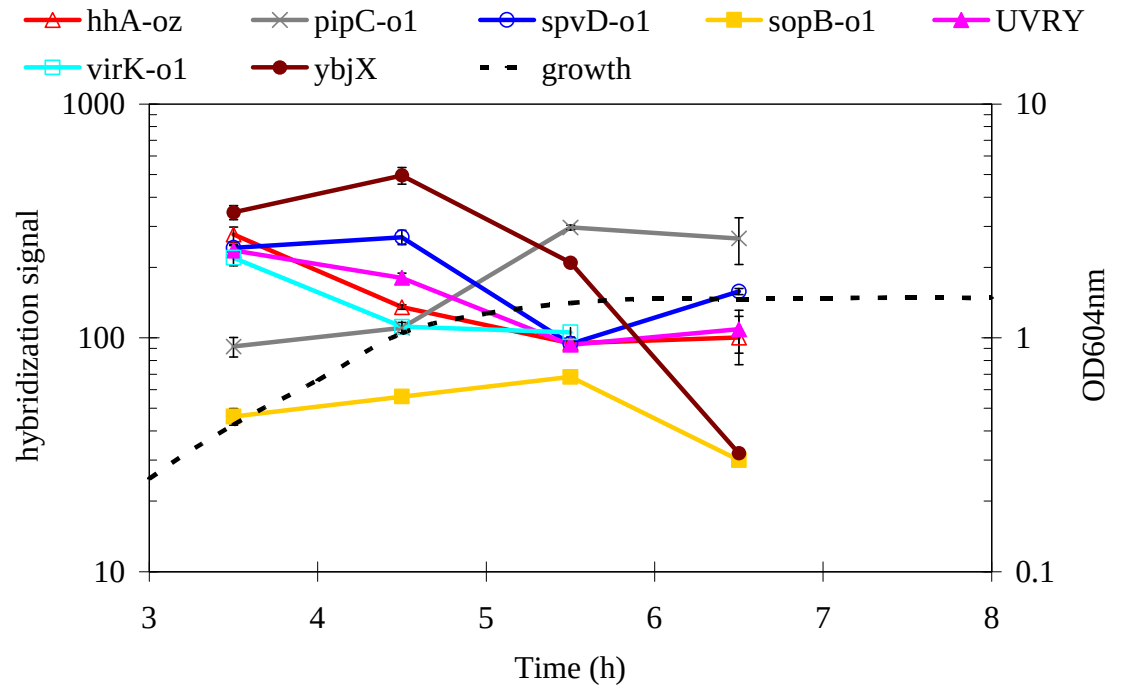


FIG. 3B in color, including standard deviations and additional genes of the same expression pattern.

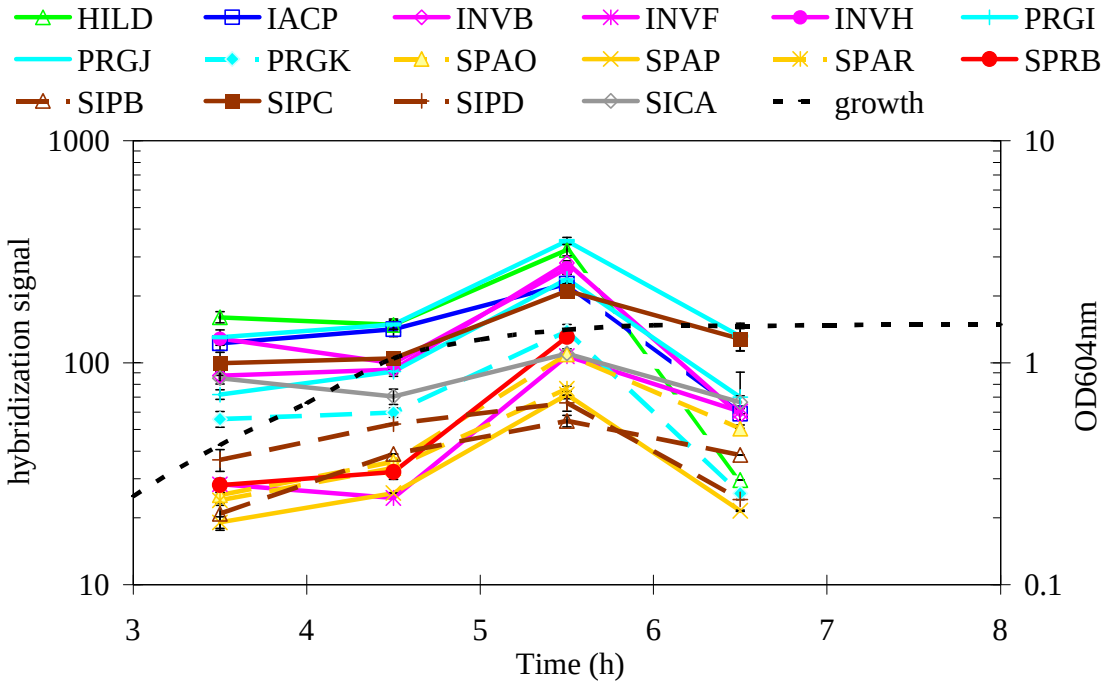


FIG. 3D in color, including standard deviations and additional genes of the same expression pattern.

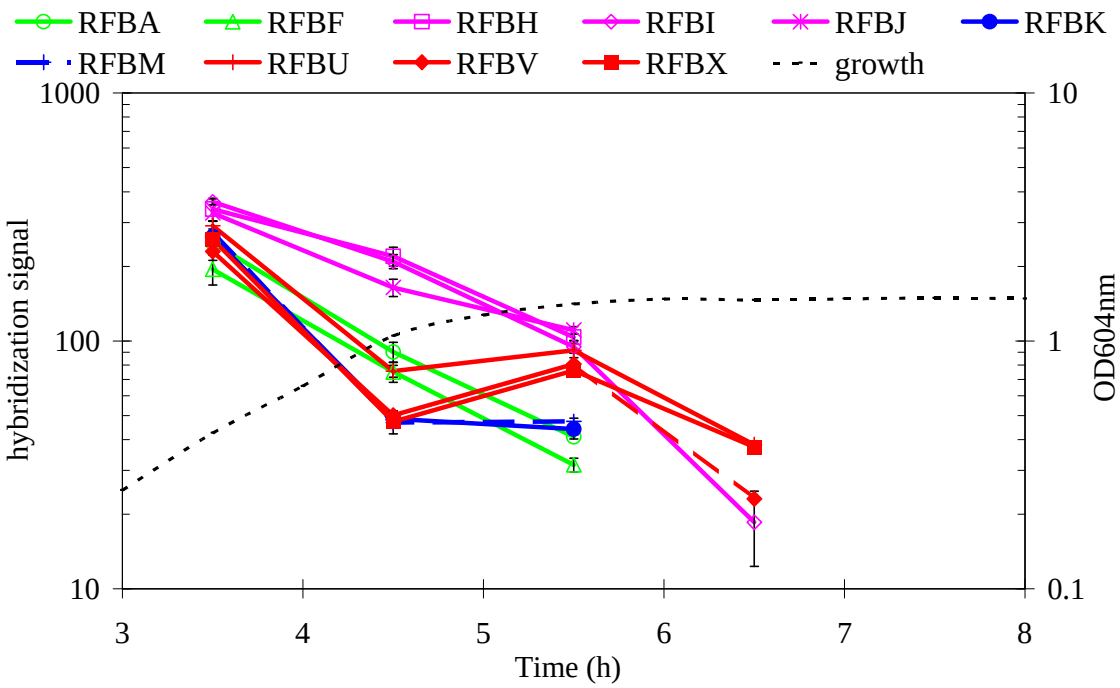


FIG. 4B in color and including standard deviations.

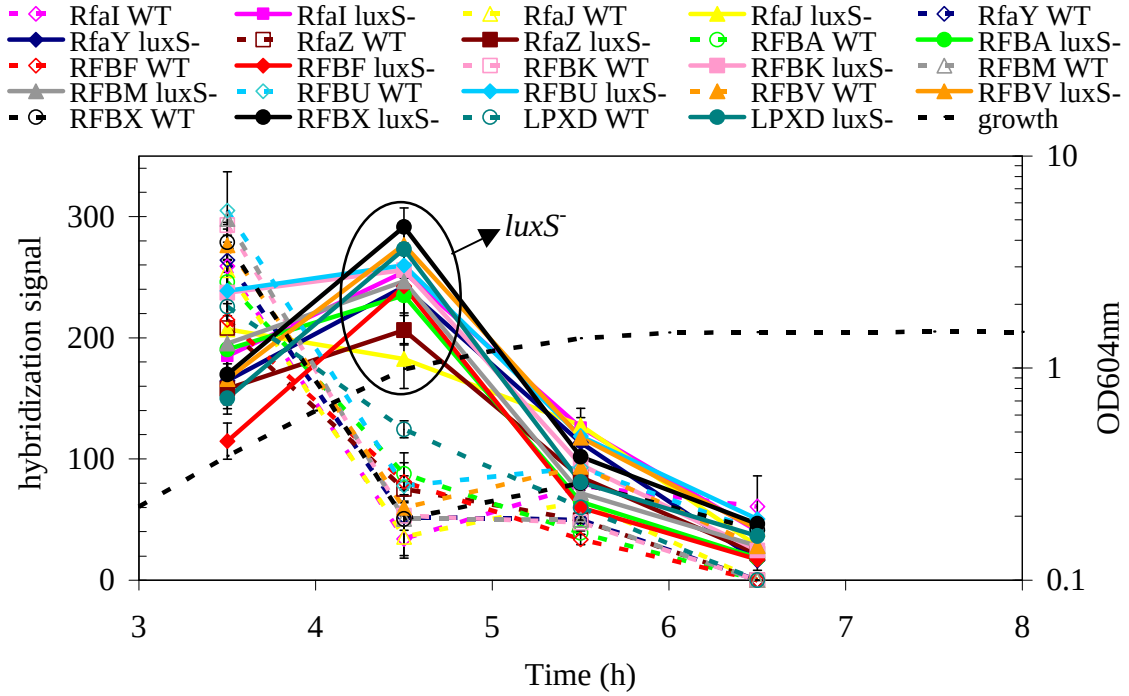
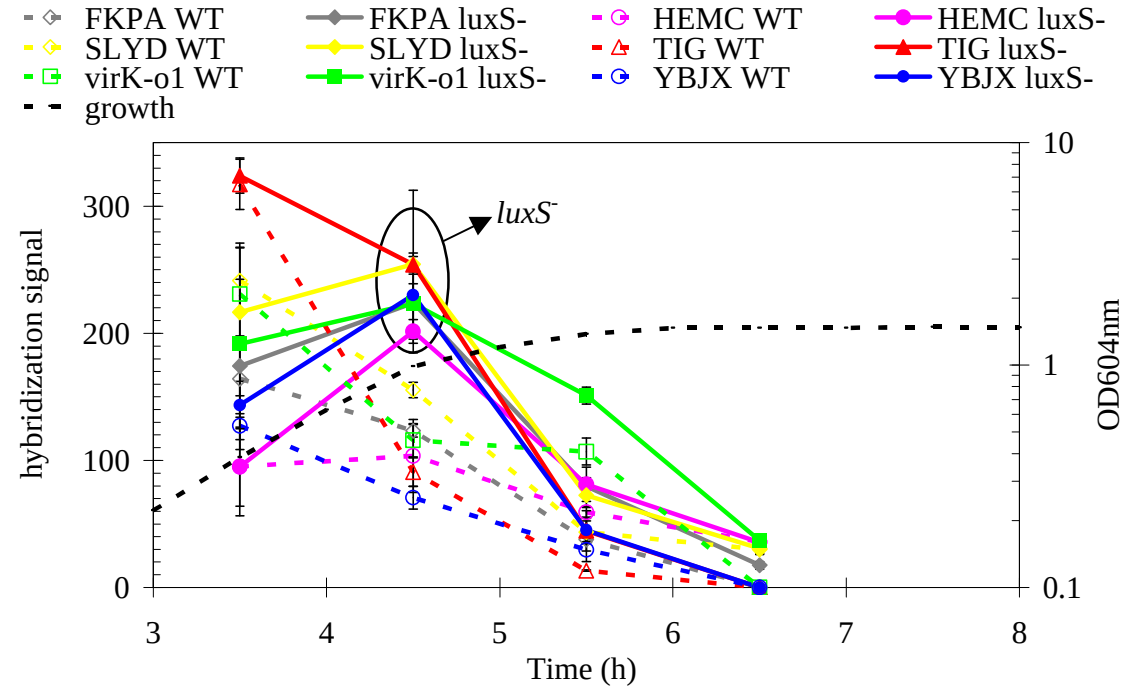


FIG. 4D in color and including standard deviations.



Supplementary material of Chapter 6

Table S1. Ratio values[a] between two conditions as depicted below exhibiting a significant (P<0.05) change between both conditions

Gene	Gene function/description	stress/non-stress for:						$-O_2/+O_2$
		Heat		H_2O_2		Acid		No stress
		$-O_2$	$+O_2$	$-O_2$	$+O_2$	$-O_2$	$+O_2$	
aada2	streptomycin/spectinomycin resistance protein			1.7		0.7	0.9	0.9
aas	bifunctional: 2-acylglycerophospho-ethanolamine acyl transferase; acyl-acyl carrier protein synthetase		0.8		0.7	0.7		
aceF	pyruvate dehydrogenase, dihydrolipoyltransacetylase component			2.4				0.4
aceK	isocitrate dehydrogenase kinase/phosphatase, also has ATPase activity							
ada	bifunctional: O6-methylguanine-DNA methyltransferase; transcription activator/repressor (AraC/Xyl family)				1.9			
adhE	alcohol dehydrogenase / acetaldehyde dehydrogenase		0.6	0.1	2.4	0.3	0.4	6.8
adi	*adiA*, arginine decarboxylase, catabolic; inducible by acid		1.8	10.3		45.1	2.3	
aidB	acyl-CoA dehydrogenase, adaptive response (transcription activated by Ada)		2.1			0.7		
alkA	3-methyl-adenine DNA glycosylase II, inducible, Ada regulated							
alkB	DNA repair system specific for alkylated DNA, interaction with ada							
argI	ornithine carbamoyltransferase 1							
atoC	putative sigma 54 dependent transcriptional regulator (=STM0652)	1.8	1.5	5.0	3.5	1.9	1.6	
atoS	sensor protein AtoS for response regulator atoC (=STM2361)	1.2		0.6		0.4	0.7	1.6
atpG	membrane-bound ATP synthase, F1 sector, gamma-subunit	1.8	0.6	0.6	0.3	0.5	0.5	0.6
avrA	putative inner membrane protein	2.1	2.5	28.0	7.2	8.7	2.3	
barA	sensory histidine kinase, transcription regulator, activator of ompR							
bigA	putative surface-exposed virulence protein							
bolA	morphogene, involved in modulating cell morphology, putative regulator of murein genes		2.8		5.1		1.5	0.3
cadA	lysine decarboxylase	1.0	1.7	1.1	1.7	3.5		1.3
cadB	lysine/cadaverine transport protein					2.6	13.7	
carA	carbamoyl-phosphate synthetase, glutamine-hydrolysing small subunit, regulated by arginine	1.3	0.4	0.1	0.1	0.1	0.1	0.7
carB	carbamoyl-phosphate synthase, large subunit, regulated by arginine	1.9	0.4	0.2			0.2	
cbpA	curved DNA-binding protein	1.0		1.7		1.6	1.4	1.7
cdsA	CDP-diglyceride synthase, sigma E regulon transcribed as a three gene operon			0.3	0.5	0.7	0.5	
cheM	methyl accepting chemotaxis protein II, aspartate sensor-receptor		0.8	1.4	2.5	0.3		
cirA	outer membrane porin, receptor for colicin I, requires TonB, putative binding site for fur							
clpB	ATP-dependent protease, Hsp 100, part of novel multi-chaperone system with DnaK, DnaJ, and GrpE	3.0	1.3	2.6	2.8	5.0		2.2
clpP	specificity component of ATP-dependent serine protease with clpX, degradates RpoS when bound to MviA	1.2		2.4	1.0	1.5		
clpX	specificity component of ATP-dependent serine protease with clpP, degradates RpoS when bound to MviA							
cobB	putative nicotinate-nucleotide dimethylbenzimidazolephosphoribosltransferase, homolog of virulence factor	0.8	0.6					
cpxP	periplasmic repressor of cpx regulon by interaction with CpxA, rescue from transitory stresses		3.5		1.2	18.1	10.9	0.9
crp	catabolite activator protein (CAP), cyclic AMP receptor protein (CRP family)	0.7	0.6	0.3	0.7	0.6	1.3	1.5
csgA	curlin major subunit, coiled surface structures, cryptic							
csgB	minor curlin subunit precursor, nucleator for assembly of adhesive surface organelles		2.8					
csiE	stationary phase inducible protein							
cspA	major cold shock protein 7.4, transcriptional activator of hns	0.4	0.5	0.6	0.2		0.5	0.0

cspC	cold shock protein, multicopy suppresses mukB mutants, putative regulator	0.5	0.7	0.2	0.2		1.3	0.6
cspD	cold shock protein, similar to CspA but not cold shock induced	2.4	1.6	0.7	1.8	0.4		
cspE	RNA chaperone, negative regulator of cspA transcription	0.3	0.3	0.2	0.4	0.3	0.4	0.5
cutC	copper homeostasis protein							
dhfrX	trimethoprim resistance protein, dihydrofolate reductase found in *Salmonella* serovar Agona			1.7				
dksA	dnaK suppressor protein, acts with ppGpp	1.3	1.5			0.5	0.8	1.4
dnaJ	chaperone protein DnaJ				1.0		0.2	2.4
dnaK	chaperone Hsp70 in DNA biosynthesis/cell division	2.6		2.1	1.2	5.8		3.5
dpS	stress response DNA-binding protein; starvation induced resistance to H_2O_2	1.2	2.1	92.5	81.6	7.9	6.3	1.6
dsbC	protein disulfide isomerase II, acting on folding of enveloppe proteins						1.3	
dsrA	a small RNA antisilencer of the H-HS-silenced rdsA gene			++	10.2			
dsrB	regulatory RNA, regulated by DsrA and HNS		1.4	10.5	4.0	3.4	1.9	0.6
emrA	multidrug resistance secretion protein, stationary phase	0.8	0.7	2.0				0.7
emrB	putative MFS superfamily, multidrug transport protein, stationary phase	0.8	0.6	0.6	0.7	1.4		
entD	enterochelin synthetase, component D			1.9	2.0		0.6	
entF	enterobactin synthetase, component F (nonribosomal peptide synthetase)							
envZ	osmolarity sensor histidine kinase in two-component regulatory system with OmpR							
fabD	malonyl-CoA-[acyl-carrier-protein] transacylase							
fadA	3-ketoacyl-CoA thiolase; (thiolase I, acetyl-CoA transferase), in complex with FadB catalyzes			2.2	3.8			
fadB	3-hydroxyacyl-coA dehydrogenase			3.3			0.8	
fadL	transport of long-chain fatty acids; sensitivity to phage T2, putative binding site for ompR	0.6	0.4	0.4			0.1	0.3
fadR	negative regulator for fad regulon and positive activator of fabA (GntR family)			2.4				
fhuA	outer membrane protein receptor / transporter for ferrichrome, colicin M, and phages T1, T5, and phi80			8.5			0.04	0.1
fic	cell filamentation protein, stationary phase induced gene, affects cell division						1.5	
fis	site-specific DNA inversion stimulation factor, represses rpoS expression	1.0	0.6	0.03	0.2	0.1	0.2	0.5
fklB	FKBP-type 22KD peptidyl-prolyl cis-trans isomerase (rotamase)	0.6	0.3	0.1	0.4	0.4	0.5	
fkpA	FKBP-type peptidyl-prolyl cis-trans isomerase (rotamase), acting on folding of enveloppe proteins	0.6	0.4	0.3	0.6	4.3	3.6	
fldA	flavodoxin 1, putative binding site for fur		0.9	0.3		0.8		0.8
flgM	anti-FliA (anti-sigma) factor; also known as RflB protein		0.9	2.7	3.5	1.1	2.3	
flhD	regulator of flagellar biosynthesis, acts on class 2 operons, putative binding site for ompR	1.6	1.6	0.2	0.7	0.8	0.5	1.4
fliC	flagellar biosynthesis, flagellin, filament structural protein, phoPQ represses transcription of fliC	1.1	1.1	3.3	1.6	1.0		
flo	chloramphenicol and florfenicol resistance protein	0.9	0.8	0.8	0.8			0.8
folA	dihydrofolate reductase type I, trimethoprim resistance	0.7	0.9	0.2		1.5		0.8
folE	GTP cyclohydrolase I		0.6	5.0	1.4		0.9	0.5
ftsA	ATP-binding cell division protein, septation process, complexes with FtsZ, junctions of inner and outer membranes	1.5			1.3		0.8	
ftsK	cell division protein, required for cell division and chromosome partitioning, regulates UspA	0.9	0.6			0.6	0.8	
ftsN	essential cell division protein		0.6	0.6				
ftsQ	cell division protein; ingrowth of wall at septum		0.9	0.7	0.6	0.6	0.7	0.6
ftsZ	tubulin-like GTP-binding protein and GTPase, forms circumferential ring in cell division		0.9		0.9			
fur	major iron regulator	0.9	0.9	3.5	1.8		0.8	0.4
gabP	APC family, gamma-aminobutyrate transport protein	1.3		1.8				
galK	galactokinase		0.9	1.1	1.0			1.4
gapA	glyceraldehyde-3-phosphate dehydrogenase A		0.4	0.4	0.6	2.5	0.9	1.9
glgB	1,4-alpha-glucan branching enzyme	1.5	1.5		1.7	1.4	2.7	

glgC	glucose-1-phosphate adenylyltransferase		1.3		2.8		1.4	1.4
glgS	glycogen biosynthesis, rpoS dependent		2.1	6.3		1.2		
gmk	guanylate kinase		0.7		0.8		0.9	0.6
groEL/intI1	GroEL/integrase fusion protein			3.6		2.1		
grpE	molecular chaparone, heat shock protein	2.0	1.1	0.6	1.0	3.4		2.2
grxB	glutaredoxin 2		1.3		1.9		1.7	1.3
gst	glutathionine S-transferase			1.8	1.7	0.6		1.2
hemC	Hydroxymethylbilane synthase			2.0	1.3	0.8		
hfq	host factor I, RNA-binding protein, essential for translation of rpoS			1.3		0.6		
hhA	hemolysin expression modulating protein (involved in environmental regulation of virulence factors)	2.6	2.0	5.5	2.1	4.4	1.9	
hilA	invasion genes transcription activator		1.3			3.8		
hilC	bacterial regulatory helix-turn-helix proteins, araC family		2.9		1.6	13.2	1.7	1.9
hilD	regulatory helix-turn-helix proteins, araC family	1.4	4.9	1.6		1.4	1.1	
hlpA	histone-like protein, located in outer membrane, acting on folding of enveloppe proteins	0.7	0.5	0.8	0.5		0.9	
hnR	Response regulator in protein turnover: mouse virulence		1.3	1.6		1.4		0.8
hns	DNA-binding protein, binds to HF-1protein, preventing rpoS translation		0.9		0.8	1.4	0.8	0.7
hscA	chaperone, member of Hsp70 protein family, believed to be involved in assembly of Fe-S clusters	0.6	0.3	0.5			0.6	0.9
hscC	putative heat shock protein, homolog of hsp70 in Hsc66 subfamily	1.4	2.4					1.3
hslU	ATPase component of the HslUV protease, rpoH controlled heat shock response	1.0		0.4		4.5		1.8
hslV	peptidase component of the HslUV protease, rpoH controlled heat shock response	1.2				4.6	0.2	1.4
htgA	positive regulator for sigma H (sigma 32) promoters, permitting growth at high temperature		1.1	4.5	5.3	2.2	2.6	
htpG	chaperone Hsp90, heat shock protein C	4.3	1.3	0.8	1.0	4.8		6.5
htrA	periplasmic serine protease Do, heat shock protein, transcribed by rpoE	1.3	1.7		0.5	10.1	7.9	1.4
hycA	transcriptional repressor of hyc and hyp operons	0.2				3.3		
hycB	hydrogenase-3, iron-sulfur subunit (part of FHL complex)	0.5	0.6	0.3				4.4
hycC	hydrogenase 3, membrane subunit (part of FHL complex)							
hycD	hydrogenase 3, membrane subunit (part of FHL complex)					0.9		
hycE	hydrogenase 3, large subunit (part of FHL complex)							
hycF	hydrogenase 3, putative quinone oxidoreductase	0.3		0.4		1.9		7.3
hycG	hydrogenase activity		1.3					
hycH	processing of HycE (part of the FHL complex)	0.4		0.6		1.5		4.7
hycI	protease involved in processing C-terminal end of HycE							
hypA	guanine-nucleotide binding protein in formate-hydrogenlyase system, nickel donor for HycE of hydrogenlyase 3	0.5		0.6		1.6		
hypB	hydrogenase-3 accessory protein, assembly of metallocenter						2.1	2.4
hypC	putative hydrogenase expression/formation protein			0.3			2.4	
hypD	putative hydrogenase expression/formation protein						1.7	
hypE	putative hydrogenase expression/formation protein		0.9	0.5		0.7		2.0
hypO	putative Ni/Fe hydrogenases, small subunit		1.9	0.1		0.2		11.4
iacP	putative acyl carrier protein, invasion	1.8	3.3		0.3		0.5	0.3
iagB	cell invasion protein	1.1	3.5					1.4
invA	invasion protein	0.6				0.7		
invB	surface presentation of antigens; secretory proteins		1.7		0.7	0.9	0.5	2.5
invC	surface presentation of antigens; secretory proteins	0.6	1.6					2.1
invE	invasion protein							

invF	invasion protein, regulates SPI-1			1.6			0.7	0.9
invG	invasion protein; outer membrane						0.6	
invH	invasion protein	0.7	0.8		0.4		0.7	
invI	surface presentation of antigens; secretory proteins						0.5	
invJ	surface presentation of antigens; secretory proteins	0.7	1.5					2.0
katE	catalase, hydroperoxidase HPII(III)	1.8	1.2	3.9	3.0	2.8	1.6	
katG	catalase, hydroperoxidase HPI(I)			184.8	170.4			1.6
kdtA	3-deoxy-D-manno-octulosonic-acid transferase (KDO transferase)		1.9	2.7		0.6		1.4
kdtB	phosphopantetheine adenylyltransferase		0.6					
lon	DNA-binding, ATP-dependent protease la; cleaves RcsA and SulA, heat shock k-protein (DNA binding activity)					1.5		1.7
lpxA	UDP-N-acetylglucosamine acetyltransferase, lipid biogenesis	0.9	1.1	0.5	0.7	0.4	0.6	
lpxD	UDP-3-O-(3-hydroxymyristoyl)-glucosamine n-acyltransferase, lipid biogenesis	0.7	0.8	0.6	0.6	0.8	0.7	0.6
lrhA	NADH dehydrogenase transcriptional repressor (LysR family), indirect control on mviA			2.0				0.8
lrp	Leucine-responsive regulatory protein, putative binding site for ompR, stationairy phase & rpoS regulator			3.1			1.1	
luxS	quorum sensing protein, produces autoinducer - acyl-homoserine lactone-signaling molecules			1.4			0.8	
lysP	cadR; APC family, lysine-specific permease	0.9	0.7	0.6	0.4	0.4	0.6	0.6
marA	transcriptional activator of defense systems (AraC/XylS family), multiple antibiotic resistance protein		3.4		0.5		5.2	
marB	multiple antibiotic resistance protein	1.9	3.3			14.9	6.5	0.8
marC	putative MarC Transporter, multiple antibiotic resistance protein				2.0			
marR	transcriptional repressor of marRAB operon, multiple antibiotic resistance protein	2.0	3.7	2.1	0.6	20.6	5.5	0.6
mdh	malate dehydrogenase			1.7	1.9	0.3	1.1	
mdoG	periplasmic glucans biosynthesis protein	0.9	0.5	0.4	0.8		1.0	
mdoH	membrane glycosyltransferase; synthesis of membrane-derived oligosaccharide (MDO)/synthesis of OPGs		0.6				0.8	0.8
metE	5-methyltetrahydropteroyltriglutamate- homocysteine S-methyltransferase			1.8				
mgtA	P-type ATPase, Mg2+ ATPase transporter	1.3	6.5			0.6		
mgtB	Mg2+ transport protein	1.1	17.9	1.3		1.7	6.8	
modE	transcriptional repressor of modABCD operon (molybdate uptake)			1.2				
mopA	chaperone Hsp60 with peptide-dependent ATPase activity, affects cell division	4.6	1.8		1.0	2.8	1.0	5.8
mopB	chaperone Hsp10, affects cell division	4.8	2.2			3.0		5.0
mreB	rod shape-determining protein; HSP70 class molecular chaperones involved in cell morphogenesis		0.8	0.2	0.4	0.6	0.6	
msgA	Macrophage survival gene; reduced mouse virulence	1.6	2.2	4.7	3.7			
mtlD	mannitol-1-phosphate dehydrogenase	1.2	1.5			1.2		1.8
mukB	kinesin-line cell division protein involved in sister chromosome partitioning		0.6	0.5	0.4	0.4	0.5	
mutS	methyl-directed mismatch repair, recognize exocyclic adducts of guanosine		1.2	2.4				
mviM	putative virulence factor		0.5				0.7	
mviN	putative virulence factor	1.5		0.7	0.8		0.8	
narV	nitrate reductase gamma chain							
narW	nitrate reductase delta chain							
narY	nitrate reductase beta chain							
narZ	nitrate reductase alpha chain						1.5	
nfnB	dihydropteridine reductase/oxygen-insensitive NAD(P)H nitroreductase	0.8	0.5					1.4
nlpB	lipoprotein-34	0.7	0.6	0.2	0.5	0.4	0.8	
nlpD	lipoprotein (upsteam of rpoS)			10.2	2.0	2.9	1.5	0.2
oafA	O-antigen five: acetylation of the O-antigen (LPS)			0.1		0.2	0.3	0.4
oat	putative acetylornithine aminotransferase				2.7		2.0	

ogt	O-6-alkylguanine-DNA/cysteine-protein methyltransferase, stationary phase		1.4	4.2	2.0	7.8	3.0	
ompC	outer membrane protein 1b (ib;c), porin		0.4		0.7	0.1	0.2	
ompF	outer membrane protein 1a (ia;b;f), porin	0.8	0.8	1.3	0.7	1.6	2.6	
ompR	stationary phase transcription response regulator in two-component regulatory system with EnvZ		1.0	2.4	0.8		0.5	0.1
orf242	putative regulatory proteins, merR family							
orf245	putative cytoplasmic protein							
orf319	putative inner membrane protein							
orf32	putative hydrolase or acyltransferase							
orf408	putative regulatory protein, deoR family							
orf48	putative amino acid permease					2.8		
orf7	putative bacterial regulatory proteins, luxR family		3.8					
orf70	putative cytoplasmic protein	0.8			0.8	0.6	0.6	0.4
orgA	putative flagellar biosynthesis/type III secretory pathway protein		0.9	0.2	0.4	1.7		0.6
osmB	osmotically inducible lipoprotein			2.0	3.4	4.9	6.0	0.8
osmC	resistance protein, osmotically inducible		1.5	2.8	3.6	2.1	1.6	
osmY	hyperosmotically inducible periplasmic protein, stationary phase	1.5	2.5	16.8	6.1	7.1	5.1	0.5
otsA	trehalose-6-phosphate synthase, stationary phase	1.4	1.3	3.4	2.6	2.4	2.0	
otsB	trehalose-6-phosphate phophatase, biosynthetic, stationary phase		2.4	6.4	6.1	3.4	3.4	
oxyR	regulatory protein sensor for oxidative stress, regulates intracellular hydrogen peroxide (LysR family)	1.4	1.1	2.4	2.1	0.9	0.5	
oxyS	stable RNA induced by oxidative stress	0.8		++	1.9			
pagC	putative outer membrane protein, virulence gene						2.1	
pagD	PhoP regulated, virulence gene							
pagK	PhoPQ-activated gene		2.5				10.2	
pagO	PhoPQ-activated gene; predicted integral membrane protein		1.7				5.6	
pagP	PhoPQ-activated gene			0.04	0.3	2.2	2.2	
pgi	glucosephosphate isomerase, synthesis of organic acids		1.2		1.5			1.7
phoP	response regulator in two-component regulatory system with PhoQ, transcribes genes expressed under low Mg+	1.5	1.9	1.4		1.7	3.2	
phoQ	sensory kinase protein in two-component regulatory system with PhoP, ligand is Mg+		0.9	0.4	0.8	1.6	2.0	0.8
phoU	regulatory gene for high affinity phosphate uptake			1.7			0.3	
pipA	Pathogenicity island encoded protein: SPI3			1.1		2.9		
pipB	Pathogenicity island encoded protein: SPI3	1.8	6.7	4.2	1.5	9.4	8.0	
STM2780	homologue of pipB, putative pentapeptide repeats (8 copies)			2.7	1.2	1.4		0.6
pipC	Pathogenicity island encoded protein: homologous to ipgE of Shigella	2.0	3.1	2.3		1.2		
pipD	Pathogenicity island encoded protein: SPI3		1.6		2.3			
pmrA (=basR)	response regulator in two-component regulatory system with BasS (OmpR family)	0.3	0.4	0.6	0.6	1.7	3.1	0.6
pmrB (=basS)	sensory kinase in two-component regulatory system with BasR	0.4	0.3	1.4	0.6	2.4		0.5
pmrD	polymyxin resistance protein B		1.5	2.1	2.8	2.4	5.6	
pmrF	putative glycosyl transferase, PhoPQ regulated via PmrAB	0.3	0.2		0.5	5.8	3.6	
polA	DNA polymerase I			1.6	1.6	0.6	0.9	
potE	APC family, putrescine/ornithine antiporter	0.3		0.5		0.5		
poxB	pyruvate dehydrogenase/oxidase FAD and thiamine PPi cofactors, cytoplasmic in absence of cofactors							
pphB	serine/threonine specific protein phosphatase 2		1.5	1.5	0.6		0.8	0.5
ppiA	peptidyl-prolyl cis-trans isomerase A (rotamase A)			0.4	0.6	2.1		0.7

ppiB	peptidyl-prolyl cis-trans isomerase B (rotamase B)	1.0	0.8	1.2		1.2	0.9	0.8
ppiC	peptidyl-prolyl cis-trans isomerase C (rotamase C)	0.6	0.7	0.5			0.6	0.4
pqaA	PhoPQ-regulated protein	1.1	1.5			8.0	9.0	0.8
prgH	cell invasion protein	0.4					0.9	
prgI	cell invasion protein; cytoplasmic	0.7	0.9			0.5	0.6	2.1
prgJ	cell invasion protein; cytoplasmic					0.7		2.1
prgK	cell invasion protein; lipoprotein, may link inner and outer membranes	0.6			0.9	0.6	0.7	2.2
proP	MFS family, low-affinity proline transporter (proline permease II)	1.4	3.0	3.1	1.3	2.7	2.2	
proV	ABC superfamily (atp_bind), glycine/betaine/proline transport protein, stationary phase	1.3			0.3	0.5		0.6
proW	ABC superfamily (membrane), glycine/betaine/proline transport protein, stationary phase			1.4				
proX	ABC superfamily (bind_prot), glycine/betaine/proline transport protein, stationary phase				0.3			0.4
pse-1	beta-lactamase Pse-1 precursor	1.4	2.2	3.1	1.5	1.5	1.5	0.7
purA	adenylosuccinate synthetase		0.6	0.5		0.6	0.8	
purD	phosphoribosylglycinamide synthetase (GAR synthetase)	0.8	1.0	0.5	0.6	0.7	0.6	0.7
ramA	putative regulatory protein, resistance against oxidative stress				0.8			
rcK	resistance to complement killing	2.2		2.0			0.7	
rcsA	positive transcriptional regulator of capsular/exo- polysaccharide synthesis (LuxR/UhpA family)	0.7	1.7	1.2	1.7	2.8	7.3	0.6
rcsB	response regulator (positive) in two-component regulatory system with RcsC (LuxR/UhpA familiy)	0.8	0.4			1.3		
rcsC	sensory histidine kinase in two-component regulatory system with RcsB, regulates colanic capsule biosynthesis	0.6				0.6		
recD	exonuclease V, alpha chain		0.8					
relA	ppGpp synthetase I (GTP pyrophosphokinase), ppGpp act as positive signal for rpoS transcription		1.3	1.1			1.6	1.2
rep	replication protein		1.2		0.5		0.7	
res (tmpr)	resolvase	1.8	5.1	1.2				0.4
rfaB	UDP-D-galactose:(glucosyl)lipopolysaccharide-1,6-D-galactosyltransferase	0.8	0.5					1.3
rfaC	heptosyl transferase I	0.8	0.4	0.2		0.4	0.6	
rfaD	ADP-L-glycero-D-mannoheptose-6-epimerase, lipid biogenesis		0.8			0.7	0.9	
rfaE	bifunctional; putative sugar nucleotide transferase domain of ADP-L-glycero-D-manno-heptose synthase			0.3		0.4		2.2
rfaF	ADP-heptose; LPS heptosyltransferase 1		0.7	0.5	0.5	0.3	0.6	
rfaG	glucosyltransferase I		1.0	0.5	0.9		0.6	0.4
rfaH	transcriptional activator affecting biosynthesis of lipopolysaccharide core, F pilin, and haemolysin			0.7		0.5	0.5	0.6
rfaI	UDP-D-galactose:(glucosyl)lipopolysaccharide-alpha-1,3-D-galactosyltransferase		1.0	0.5	0.8		0.5	0.4
rfaJ	UDP-D-glucose:(galactosyl)lipopolysaccharide glucosyltransferase		0.9	0.4	0.7	0.6	0.6	0.4
rfaK	putative hexose transferase, lipopolysaccharide core biosynthesis	0.8	0.9	0.5	0.7	0.4	0.8	
rfaL	O-antigen ligase	0.7	0.8	0.3	0.9	0.6	1.0	0.7
rfaP	lipopolysaccharide core biosynthesis; phosphorylation of core heptose	0.8	0.6	0.5	0.7		0.7	0.6
rfaQ	lipopolysaccharide core biosynthesis; modification of heptose region of core		0.6	0.3		0.7	0.6	0.6
rfaY	lipopolysaccharide core biosynthesis; modification of heptose region of the core	0.8	0.7	0.2	0.6		0.6	0.5
rfaZ	lipopolysaccharide core biosynthesis		0.7		0.6			0.5
rfbA	dTDP-glucose pyrophosphorylase	0.6	0.6	0.3	0.4	0.6	0.4	0.5
rfbB	dTDP-glucose 4,6 dehydratase	0.8	0.6	0.2	0.5	0.4	0.5	0.6
rfbC	dTDP-4,deoxyrhamnose 3,5 epimerase	0.7	0.8	0.3	0.4	0.3	0.5	0.6
rfbD	TDP-rhamnose synthetase	0.7	0.5	0.4	0.4	0.4	0.5	0.7
rfbF	LPS side chain defect: glucose-1-phosphate cytidylyltransferase	0.5	0.6	0.5	0.3	0.4	0.4	0.6
rfbG	LPS side chain defect: CDP glucose 4,6-dehydratase	0.7	0.7	0.5	0.4	0.3	0.5	0.7
rfbH	LPS side chain defect: CDP-6deoxy-D-xylo-4-hexulose-3-dehydrase	0.7	0.8	0.7	0.3	0.6	0.6	

rfbI	LPS side chain defect: CDP-6-deoxy-delta3,4-glucoseen reductase	0.8	0.8	0.2	0.3	0.3	0.4	0.6
rfbJ	LPS side chain defect: CDP-abequose synthase	0.7		0.6	0.4		0.6	
rfbK	LPS side chain defect: phosphomannomutase	0.8	0.7	1.6	0.9	0.7	0.8	0.6
rfbM	LPS side chain defect: mannose-1-phosphate guanylyltransferase	0.7	0.6	0.7	0.5		0.6	0.5
rfbN	LPS side chain defect: rhamnosyl transferase	0.8	0.8		0.6	0.6	0.7	0.4
rfbP	LPS side chain defect: bifunctional enzyme, undecaprenol-phosphate galactosephosphotransferase/O-antigen transfer	0.7	0.6	0.6	0.6	0.7	0.6	0.5
rfbU	LPS side chain defect: mannosyl transferase		0.9	1.4	0.7	0.7	0.7	0.4
rfbV	LPS side chain defect: abequosyltransferase	0.9	1.0	0.8	0.7		0.9	0.4
rfbX	LPS side chain defect: putative O-antigen transferase			0.8	0.8	0.7	0.8	0.4
rpoA	DNA-directed RNA polymerase subunit alpha	0.9	1.1	0.5	0.3	0.1	0.3	0.8
rpoB	RNA polymerase, beta subunit	0.9	0.7	0.4	0.4	0.3	0.4	
rpoC	RNA polymerase, beta prime subunit	0.7	0.8	0.5	0.3	0.3	0.7	
rpoD	sigma D (sigma 70) factor of RNA polymerase, major sigma factor during exponential growth		0.4	0.7		5.8		
rpoE	sigma E (sigma 24) factor of RNA polymerase, response to periplasmic stress, also important in stationary phase	0.9	0.9		1.6	5.5	2.8	1.2
rpoF (=fliA)	sigma F (sigma 28) factor of RNA polymerase		0.3	1.8		0.4	0.7	2.2
rpoH	sigma H (sigma 32) factor of RNA polymerase	1.7	1.8	1.5	1.5	2.4	2.3	1.4
rpoN	sigma N (sigma 54) factor of RNA polymerase		0.4	0.4	0.6	1.4		0.6
rpoS	sigma S (sigma 38) factor of RNA polymerase, major sigmafactor during stationary phase	1.4	1.3	4.0	1.5	1.3	1.2	0.4
rpoZ	RNA polymerase, omega subunit		0.4	0.6	0.6	0.7	0.7	
rprA	regulatory RNA							
rsD	regulator of sigma D, has binding activity to the major sigma subunit of RNAP		1.0		6.1		2.1	
rseA	anti sigma E (sigma 24) factor, negative regulator	0.9	0.7			8.0	4.6	1.2
rseB	anti sigma E (sigma 24) factor, negative regulator			1.5		3.0	1.7	1.6
rseC	regulator of sigma E (sigma 24) factor	0.8	0.8		1.6	1.7	1.5	0.7
rt	reverse transcriptase				0.3		0.6	1.3
rtcR	sigma N (sigma 54)-dependent regulator of rtcBA expression (EBP familiy)	2.3		4.5	++	2.4	1.4	
sdiA	transcriptional regulator of ftsQAZ gene cluster (LuxR/UhpA family), regulator of rck operon on virulence plasmid		0.5	5.6	2.3	0.5		0.6
sicA	surface presentation of antigens; secretory proteins		1.3	3.1				1.8
sicP	chaparone, related to virulence on SPI, acts as a specific chaperone for SptP	1.8	3.6	1.2	0.3		0.5	0.3
sipA	cell invasion protein		1.8				0.7	2.2
sipB	cell invasion protein	1.6	1.2	1.9		0.6	0.6	2.5
sipC	cell invasion protein		1.5	1.7	1.3		0.9	4.4
sipD	cell invasion protein		1.6		0.8	0.6	0.7	3.0
sitA	Salmonella iron transporter: fur regulated		0.2	310.1	31.3		0.5	
sitB	Salmonella iron transporter: fur regulated			++	20.5			
sitC	Salmonella iron transporter: fur regulated			++	++		0.6	
sitD	Salmonella iron transporter: fur regulated	0.6	0.7	4.4	5.2			0.8
slyD	FKBP-type peptidyl prolyl cis-trans isomerase (rotamase)	0.6	0.5	0.8	0.9			
sodA	superoxide dismutase, manganese, putative binding site for fur		0.3	159.0	3.6	2.7		0.0
sodB	superoxide dismutase, iron, underexpressed	1.3			0.2	1.3	1.6	
sodC	Gifsy-2 prophage: superoxide dismutase precursor (Cu-Zn), copper/zinc superoxide dismutase		1.3	1.6	1.4	2.1	4.3	1.6
sopB	Salmonella outer protein: homologous to ipgD of Shigella		2.4	2.2			0.7	

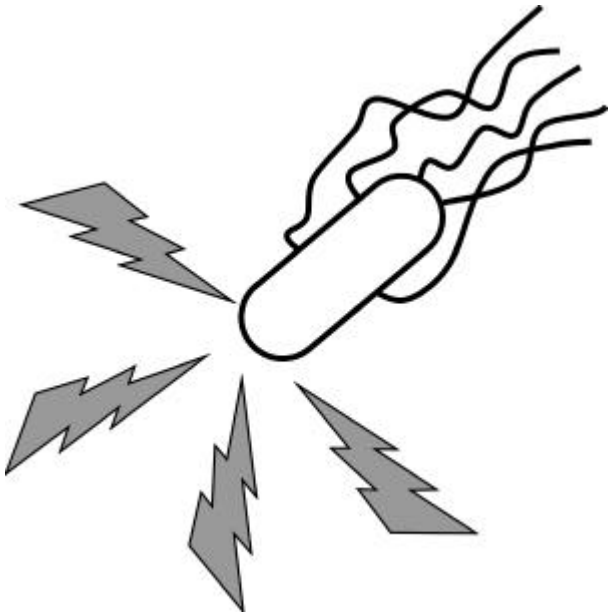
sopE2	TypeIII-secreted protein effector: invasion-associated protein	1.1	1.4	2.4	1.9			
spaO	surface presentation of antigens; secretory proteins		1.8					
spaP	surface presentation of antigens; secretory proteins	0.7	3.1				0.6	1.7
spaQ	surface presentation of antigens; secretory proteins							
spaR	surface presentation of antigens; secretory proteins				0.5		0.6	
spaS	surface presentation of antigens; secretory proteins							
speF	ornithine decarboxylase isozyme, inducible	0.5	2.1		1.5	4.2	1.2	2.7
spoT	bifunctional : ppGpp synthetase II; also guanosine-3',5'-bis pyrophosphate 3'-pyrophosphohydrolase		0.4	0.2	0.4	0.5	0.4	
sprB	transcriptional regulator		2.9	1.3	1.8	4.6	1.7	1.8
sptP	protein tyrosine phosphate	2.3	2.2		0.3	0.7	0.4	0.4
spvA	Salmonella plasmid virulence: outer membrane protein			4.6				0.1
spvB	Salmonella plasmid virulence: hydrophilic protein			2.9				
spvC	Salmonella plasmid virulence: hydrophilic protein							
spvD	Salmonella plasmid virulence: hydrophilic protein	1.0	0.6	1.7				
spvR	Salmonella plasmid virulence: regulation of spv operon, lysR family		12.1	2.0				
srgA	sdiA-regulated gene; putative thiol-disulfide isomerase or thioredoxin							
srgB	sdiA-regulated gene; putative outer membrane protein		1.6					
srgC	sdiA-regulated gene;putative bacterial regulatory helix-turn-helix proteins, araC family	1.6	4.0	2.7	1.7	1.3	1.8	
ssaB	Secretion system apparatus	0.7					0.7	
ssaC	Secretion system apparatus			7.6				
ssaD	Secretion system apparatus	0.6						
ssaE	Secretion system effector		3.4					
ssaG	Secretion system apparatus			1.7				
ssaH	Secretion system apparatus							
ssaI	Secretion system apparatus							
ssaJ	Secretion system apparatus: homology with the yscJ/mxiJ/prgK family of lipoproteins							
ssaK	Secretion system apparatus			1.6				
ssaL	Secretion system apparatus						0.7	
ssaM	Secretion system apparatus							
ssaN	Secretion system apparatus: homology with the YscN family of proteins							
ssaO	Secretion system apparatus							
ssaP	Secretion system apparatus	1.2	++	1.3				0.7
ssaQ	Secretion system apparatus	1.2			0.8		0.8	0.3
ssaR	Secretion system apparatus: homology with YscR of the secretion system of *Yersinia*	2.2	3.2					
ssaS	Secretion system apparatus: homology with YscS of the secretion system of *Yersinia*		++					
ssaT	Secretion system apparatus: homology with YscT of the secretion system of *Yersinia*		2.6			1.4		
ssaU	Secretion system apparatus: homology with YscU of the secretion system of *Yersinia*		++					
ssaV	Secretion system apparatus: homology with the LcrD family of proteins		2.2	2.5		1.4		
sscA	Secretion system chaparone		++	1.5				
sscB	Secretion system chaparone		++					
sseA	Secretion system effector		4.5					
sseB	Secretion system effector, enhances serine sensitivity	2.5	3.2	3.2		3.1		
sseC	Secretion system effector	1.0	4.9			1.5		0.5
sseD	Secretion system effector	1.6	2.5	3.9				
sseE	Secretion system effector	1.5	2.6			1.5		1.2

sseF	Secretion system effector							
sseG	Secretion system effector		++					
sspA	stringent starvation protein A, regulator of transcription		0.8	0.6		0.6		
sspB	stringent starvation protein B	0.8	0.6	0.5	0.7		0.9	
sspH2	Leucine-rich repeat protein, induced by the SPI-2 regulator ssrA/B							
sspJ (=yfgL)	putative serine/threonine protein kinase, necessary for resistance against superoxide and replication within macrophages	0.7	0.5	0.5	0.5	0.5	0.5	
ssrA	secretion system regulator,sensor component, regulates SPI-2		2.2				2.4	
ssrB	secretion system regulator, transcriptonal activator, homologous with degU/uvrY/bvgA, regulates SPI-2	2.2	3.0	5.1		3.7	4.7	0.7
stiA	putative fimbrial subunit				1.6			
stiB	putative fimbrial chaparone							
stiC	putativie fimbrial usher	0.7	0.8	0.7	0.4	0.6	0.6	
STM4463	putative arginine repressor							
STM4464	putative arginine repressor							
STM4465	putative ornithine carbamoyltransferase							
STM4466	putative carbamate kinase							
STM4467	putative arginine deiminase							
sul1	sulfonamide resistance protein			0.9		0.6	1.6	
surA	peptidyl-prolyl cis-trans isomerase, survival protein, acting on folding of enveloppe proteins		0.6	2.0			0.8	
tet(G)	tetracycline resistance protein		1.2		1.3			
tetR	tetracycline resistance regulator protein	1.4		3.7		2.4		
tig	peptidyl-prolyl cis/trans isomerase, trigger factor; a molecular chaperone involved in cell division	0.6	0.6	0.4	0.4	0.4	0.4	0.8
tnpa	transposase		2.4		1.8		1.4	0.7
tonB	energy transducer; uptake of iron, cyanocobalimin; sensitivity to phages, colicins, putative binding site for fur		0.2	2.9			0.3	
tpiA	triosephosphate isomerase		0.5	0.3	0.7	1.4		1.5
treA	trehalase, periplasmic, stationary phase	1.6	1.9		2.5	0.5	1.6	
ttrA	Tetrathionate reductase complex, subunit A							
ttrB	Tetrathionate reductase complex, subunit B		4.2					
ttrC	Tetrathionate reductase complex, subunit C			0.6				
ttrR	Tetrathionate reductase complex: response regulator	0.5						
ttrS	Tetrathionate reductase complex: sensory transduction histidine kinase	0.6						
uhpA	response regulator (repressor) in two-component system with UhpB, regulates uhpT operon (LuxR/UhpA family)			0.5		0.3	0.3	0.6
uhpB	sensory histidine kinase in two-component regulatory sytem with UhpA	0.8						
uhpC	membrane protein, regulator of uhpT expression							
uhpT	MFS family, hexose phosphate transport protein							
upps	undecaprenyl pyrophosphate synthetase, sigma E regulon transcribed as a three gene operon		0.6	0.3	0.6	0.6	0.4	
urt	hypothetical protein	1.6	1.6	1.5				
uspA	universal stress protein A	1.9	1.6	7.2	3.1	2.3	2.1	1.6
uspB	universal stress protein B, involved in stationary-phase resistance to ethanol	1.7	2.1	24.6	6.1	3.3	2.9	0.5
uvrY	putative response regulator (LuxR/UhpA familiy)	0.8	0.8	0.4	0.8	1.6	2.0	1.3
vacB	putative exoribonuclease		2.0	4.2	2.6	1.5	1.6	
virK	virulence gene; homologous sequence to virK in Shigella	1.6	2.9	0.1		21.6	25.2	0.8
waaD hom	Waad (=HldD) homologue in phage type DT104: putative lipopolysaccharide core biosynthesis enzyme							

wraB (=wrbA)	trp-repressor binding protein, stationairy phase protein (trp = tryptophan pathway)	1.6	2.3	2.4		5.8	3.9	2.0
xthA	exonuclease III, may repair singlet oxygen induced lesions (stationary phase oxygen stress resistance)	0.8	1.4	7.9	5.1	1.1	1.4	0.9
yaeL	putative membrane-associated Zn-dependent protease, sigma E regulon, down-regulates rpoH and rpoE	0.7	0.7	0.3			0.6	0.1
yahO	putative periplasmic protein	1.1	2.2	2.2	3.1	2.5	2.1	
ybdQ	putative Universal stress protein UspA	1.6	1.9	7.6	3.4	1.8	2.0	1.6
ybiI	putative DnaK suppressor protein	1.2	1.6	9.5	6.2		3.7	
ybjX	virulence gene virK homologue	0.6	0.5	0.2	0.3	0.6	0.5	0.4
ycgB	putative cytoplasmic protein		1.1	4.6	6.7		3.3	
yciF	putative cytoplasmic protein	1.9	3.1	1.2	0.4		0.5	0.4
ydaA	putative universal stress protein	1.5		4.0	2.9	1.7	1.7	
ydiD	homologue of a plant pathogenicity factor		0.6				0.5	
yeaG	putative Ser protein kinase			++	4.8	++	3.9	
yecG	putative universal stress protein	1.6	1.5	11.0	6.2	2.3		
yegD	putative heat shock protein (Hsp70/DnaK)							
yehY	putative ABC-type proline/glycine betaine transport systems, permease component			1.9	1.6		1.5	
yfcZ	putative cytoplasmic protein, putative binding site for ompR		1.1	0.4	2.1	2.0		6.2
yfiA	ribosome associated factor, stabilizes ribosomes against dissociation	1.5	2.1	29.1	64.1	18.7	13.1	2.3
ygaU	putative LysM domain	1.1	2.0	4.2	5.9	6.6	3.3	0.8
yggN	putative periplasmic protein	0.7		1.6		1.9	2.6	0.7
yhbH	putative sigma N modulation factor	2.0	2.0	5.9	4.6	3.5	2.8	0.5
yhbL	sigma cross-reacting protein 27A to sigma D and sigma H (SCRP-27A)	1.2	1.1	2.7	2.5	1.3	1.4	
yhhP	small ubiquitous protein required for normal growth			0.5				
yhjB	putative transcriptional regulator (LuxR/UhpA familiy)			2.6		2.1		
yhjC	putative transcriptional regulator, LysR family				0.7	1.3	1.0	
yhjD	putative tRNA-processing ribonuclease							
yhjE	putative MFS family transport protein	0.8		1.7		1.4		
yhjG	putative inner membrane protein							
yhjH	putative diguanylate cyclase/phosphodiesterase domain 3, flagellar regulon related						1.5	
yhjY	putative lipase		2.7					
yibR	putative inner membrane protein	1.3				1.5		1.3
yicC	putative stress-induced protein	0.8	0.7	1.5	1.9		0.9	
yidY	putative drug translocase							
yjfJ	putative Phage shock protein A, IM30, suppresses sigma 54-dependent transcription	1.7	3.2	2.4				
yjgB	putative alcohol dehydrogenase		1.6	2.0		1.4	1.7	
ynaF	putative universal stress protein	2.0	1.5	2.2	9.2	0.4	0.9	3.5
yncC	putative regulatory protein, gntR family		2.6	1.4	1.3	1.8	2.3	
yodD	putative cytoplasmic protein			3.5		2.0		
yohF	putative oxidoreductase							
ytfJ	putative transcriptional regulator	1.3				2.5		

[a] Empty slot indicates no significant change between the non-stressed and stressed condition; ../-, induced/repressed in stressed condition; ++, expression in non-stressed condition below detection limit and in stressed condition highly up-regulated

Samenvatting



Dit proefschrift beschrijft de identificatie van nieuwe *Salmonella* serovar Typhimurium faagtype DT104-sequenties en de distributie ervan in andere faagtypes. De analyse van de expressie van stress-response- en virulentiegenen in deze pathogeen bij verschillende groei- en stresscondities wordt ook beschreven in dit proefschrift. *Salmonella* serovar Typhimurium DT104 werd bestudeerd, omdat gedurende de afgelopen decennia het aantal infecties veroorzaakt door serovar Typhimurium-isolaten is toegenomen in vele delen van de wereld. Daarnaast is faagtype DT104 multi-antibiotica-resistent en wordt dit faagtype gezien als een gevaarlijke voedselgerelateerde pathogeen. Voor isolaten van humane oorsprong is in Nederland het percentage DT104 binnen de groep van *Salmonella* serovar Typhimurium gestegen van 7 procent in de periode van 1990-1995 naar 29 procent in 1996-2001 en naar 32 procent in 2002-2005.

Door gebruik te maken van de moleculaire methode genaamd “genomic subtractive hybridization”, werd het chromosomaal DNA van de *Salmonella* serovar Typhimurium LT2-stam met bekende genoomsequentie afgetrokken van het chromosomaal DNA van een *Salmonella* serovar Typhimurium DT104-isolaat (**Hoofdstuk 2**). Deze subtractie zou kunnen resulteren in DT104-specifieke sequenties die kunnen coderen voor delen van genen of hele genen die mogelijk een rol spelen bij stress-response of virulentie. De subtractie resulteerde voornamelijk in de isolatie van DNA-fragmenten die leken op bacteriofaag ST64B, ST104 of P27-sequenties. Tevens werden met de subtractie twee fragmenten geïsoleerd die mogelijke virulentiefactoren zouden kunnen zijn. Het ene fragment bleek identiek te zijn aan het *irsA* gen van *Salmonella* serovar Typhimurium ATCC 14028, waarvan wordt gesuggereerd dat dit gen een rol speelt bij de overleving van deze pathogeen in de macrofaag. Het andere fragment bleek homologie te vertonen met HldD, een *Escherichia coli* O157:H7 eiwit dat betrokken is in een specifieke route voor de aanmaak van lipopolysacharides (LPS). Om te bepalen of deze fragmenten DT104-specifiek waren, werden de *irsA* en HldD-homoloogfragmenten en drie andere fragmenten, die sequentiegelijkenis vertoonden met profagen, getest op aanwezigheid in de genomen van 17 DT104 en 27 niet-DT104-isolaten met behulp van PCR. Let wel, de sequenties van de drie profaagfragmenten werden niet teruggevonden in andere openbare *Salmonella*-genomen. Alle vijf de geselecteerde DNA-fragmenten bleken *Salmonella* serovar Typhimurium DT104-specifiek te zijn en de detectie van deze DNA-fragmenten zou een bijdrage kunnen leveren om *Salmonella* serovar Typhimurium DT104 beter te detecteren en te typeren.

Later in ons onderzoek konden we complete profagen aantonen in het genoom van DT104 in plaats van alleen maar kleine profaagfragmenten, zoals hierboven beschreven. Dit konden we doen aan de hand van onze geïsoleerde subtractie DNA-fragmenten, nieuwe profaagsequenties uit openbare databanken en een onvoltooide genoomsequentie van een *Salmonella* serovar Typhimurium DT104-isolaat (Sanger Institute). De profagen die wij konden aantonen waren profagen ST104 en ST64B, en een niet eerder beschreven deel van een profaag waar de HldD-homoloog op lag. Deze laatste profaag hebben wij profaag ST104B genoemd. De distributie van deze drie DT104-profagen en profagen Gifsy-1, Gifsy-2, Fels-1 en Fels-2 die in het genoom *Salmonella* serovar Typhimurium LT2 aanwezig zijn, werden getest voor 19 DT104 en 23 niet-DT104-isolaten. Onder deze DT104-isolaten waren ook isolaten van de DT104-subtypes DT104A, DT104B low en DT104L, en van het DT104-verwante faagtype U302 (**Hoofdstuk 3**). Tevens werd de aanwezigheid van de vijf meest voorkomende DT104-antibiotica-resistentiegenen *aadA2, floR, pse-1, sul1,* en *tet(G)*

bestudeerd. De geteste isolaten konden in 12 groepen verdeeld worden op basis van de verschillen in profaagaanwezigheid in het genoom. Hoewel geen duidelijke relatie gevonden werd tussen faagtype en aanwezigheid van profagen, konden we de verschillende DT104-subtypes onderscheiden op basis van aan- of afwezigheid van profagen ST104, ST104B, en ST64B. De ST104B-profaag, inclusief de HldD-homoloog, konden we aantonen alleen in de 14 DT104L-isolaten en in de isolaat van het DT104-verwante faagtype U302. Uit dit deel van ons onderzoek konden we concluderen dat de aanwezigheid van de vijf meest voorkomende DT104-antibiotica-resistentiegenen, de ST104B-profaag inclusief de HldD-homoloog en sub-faagtype DT104L, de kenmerken zijn behorende bij de gevaarlijke variant van *Salmonella* serovar Typhimurium DT104.

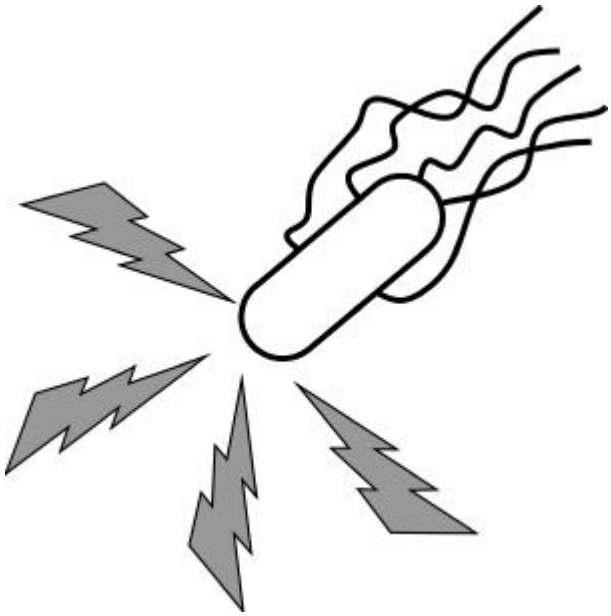
Om de expressie te bestuderen van genen betrokken bij stress-response en virulentie, werd een thematische microarray ontwikkeld voor *Salmonella* serovar Typhimurium DT104 (**Hoofdstuk 4**). Hierbij werd uitgegaan van de genoomsequentie van *Salmonella* serovar Typhimurium LT2 en bekende stress-response- en virulentiegenen. Deze microarray werd aangevuld met bekende *Salmonella* serovar Typhimurium DT104-genen, zoals de vijf meest voorkomende DT104-antibiotica-resistentiegenen. In totaal werden voor 425 geselecteerde genen oligo's ontwikkeld. De ontwikkelde oligo's gebaseerd op de LT2 genoomsequentie bleken ook bruikbaar te zijn voor DT104, omdat chromosomaal DNA van LT2 en DT104 dat gehybridizeerd was op de thematische microarray vergelijkbare resultaten opleverde. Daarnaast werden een aantal verschillende microarray-parameters gevarieerd. Hieruit bleek dat het gebruik van epoxy-gecoate microarrays en natriumfosfaat spottingbuffer de gevoeligste microarrays opleverde. Zodoende konden genen die laag tot expressie komen ook aangetoond worden, resulterend in de detectie van expressie van meer genen. Tevens werd de expressie bestudeerd van zes genen coderend voor verschillende universele stresseiwitten en paralogen en vijf virulentiegenen *spvRABCD* die op het plasmide liggen. De expressie van deze genen werd gemeten in response op stress veroorzaakt door een tekort aan nutriënten voor deze pathogeen tijdens groei bij pH 5.0 en pH 7.0. De bestudeerde genen bleken dezelfde response te geven voor beide pH-condities. Uit de resultaten van de ontwikkeling van de thematische microarray konden we concluderen dat deze microarray klaar was voor gebruik om expressie te meten van stress-reponse- and virulentiegenen in *Salmonella* serovar Typhimurium DT104.

In een eerste expressiestudie hebben we de genexpressies gemeten in de tijd tijdens de groei van *Salmonella* serovar Typhimurium DT104-wildtype en een *luxS*-deletiemutant (**Hoofdstuk 5**). LuxS zou in andere bepaalde Gram-negatieve bacteriën een mogelijke rol spelen in de regulatie van biofilmaanmaak, beweeglijkheid, ijzeropname, of virulentie. LuxS speelt hoogstwaarschijnlijk in *Salmonella* serovar Typhimurium een rol in stress-response en virulentie. De expressiegegevens van *Salmonella* serovar Typhimurium DT104-wildtype en de *luxS*-deletiemutant werden vergeleken voor verschillende groeifasen. Uit de wildtype expressiepatronen in de tijd was duidelijk op te maken dat de expressie van stress-response- en virulentiegenen erg groeifase afhankelijk was. Bijvoorbeeld, de genen die onder controle staan van de algemene stressregulator RpoS, het *rpoS* gen en de genen coderend voor universele stresseiwitten lieten de hoogste expressie zien in de stationaire groeifase. De genen coderend voor eiwitten die betrokken zijn in remming van RpoS-aanmaak (H-NS, Fis, Lrp, and Hfq) kwamen laag tot expressie in de stationaire groeifase in vergelijking tot de exponentiële groeifase. De SPI-1-invasiegenen hadden de

hoogste expressie aan het eind van de exponentiële groeifase. Dit kwam overeen met de hoogste Caco-2-invasiecapaciteit van DT104 cellen van diezelfde groeifase in vergelijking tot cellen van andere groeifasen. Uit de vergelijking tussen de expressiepatronen in de tijd van de wildtype en de *luxS*-deletiemutant was het voornaamste effect zichtbaar aan het eind van de exponentiële groeifase voor de expressie van 15 LPS-genen. In dit deel van de groei werd tevens een hogere expressie gemeten voor het *rpoE*-gen coderend voor de periplasmatische stresssigmafactor en voor de genen onder controle van RpoE. Hieronder vallen een aantal LPS en chaperons coderende genen en de hittestresssigmafactor *rpoH* gen. Verder werd er een hogere Caco-2-adhesie en invasiecapaciteit waargenomen voor de *luxS*-deletiemutant, hoewel de expressieniveaus van de SPI-1-invasiegenen gelijk waren voor beide stammen. Uit dit deelonderzoek concludeerden wij dat het verlies van *luxS* resulteert in overexpressie van LPS-genen en waarschijnlijk ook in overexpressie van LPS-moleculen, waarbij de *in vitro* virulentie-eigenschappen van de DT104 *luxS*-deletiemutant werden beïnvloed.

In een tweede expressiestudie werd de wildtype DT104-stam gekweekt onder aërobe en anaërobe groeicondities en erna blootgesteld aan hitte-, oxidatieve- of zuurstress (**Hoofdstuk 6**). Opnieuw werden de verschillende verkregen expressieresultaten vergeleken. De genen coderend voor de stresstranscriptieregulators Fur, OmpR, en RpoS en de oxidatieve stress-responsegenen kwamen hoger tot expressie onder de aërobe conditie wanneer we de niet-gestresste cellen vergeleken. Daarnaast kwamen voor niet-gestresste cellen de genen coderend voor de universele stresseiwitten en hitteshockchaperons hoger tot expressie onder de anaërobe conditie. Voor de virulentiegenen betrokken bij LPS en SPI-1 was de expressie hoger onder aërobe condities voor niet-gestresste cellen. Wanneer de cellen wel aan stress blootgesteld werden, bleken stressgenen van het RpoS en PhoPQ regulon, chaperons en universele stresseiwitten voornamelijk vergelijkbaar omhoog- of omlaaggereguleerd te worden voor beide groeicondities. Daarnaast bleken de virulentie(-geassocieerde) LPS, PhoPQ, Spv, SPI-1, en SPI-2-genen verschillend gereguleerd te worden onder de verschillende stresscondities. Concluderend biedt de ontwikkelde thematische microarray ons de mogelijkheid om het effect op de expressie van stress-response- en virulentiegenen te bepalen voor verschillende combinaties van stresscondities voor *Salmonella* serovar Typhimurium DT104.

Dankwoord



Na een periode van bijna twee jaar onderzoek tijdens afstudeervakken en stages, werd het me steeds duidelijker dat ik na mijn studie verder wilde in het onderzoek. Werkzaam zijn op een instituut zoals het RIKILT en gebruik maken van de destijds nieuwe microarray-technologie sprak mij erg aan. Verder vond ik stressoverleving van *Salmonella* ook wel tot de verbeelding spreken. Het resultaat mag er wezen, "mijn proefschrift". Het heet dan wel mijn proefschrift, maar uiteraard had dit er nu niet zo gelegen zonder de hulp van verschillende personen die ik allen graag wil bedanken.

Allereerst wil ik mijn begeleiders bedanken: Henk Aarts, Tjakko Abee en Marcel Zwietering. Henk, jij hebt mij in het bijzonder de eerste jaren geholpen om een goede basis voor mijn promotie te leggen. Ik denk hieraan vooral aan de hulp en discussies over de "fancy" moleculaire tools, zoals de genomische subtractie, kwantitatieve PCR en de microarray. Tjakko, jij hebt mij de laatste jaren vooral geholpen en geleerd om leuke resultaten om te zetten in wetenschappelijke publicaties. Sinds kort ben je professor, veel succes in deze nieuwe functie. Marcel, jij deed je intrede in mijn onderzoek toen ik al een tijdje bezig was. Ik was erg blij om een promotor erbij te hebben en dan vooral eentje die lette op de vertaalslag van mijn onderzoek naar praktijkonderwerpen door er vragen over te stellen zoals: "Hoe groeien pathogenen nu in een voedselproduct, exponentieel of stationair?" of "Hoeveel *Salmonella*'s zitten er op of in een product?". Hiervoor mijn dank.

Daarnaast hebben verschillende studenten en stagiaires een grote bijdrage geleverd aan dit proefschrift. Hoewel dan wel wordt gezegd dat ik de resultaten heb omgezet tot dit proefschrift, ben ik altijd van mening geweest dat er zonder resultaten ook geen proefschrift was geweest. Graag wil ik jullie één voor één bedanken. Stijn, met jou heb ik samen gepionierd. We hebben de eerste microarray-protocollen en -experimenten voor bacterieonderzoek opgezet binnen het RIKILT en daarnaast vanuit de literatuur relevante genen geselecteerd voor de latere thematische microarray. Maarten, door jouw langere stageperiode hebben we nog eens kritisch naar de microarray-procedure gekeken wat leidde tot betere en gevoeligere microarrays en ben je co-auteur van hoofdstuk 5 over LuxS. Ook hebben we de nodige uurtjes gediscussieerd over al het andere wat ons bezig hield in het onderzoek. Erg leuk vind ik het dan ook dat Maarten en Stijn beide zijn doorgegaan in soortgelijk onderzoek: Stress in *Bacillus cereus* en Stress in *Listeria monocytogenes*. Annelien, jij begon met vele sequenties die uit de subtractie voortvloeiden en na veel gepuzzel en ge-BLAST wordt wellicht mede dankzij jouw inzet ooit een profaagtyperingsmethode ontwikkeld. Verder heb jij je de vele nieuwe aspecten rondom moleculaire biologie snel eigengemaakt. Sükrü, ook voor jou was alles nieuw. Je hebt prima werk verricht, en hopelijk heb je veel van mij geleerd. Alexander, jij werkt nu nog steeds bij het RIKILT. Jij hebt dan ook meegemaakt hoe alle onderzoeksresultaten uiteindelijk leidden tot dit proefschrift. Ik hoop dat je in de toekomst meer moleculair biologisch onderzoek zal gaan doen. Emmy, ondanks dat je een andere richting opwilde na je studie, heb je jezelf toch door de complexe materie heen weten te slaan. Samen met het werk van Sükrü en Annelien hebben we alle resultaten compleet kunnen maken voor het profaagonderzoek, zoals beschreven in hoofdstuk 3.

Onderzoek doen bij het RIKILT is niet altijd even gemakkelijk gebleken. Mede dankzij de steun en vertrouwen van mijn collega's heb ik de finish gehaald. Bedankt cluster Microbiologie en Novel Foods en de RIKILT-AIO's. Verder, wil ik in het bijzonder Wendy, Angela en Jeroen bedanken voor hun hulp of discussies binnen mijn onderzoek. Daarnaast

Petra, Rob en Jasper bedankt voor de prettige samenwerking binnen het NWOVV project. Marco de Boer, die destijds werkzaam was bij Isogen Life Science, is altijd zeer behulpzaam geweest en probeerde altijd mijn vaak moeilijke vragen te beantwoorden over de microarray-ontwikkeling. Thanks to Andreas Schroeter and Burkhard Malorny from the Federal Institute for Risk Assessment (BfR) in Germany for advice and help about *Salmonella* serovar Typhimurium phage-typing. Ook Max Heck en Wim Wannet van het RIVM bedankt voor hulp en advies over faagtypering. Naast mijn promotie heb ik ook in de RIKILT-ondernemingsraad gezeten. Hiervan heb ik veel geleerd dankzij mijn mede-OR-leden Constant, Gea, Harry, John, Maryvon, Theo, Wim en Nico en de beide directeuren Andre en Kees.

Ook mijn paranimfen, Alexander Elferink en Linette Pellis, wil ik graag bedanken voor hun steun en betrokkenheid bij mijn onderzoek tijdens mijn promotie, maar vooral ook tijdens de verdediging van mijn proefschrift.

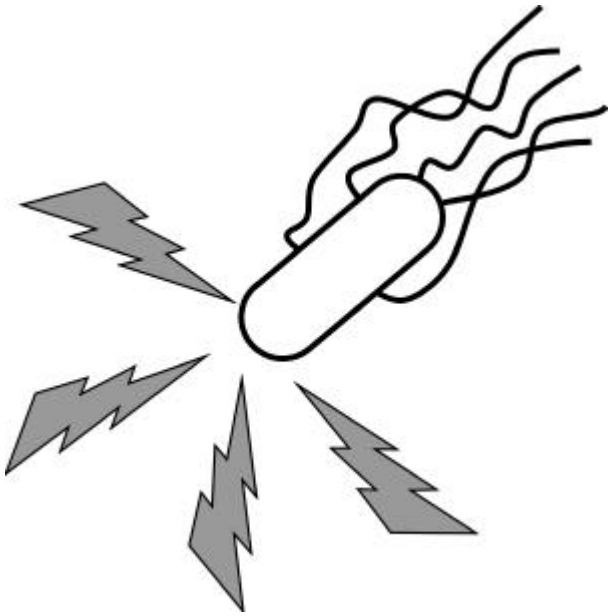
Beste Pap en Mam, mijn schoonouders Wil en Jetty, Maurice, Wanda, Ruben, Tessa, en Jeroen. Hoewel mijn onderzoek vrij ingewikkeld is, genoot ik ervan om jullie regelmatig bij te praten over de vorderingen. Mede dankzij jullie steun en luisterend oor heb ik dit proefschrift kunnen afronden. "De derde doctor in de familie". Bedankt!

Sonja, zoals ook jij al in je proefschrift vermeldde zouden we samen wel een boek kunnen schrijven over promoveren. Dit geeft duidelijk weer dat onze promotieonderzoeken een belangrijke rol speelden in ons dagelijks leven. Jij hebt die jaren mij altijd weer inspiratie gegeven en me liefdevol gesteund. Mooi is het dat we samen onze dromen hebben laten uitkomen: ons eigen huisje en een gezinnetje. Ja jij, Cécile, jij hebt het laatste duwtje in de rug gegeven zodat pappa het proefschrift kon afronden en je hebt je beide ouders geleerd om meer te relativeren.

Armand

P.S. Zoek de overeenkomsten tussen bobsleeën en promoveren.

List of publications



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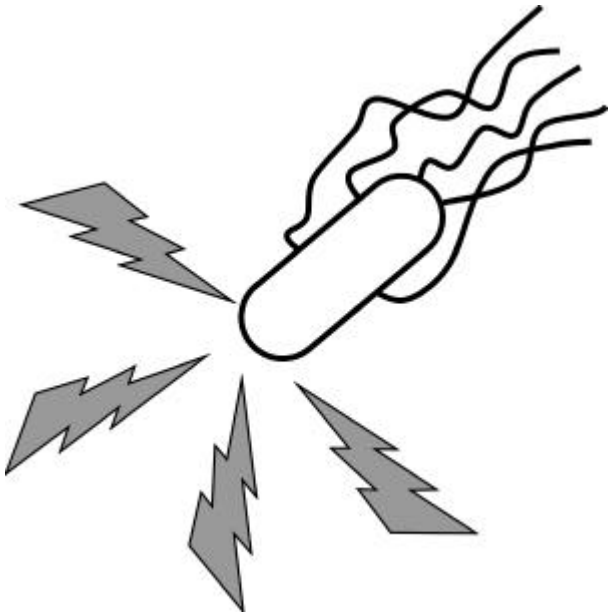
Curriculum vitae



Armand Petrus Henricus Maria Hermans werd geboren op 4 oktober 1976 in Maastricht. In 1995 behaalde hij zijn VWO-diploma aan het Trichter College in Maastricht. Na een niet alledaagse zomervakantie in het ziekenhuis startte hij in datzelfde jaar met de studie Bioprocestechnologie aan de Wageningen Universiteit. Tijdens deze studie schreef hij vier afstudeer- en stageverslagen. Bij de leerstoelgroep Microbiologie aan de Wageningen Universiteit over het onderwerp getiteld “Microbial ecology of anaerobic sludge: molecular monitoring of microbial diversity in anaerobic wastewater treatment systems”. Verder bij NIZO food research over de bacteriële genetica-onderwerpen getiteld “Bio-informatics based tetracycline resistance in *Lactobacillus plantarum* WCFS 1” en “*In vitro* promoter screening of *Lactobacillus plantarum* WCFS 1” en over het procestechnologie-onderwerp getiteld “Fermentative folic acid production of *Lactococcus lactis*, reactor optimization and metabolic flux engineering”.

In september 2001 rondde hij zijn universitaire studie af, waarna hij begon met zijn promotieonderzoek bij het RIKILT - Instituut voor Voedselveiligheid te Wageningen vanuit de leerstoelgroep Levensmiddelenmicrobiologie van de Wageningen Universiteit. Het onderwerp van zijn promotieonderzoek was het bestuderen van stress-response en virulentie in *Salmonella* serovar Typhimurium DT104 door gebruik te maken van genomicstechnieken. Een groot deel hiervan staat weergegeven in dit proefschrift. Dit promotieonderzoek werd begeleid door Prof. Dr. Tjakko Abee, Prof Dr. Ir. Marcel Zwietering en Dr. Henk Aarts. Tijdens deze periode heeft hij meerdere cursussen gevolgd onder andere van de onderzoeksschool Voeding, Levensmiddelentechnologie, Agrobiotechnologie en Gezondheid (VLAG) en zijn onderzoeksresultaten gepresenteerd op (inter)nationale bijeenkomsten en in wetenschappelijke tijdschriften. Tijdens zijn promotie heeft hij deeluitgemaakt van de RIKILT-ondernemingsraad en was hij medeorganisator van een 10-daagse studiereis voor promovendi naar de Verenigde Staten.

Vanaf oktober 2006 werkt hij als hoofd microbiologie en hygiënist bij Nestlé in Nunspeet voor het “Nestlé Nutrition Quality Assurance Centre”, waar men de microbiologische veiligheid waarborgt van voornamelijk producten uit Nederland, Scandinavië en het Midden-Oosten.

The research presented in this thesis was financially supported by the Netherlands Organization for Health Research and Development, program “Nutrition: Health, Safety and Sustainability” and RIKILT Institute of Food Safety. Printing of this thesis was financially supported by RIKILT Institute of Food Safety.

Cover design & lay-out: Armand Hermans

Printed by: Grafisch Service Centrum, Wageningen