Molecular Diagnostics of Foodborne Pathogens

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Preface and acknowledgement

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The main supervisor of the project was Professor Jeffrey Hoorfar, Division of Food Microbiology, National Food Institute, Technical University of Denmark. Co-supervisors were Assistant Professor Charlotta Löfström, Division of Food Microbiology, National Food Institute, Technical University of Denmark, Associate Professor Håkan Vigre, Division of Epidemiology and Microbial Genomics, National Food Institute, Technical University of Denmark and Assistant Professor Leise Riber, Department of Biology, Faculty of Science, University of Copenhagen.

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Summary

Illness caused by foodborne pathogens represents an important economic and public health burden worldwide. In order to minimize the occurrence of foodborne pathogens in the food production chain and thereby increase the food safety, better detection methods and knowledge about the behavior of pathogens are needed. The introduction of the molecular diagnostics methods based on detection of the organisms nucleic acids have made detection, identification and characterization of foodborne pathogens faster and with greater specificity and sensitivity.

The objectives of research in this thesis were to investigate the use of different nucleic acid based methods for molecular diagnostics of foodborne pathogens focusing on *Salmonella* and *Bacillus cereus* with respect to improve food safety. The work represents two parts of molecular diagnostics; the characterization *Salmonella* for better understanding of its behavior in pork processing environments, and detection of *B. cereus* in food, feed and water samples without prior cultivation.

The persistence of *Salmonella* in food production chains has been suggested to be a result of bacterial attachment and surface colonization. It was found that the physiological state of *Salmonella* has an impact on the ability of *Salmonella* to attach to a pork meat surface and subsequently the possibility of contributing to cross contamination in the slaughter-line. Cells that were grown immobilized prior application on a pork meat surface were found to be more easily removed. In the pork processing, *Salmonella* might appear in an immobilized state on the pork surfaces where low attachment ability might pose a risk for cross contamination. A stronger attachment to a surface makes on the other hand decontamination steps more difficult. The attachment ability of *Salmonella* could to some extend be connected to specific genes. Deletion of either of the operons *prg* or *flhDC* in *S.* Typhimurium resulted in lower attachment ability to the pork meat surface. In addition, was it found that a *S.* Rissen isolate with low attachment ability after immobilized growth lacked two fimbriae genes, *safC* and *lpfD,* important for the adhesion and biofilm formation. It was further found that *S.* Typhimurium exposed to a heat shock was more resistance to heat and acid inactivation conditions, which might make later decontamination steps more difficult and subsequently lead to a higher risk of contamination of food products.

Deliberate or accidental contamination of food, feed and water supplies pose a threat to human health worldwide and the need for generic detection methods that can screen for many pathogens at the time are highly desirable. A metagenomics based direct 16S rDNA sequencing approach was evaluated as a diagnostic tool for screening of unknown bacteria in bottled water without prior cultivation. *B. cereus* artificially inoculated in bottled water was used as a model. The results revealed that the method was able to detect *B. cereus* at levels of 10^5-10^6 CFU/L, a detection level low enough for detection in outbreaks situations. Consequently, the method was found to be a good candidate as a method for detection of *B. cereus* and for screening of other bacterial contaminants in water samples. The capability of the method was further evaluated on a variety of food and feed model samples. Before the method could be adapted to these types of samples, an optimization of the total DNA extraction step was applied. Five different commercial available DNA extraction kits were evaluated and the MasterPure DNA Purification Kit was found to be suitable for the food and feed samples. The detection of *B. cereus* in food and feed samples was found to be more complicated and for the method to be used for this type of samples, additional optimizations have to be conducted.

In conclusion, the work present in this thesis contributes to the better understanding of the behavior of *Salmonella* in the pork processing and which factors that might influence the persistence and adaption. The information can be used for control of *Salmonella* by contributing to developments of more specific control measures and treatments within the food production-line and thereby improve the food safety. In addition, the method for direct detection of *B. cereus* in different biological matrices was found promising with the potential to be adapted for screening of bacterial contamination. This makes the method useful in outbreaks situations where the causing agent might be unknown.

Dansk sammendrag (Danish summary)

Sygdomme forårsaget af fødevarebårne patogener udgør en vigtig økonomisk og sundhedsmæssig belastning på verdensplan. For at minimere forekomsten af fødevarebårne patogener i fødevareproduktionen og dermed øge fødevaresikkerheden, er metoder til påvisning og karakterisering af patogener nødvendig. Indførelsen af molekylær diagnostik metoder baseret på påvisning af organismers nukleinsyrer har gjort påvisning, identifikation og karakterisering af fødevarebårne patogener hurtigere og med større specificitet og sensitivitet.

Fokus i denne afhandling har været at undersøge brugen af forskellige nukleinsyre baserede metoder til molekylær diagnostik af fødevarebårne patogener med fokus på *Salmonella* og *Bacillus cereus* med henblik på at forbedre fødevaresikkerheden. Forskningen i afhandlingen beskæftiger sig med to dele af molekylær diagnostik: Karakterisering af *Salmonella* for en bedre forståelse af adfærden i svinekødsproduktionsmiljøer og påvisning af *B. cereus* i fødevare-, foder- og vandprøver uden forudgående dyrkning.

*Salmonella*s evne til at overleve i fødevareproduktionskæden menes at skyldes fasthæftelse på udstyrs- og kødoverflader, samt den efterfølgende kolonisering. I et studie i denne afhandling blev det vist, at vækstilstanden for *Salmonella* har betydning for fasthæftelsen på en svinekødsoverflade, hvilket yderligere har betydning for at kunne forårsage krydskontaminering i slagtekæden. *Salmonella* celler, der blev dyrket immobiliseret havde lettere ved at slippe kødoverfladen. I slagteprocessen kan *Salmonella* findes som immobiliseret på svinekød, hvilket gør at celler med lav fasthæftningsevne har en større risiko for krydskontaminering. Derimod vil en stærkere fasthæftning gøre dekontamineringstiltag mere vanskelige. *Salmonella*s evne til fasthæftelse kunne til en vis grad knyttes til specifikke gener. Deletionen af operonerne for enten *prg* eller *flhDC* i *S.* Typhimurium resulterede i en lavere fasthæftningsevne til en svinekødsoverflade. Desuden blev det fundet at en *S.* Rissen isolat med svag fasthæftning efter immobiliseret vækst manglede to fimbriae gener, *safC* og *lpfD*, som er vigtige for fasthæftning og dannelse af biofilm. Derudover blev det fundet at *S.* Typhimurium udsat for et varmechok blev mere modstandsdygtig overfor efterfølgende varme- og syrepåvirkninger, hvilket kan gøre efterfølgende dekontamineringstiltag vanskeligere, og dermed føre til en højere risiko for forurening af fødevarer.

Bevidst eller utilsigtet forurening af fødevarer, foder og vandforsyning udgør en trussel mod folkesundheden på verdensplan, og behovet for generiske påvisningsmetoder, som kan screene for mange patogener på samme tid er ønskelig. En metagenom-baseret direkte 16S rDNA sekventeringsmetode blev evalueret som et redskab til screening af ukendte bakterier uden forudgående dyrkning. *B. cereus* i flaskevand blev brugt som model for metoden. Resultaterne viste, at metoden kunne påvise *B. cereus* i koncentrationer af 10^5-10^6 CFU/L, hvilket er et niveau som kan bruges til påvisning i udbrudssituationer. Som følge heraf blev metoden fundet til at være en god kandidat til påvisning af *B. cereus* og til screening for bakteriel forurening i vandprøver. Metoden blev yderligere testet på en række fødevare- og foderprøver. Før metoden kunne benyttes til denne type prøver, blev fem forskellige kommercielt tilgængelige DNA ekstraktions kits evalueret, og MasterPure DNA Purification Kit blev fundet at være det bedst egnede til fødevare-og foderprøver. Påvisning af *B. cereus* i disse prøver blev fundet til at være mere kompliceret sammenlignet med vandprøverne, og for at metoden skal kunne anvendes på denne type af prøver, skal yderligere optimeringer udføres.

Denne afhandling bidrager til bedre forståelse af adfærden af *Salmonella* i svinekødsproduktionen og hvilke faktorer, der påvirker tilpasningen og overlevelsen. Disse fund kan bruges til bedre kontrolforanstaltninger for *Salmonella* ved at bidrage til udviklingen af mere hensigtsmæssige produktionsmiljøer indenfor fødevareproduktion, og som kan være med til at forbedre fødevaresikkerheden. Desuden blev metoden til direkte påvisning af *B. cereus* i forskellige biologiske matricer fundet lovende med potentiale til at blive kunne bruges til screening af patogener. Metoden vil derfor være anvendelig i udbrudssituationer, hvor den forårsagende patogen kan være ukendt.

Abbreviations

ANOVA	Analysis of variance
cDNA	Complementary deoxyribonucleic acid
CFU	Colony forming units
C_T	Threshold cycle
DNA	Deoxyribonucleic acid
dsDNA	Double stranded deoxyribonucleic acid
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assays
EU	European Union
FDA	US Food and Drug Administration
IAC	Internal amplification control
ISO	International Organization for Standardization
kb	Kilobase
LOD	Limit of detection
MCR	Multivariate curve resolution
mRNA	Messenger ribonucleic acid
NGS	Next generation sequencing
PCA	Principal component analysis
PCR	Polymerase chain reaction
qRT	Quantitative reverse transcriptase
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
ssDNA	Single stranded deoxyribonucleic acid
UV	Ultraviolet
WHO	World Health Organization

List of papers

Paper 1

Hansen, T., Riber, L., Nielsen, M.-B., Vigre, H., Thomsen, L. E., Hoorfar, J., Löfström, C. (2013) Influence of contact time and pre-growth conditions on the attachment of *Salmonella enterica* serovar Typhimurium to pork meat. Submitted to Food Microbiology

Paper 2

Hansen, T., Löfström, C. (2013) Gene content and attachment ability of *Salmonella* isolated from a pork slaughter-line. Paper in preparation

Paper 3

Pin, C., **Hansen, T.**, Muñoz-Cuevas, M., de Jonge, R., Rosenkrantz, J. T., Löström, C., Aarts, H., Olsen, J. E. (2012) The transcriptional heat shock response of *Salmonella* Typhimurium shows hysteresis and heated cells show increased resistance to heat and acid stress. PLoS ONE, 7(12): e51196

Paper 4

Hansen, T., Skånseng, B., Hoorfar, J., Löström, C. (2013) Evaluation of direct 16S rDNA sequencing as a metagenomics based approach for screening of bacteria in bottled water. Accepted in Biosecurity and Bioterrorism: Biodefense Strategy, Practice, and Science

Paper 5

Hansen, T., Löfström, C. (2013) Evaluation of a direct 16S rDNA sequencing approach for screening of *Bacillus cereus* in feed and food. Paper in preparation

Not included in this thesis

Josefsen, M. H., Löfström, C., **Hansen, T.**, Reynisson, E., Hoorfar, J. (2012) Instrumentation and fluorescent chemistries used in qPCR. In M. Filion (Ed.) Quantitative Real-time PCR in Applied Microbiology (pp. 27-52) Caister Academic Press

Karlsson, O., **Hansen, T.**, Knutsson, R., Löfström, C., Granberg, F., Berg, M. (2013) Metagenomic detection methods within biopreparedness outbreak scenarios. Accepted in Biosecurity and Bioterrorism: Biodefense Strategy, Practice, and Science

Anniballi, F., Fiore, A., Löfström, C., Skarin, H., Auricchio, B., Woudstra, C., Bano, L., Segerman, B., Koene, M., Båverud, V., **Hansen, T.**, Fach, P., Åberg, A. T., Hedeland, M., Engvall, E. O., De Medici, D. (2013) Management of Animal Botulism Outbreaks: from Clinical Suspicion to Practical Countermeasures to Prevent or Minimize Outbreaks. Accepted in Biosecurity and Bioterrorism: Biodefense Strategy, Practice, and Science

Skarin, H., Åberg, A. T., Woudstra, C., **Hansen, T.**, Löfström, C., Koene, M., Bano, L., Hedeland, M., Anniballi, F., De Medici, D., Engvall, E. O. (2013) The outcome of the Workshop "Animal botulism in Europe". Accepted in Biosecurity and Bioterrorism: Biodefense Strategy, Practice, and Science

Woudstra, C., Åberg, A. T., Skarin, H., Anniballi, F., De Medici, D., Bano, L., Koene, M., Löfström, C., **Hansen, T.**, Hedeland, M., Fach, P. (2013) Animal botulism research and development summary within the European project AniBioThreat. Accepted in Biosecurity and Bioterrorism: Biodefense Strategy, Practice, and Science

Woudstra, C., Skarin, H., Anniballi, F., Auricchio, B., De Medici, D., Bano, L., Drigo, I., **Hansen, T**., Löfström, C., Hamidjaja, R., van Rotterdam, B. J., Koene, M., Bäyon-Auboyer, M.-H., Buffereau, J.-P., Fach, P. (2012)Validation of a real-time PCR based method for detection of Clostridium botulinum types C, D and their mosaic variants C-D and D-C in a multicenter collaborative trial. Anaerobe, In press

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Chapter 1

Introduction and Objectives

1 Introduction and objectives

Illness caused by foodborne pathogens represents an important economic and public health burden worldwide. Hundreds of outbreaks of foodborne pathogens occur around the world. In the European Union (EU), a total of 5,648 foodborne outbreaks were reported in 2011, affecting 69,553 people (EFSA, 2013). Most foodborne pathogens are transmitted from the environment or animals through food products to humans. To minimize the occurrence of foodborne pathogens in the food production chain and thereby increase the food safety, knowledge of the behavior of the pathogens and detection methods are needed. The introduction of the molecular diagnostic methods based on detection of the organism's nucleic acids have made detection, identification and characterization of foodborne pathogens faster and with greater specificity and sensitivity (Dwivedi & Jaykus, 2011; Weile & Knabbe, 2009).

The objectives of the research in this thesis were to investigate the use of different nucleic acid based method for molecular diagnostics of foodborne pathogens focusing on *Salmonella* and *Bacillus cereus* with respect to improve food safety. The work represents two parts of molecular diagnostics: (i) Characterization *Salmonella* for better understanding of the behavior in the pork processing environment (Paper I-III), and (ii) detection of *B. cereus* in food, feed and water samples without prior cultivation (Paper IV-V).

The first part of the thesis gives a general introduction to *Salmonella* and *B. cereus*, including a description of classification, phylogeny and their occurrence in the food production chain. Secondly, a description of different nucleic acid based diagnostic methods with respect to food safety will be addressed. Additional, different considerations with regard to the use of nucleic acids based methods are described. This is followed by five papers that present the experimental work of this thesis using the different molecular diagnostic methods. The last part contains the discussion and conclusions based on the experimental work of this thesis.

Chapter 2

Foodborne Pathogens

2 Foodborne pathogens

2.1 Salmonella

2.1.1 Classification and biology

Salmonella are rod-shaped motile Gram-negative bacteria. They are facultative anaerobic and non-spore forming (Popoff & Le Minor, 2005). The *Salmonella* genus is a member of the family Enterobacteriaceae and contains two species, *Salmonella enterica* and *Salmonella bongori*, each consisting of multiple serotypes. *S. enterica* is subdivided into six subspecies; *S. enterica* subsp. *enterica* (I), *S. enterica* subsp. *salamae* (II), *S. enterica* subsp. *arizonae* (IIIa), *S. enterica* subsp. *diarizonae* (IIIb), *S. enterica* subsp. *houtenae* (IV) and *S. enterica* subsp. *indica* (VI). *S. bongori* was formerly classified as subspecies V (Tindall et al., 2005). *S. enteria* subsp. *enterica* is mainly isolated associated with humans and warm-blooded animals, whereas the other subspecies are found in cold-blooded animals and the environment (Grimont & Weill, 2007).

The subspecies of *Salmonella* are further separated into serovars according to the Kauffman-White scheme (Grimont & Weill, 2007). The separation of serovars is based on antigenic polymorphisms of lipopolysaccharide (O-antigens), flagellar proteins (H-antigens) and capsular polysaccharides (Vi-antigens). Currently there are more than 2,500 known serovars of *Salmonella*, where most of the serotypes belong to *S. enterica*. For simplicity, the *Salmonella* strains mentioned in this thesis are abbreviated by *S.* followed by the serovar name, e.g. *Salmonella enterica* subsp. *enterica* serovar Typhimurium is abbreviated as *S.* Typhimurium.

Salmonella can be found in natural environments, such as water, soil and plants, but it does not seem to grow significantly there. However, since *Salmonella* are known to be quite resistant towards different environmental factors, it can survive for weeks in water and several years in soil, as long as the conditions are favorable (Jensen et al., 2006; Tietjen & Fung, 1995). The optimal growth temperature for *Salmonella* is 37°C, but can grow in a quite broad range (7-45°C). *Salmonella* growth in food has been observed at 2-4°C (Ferreira & Lund, 1987).

S. enterica is a common agent of foodborne infections and the human infections can range from enteric fever (typhoidal fever) to nontyphoidal salmonellae, which normally cause gastroenteritis (Fierer & Guiney, 2001). Under certain conditions salmonellae can cause invasive (systemic) disease, where the salmonellae enter the blood stream and spread throughout the body. The nature and severity of *Salmonella* infections depends on several factors such as the infecting *Salmonella* serotype, virulence of the strain, infection dose and the host. Gastrointestinal infections are primarily caused by serovars such as Enteritidis and Typhimurium (Jantsch et al., 2011). Usually the *Salmonella* colonize the intestine by attaching to the epithelia cells using fimbria, followed by invasion of the intestinal mucosa and multiplication in the gut-associated lymphoid tissue (Gahring et al., 1990; Galán & Curtiss, 1989; Ginocchio et al., 1992).

2.1.2 Epidemiology

Salmonella is found worldwide and is an important zoonotic pathogen. It is estimated that *Salmonella* is responsible for 93.8 million cases of human gastroenteritis globally each year and of these 80.3 million cases are estimated to be foodborne (Majowicz et al., 2010). In EU, 95,548 confirmed cases of human *Salmonella* infections were reported to the European Food Safety Authority (EFSA) in 2011 (EFSA, 2013). The two serotypes, *S.* Enteritidis and *S.* Typhimurium, are the most frequent reported serovars, together accounting for almost 70% of all human *Salmonella* infections in Europe. Human *S.* Enteritidis cases are mostly associated with the consumption of contaminated eggs and poultry meat, whereas *S.* Typhimurium cases most commonly are linked with the consumption of contaminated pork, beef and poultry meat (EFSA, 2013).

The routes where *Salmonella* are transmitted to humans include the environment, contact with animals and human-to-human contact. In industrialized countries, the main source of *Salmonella* infections are contaminated animal-derived food product, notably fresh meat and eggs. In developing countries, a large proportion of the human cases are from contaminated vegetables, water and human-to-human transmission (Wegener et al., 2003). In Denmark, domestic pork has been identified as the most important food source of salmonellosis. The second most important source is imported pork (Anonymous, 2012). In the following sections the focus will primarily be on pig processing as this is most relevant for this thesis.

2.1.3 Detection and enumeration

The conventional methods for isolation and detection of *Salmonella* in food, feed and environmental samples usually contains a low number of bacteria and the detection procedures usually consists of multiple steps. The standard methods recommended by e.g. the International Organization for Standardization (ISO) and US Food and Drug Administration (FDA) are built upon a similar procedure (Andrews et al., 2011; Anonymous, 2002). The conventional routine analysis consists of four basic steps: (i) pre-enrichment in non-selective media, (ii) enrichment in selective media, (iii) isolation on selective plates and (iv) conformation of presumptive *Salmonella* isolates (van der Zee & Huis in't Veld, 2000). In the final step several biochemical procedures are employed, where suspect colonies are verified serologically by determining the O- and H-antigens (Grimont & Weill, 2007). The conventional methods for detection of *Salmonella* are slow and labor-intensive as they usually takes 4-7 days to complete, and they suffer in sufficient detection of sublethally injured cells (Gracias & McKillip, 2004).

The use of molecular methods instead of the culture-based methods such as enzyme-linked immunosorbent assays (ELISA), DNA microarray, PCR and real-time PCR enables faster detection (van der Zee & Huis in't Veld, 2000). As many of the techniques have a lower detection level, the enrichment step can be either reduced in time or even omitted. Several published studies using molecular methods for detection of *Salmonella* has been reported (Eriksson & Aspan, 2007; Malorny et al., 2007; Uyttendaele et al., 2003).

2.1.4 *Salmonella* in the pork production chain

Salmonella can enter at any stage of the food chain, from the livestock feed, at the slaughterhouse or packing plant, in manufacturing, processing and retailing of food, through catering and cooking at home (Lo Fo Wong et al., 2002). Infected pigs constitute the main reservoir for carcass contamination in slaughterhouses and many of the carcasses harbor *Salmonella* on the skin or in the rectum (Hald et al., 2003; van Hoek et al., 2012; Visscher et al., 2011). However, the slaughter-line is an open process with many possibilities for cross contamination of both *Salmonella* positive and negative carcasses (van Hoek et al., 2012). Carcasses can throughout the slaughter-line be contaminated from tools and equipment used for the slaugh-

tering and since the equipment can be very difficult to clean and sterilize, the *Salmonella* has the possibility to colonize and form biofilms (Zottola & Sasahara, 1994).

Adaptation and persistence of *Salmonella* in food production chains have been suggested to be a result of bacterial attachment and surface colonization (Swanenburg et al., 2001; Vestby et al., 2009). When cells have attached to a surface, colony formation will occur. Colonization of *Salmonella* on surfaces in contact with food provides a reservoir of bacteria that can increase the risk of contamination in the production line. *Salmonella* present in biofilm are generally more resistant towards environmental stresses, such as disinfections, organics acids and heat compared with their planktonic counterparts (Morild et al., 2011; Nielsen et al., 2013; Scher et al., 2005; H. S. Wong et al., 2010). *Salmonella* has the capability to form biofilms on different surfaces used in the food production chain, which includes plastics (Joseph et al., 2001), glass (Prouty & Gunn, 2003) and stainless steel (Hood & Zottola, 1997; Joseph et al., 2001). Besides attaching to the materials used in the production line, *Salmonella* can also attach to meat surfaces. Common for all the surfaces are that the ability of the *Salmonella* to attach to the surface are influenced by different factors such as the serovars, the surface, contact time and the growth state (Hood & Zottola, 1997; Joseph et al., 2001; Oliveira et al., 2006)(Paper I and Paper II).

In Paper I, it was found that the longer the *S.* Typhimurium cells were allowed to attach to a pork meat surface, the stronger attachment was achieved. The impact of the contact time was further influenced by the growth state, i.e. the effect of the contact time seemed to be more pronounced for the planktonic bacteria compared with their immobilized counterparts. This suggests that a liquid contamination might contribute to a higher occurrence of bacterial attachment to the meat surface.

The *Salmonella* serovars also seems to impact the attachment ability, together with a variation between strains of the same serotype. In a study by Oliveira et *al*. it was found that the difference in adhesion ability of different *S.* Enteritidis strains, on materials used in kitchens, where strongly strain dependent (Oliveira et al., 2006). The adhesion also varied between the different contact surfaces, i.e. polyethylene, polypropylene and granite. In Paper II some difference in attachment ability was observed for different *S. enterica* serovars on a pork meat surface. In addition, the effect of the growth state, i.e. planktonic and immobilized, varied between the serovars tested.

Overall, the different factors that influences the attachment ability of *Salmonella* on surfaces in the food production line, impacts the cleaning procedures that are needed. Optimal cleaning reduces the occurrence of cross contamination in the production line, which thereby can improve the food safety.

2.2 Bacillus cereus

2.2.1 Classification and biology

The genus *Bacillus* is a rod-shaped anaerobic Gram-positive bacterium producing endospores (Logan & De Vos, 2009). The *Bacillus cereus* group consists of seven closely related species; *Bacillus cereus*, *B. thuringiensis*, *B. anthracis*, *B. mycoides*, *B. pseudomycoides, B. weihenstephanensis* and *B. cytotoxicus* (Guinebretière et al., 2013; Lapidus et al., 2008). The species are closely related, and the strains of *B. cereus*, *B. anthracis* and *B. thuringiensis* share highly conserved chromosomes but differ in their virulence encoding plasmids (Rasko et al., 2005). Where *B. thuringiensis* is pathogenic to insects, *B. cereus* is known mainly as a food-poisoning bacterium that causes diarrhea and vomiting. *B. anthracis*, the causing agent of anthrax, is found worldwide and can infect both animals and humans (Drobniewski, 1993). The three species have specific phenotypes and pathogenicity, which often are plasmid-encoded, such as the insecticidal crystal proteins in *B. thuringiensis* (Berry et al., 2002), the cereulide synthesis of emetic *B. cereus* strains (Ehling-Schulz et al., 2006) and the toxins and capsule of *B. anthracis* (Keim et al., 2009). *B. cereus* is the most important foodborne pathogen in the *B. cereus* group and has been associated with food-poisoning illnesses in humans and other kinds of clinical infections (Bottone, 2010). As *B. cereus* is very closely related to *B. anthracis*, and is sometimes used as a model organism for development and validation of new methods in order to minimize the risk arising from working with *B. anthracis* (Fricker et al., 2011). In the following the focus will be on *B. cereus* as this is most relevant for this thesis.

B. cereus is widely spread in the environment including soil, sediments, water and plants (Kotiranta et al., 2000; Schoeni & Wong, 2005; von Stetten et al., 1999; Østensvik et al., 2004). *B. cereus* has optimal growth of 28-35°C, but can grow over a wide temperature range from 4-5°C to 55°C. It has the ability to survive harsh environments because it can form

spores that are resistant to heat, dehydration and other physical stresses (Stenfors Arnesen et al., 2008).

Food-poisoning by *B. cereus* can be caused by either infection or intoxication, which leads to a diarrheal or an emetic type of disease, respectively. The diarrheal syndrome is caused by enterotoxins produced by vegetative cells during the growth in the small intestine after consumption of contaminated foods (Schoeni & Wong, 2005). The emetic syndrome is associated with the production of the cereulide toxin in foods before consumption (Ehling-Schulz et al., 2004; Stenfors Arnesen et al., 2008). Both diseases are relative mild and do usually not last more than 24 hours. For both types of food-poisoning, the contaminated foods have usually been heat-treated, which triggers the spore germination, i.e. transfer from spore to vegetative cell, and the absence of the other bacterial flora, gives the opportunity for *B. cereus* to grow well (Granum & Lund, 1997).

2.2.2 Epidemiology

According to a report from EFSA, *Bacillus* toxins were responsible for only 3.9% of the reported foodborne outbreaks in the EU in 2011, which corresponds to 220 outbreaks, where 47 were with strong evidence caused by *B. cereus* (EFSA, 2013). The two main sources of *B. cereus* infection, accounting for more than 42.5% of the outbreaks, were from mixed foods and cereal products. However, the low number of reported *B. cereus* outbreaks might be a result of underestimation. Due to the mild and transient symptoms of the illness, many cases might not be identified. In addition, *B. cereus* is not classified as a zoonosis by EFSA and has therefore received less attention in surveillance and reporting compared with e.g. *Salmonella* and *Campylobacter* (Ceuppens et al., 2013).

Soil is the primary source of contamination of foods with spores of *B. cereus*. As a result, various raw food ingredients such as vegetables, potatoes, milk, herbs and spices are often contaminated with *B. cereus* spores (EFSA, 2005). The disease of *B. cereus* in humans arises as a direct result of the survival of the *B. cereus* spores under normal cooking procedures and improper storage conditions after cooking, the spores germinate and the vegetative cells multiply (Logan, 2012). The two types of food-poisoning from *B. cereus* are often related to different types of food products. The diarrheal disease is often related to meat products, soups, vegetables, sauces and dairy products, whereas the emetic type is related to main-

ly to rice and pasta (Abee et al., 2011; Schoeni & Wong, 2005); however, emetic strains have been isolated from milk, pudding and ice cream (Messelhäusser et al., 2010). Due to the difference in food sources, different distributions between countries are observed for the emetic and diarrheal diseases. The emetic disease dominates in Japan and the UK, whereas in Northern Europe and North America, the diarrheal disease seems to be more prevalent (Kotiranta et al., 2000).

2.2.3 Detection and enumeration

The standard detection and enumeration of *B. cereus* from foods, the environment or clinical samples, are routinely performed using conventional selective plating media such as the mannitol–egg yolk–polymyxin agar. The media is recommended for the identification and enumeration of presumptive *B. cereus* colonies by ISO (Anonymous, 2005). However, not only are the culture-dependent methods time-consuming and unable to indicate toxin production capacity but the use of selective media has been shown to pose other problems. It has been found that not all *B. cereus* strains display the typical reactions, which might lead to misclassifications or missed when using selective media (Fricker et al., 2008). In addition, presumptive *B. cereus* isolates must be tested by several biochemical and microscopic procedures to confirm that it in fact is a true *B. cereus* due to the close relationship with the other *Bacillus* species (Brusetti et al., 2009).

The molecular methods, which include DNA microarray and PCR techniques, for detection of *B. cereus* can provide more accurate and rapid diagnostics compared with the culture-based methods. PCR and real-time PCR methods are mainly used for the detection of the different toxin genes of the emetic and diarrheal strains of *B. cereus*. For detection and quantification of *B. cereus* several PCR methods have been published (for some examples see Reekmans et al., 2009, Martínez-Blanch et al., 2009 and Fricker et al., 2007).

2.2.4 *Bacillus cereus* in the food production

Because of its abundance and the resistance of its spores, *B. cereus* can contaminate most agricultural products and plays a major role in the contamination and spoilage of food products.

B. cereus is also frequently present in the food production environments due the adhesion ability of the spores (Andersson & Rönner, 1998; Stenfors Arnesen et al., 2008). In

addition to the resistance of the spores, *B. cereus* can also form biofilms on stainless steel (Lindsay et al., 2000; Peng et al., 2002). In contrast to single cell attachment and planktonic cells, the biofilms of *B. cereus* have been found to be more resistant to sanitizers (Peng et al., 2002; Ryu & Beuchat, 2005). A significant increase in heat resistance of *B. cereus* spores attached to stainless steel surfaces has also been observed (Simmonds et al., 2003). Studies have shown that *B. cereus* biofilms in the air-liquid interface contain up to 90% spores, which contributes to the spread of spores in food processing areas and production-line (Wijman et al., 2007).

The spore germination plays a key role in the first stage of food spoilage and thereby the possibility of foodborne infections, as it initiates the transfer from spore to vegetative cell. A strategy to reduce or even deplete *B. cereus* in food products are to promote the spore germination before applying the food preservation methods, as the vegetative cells are less resistant compared with the spores and thereby relatively easy to kill (Abee et al., 2011).

Chapter 3

Molecular Diagnostic Methods for Foodborne Pathogens

3 Molecular diagnostic methods for foodborne pathogens

Detection and characterization of foodborne pathogens in foods are important parts of any quality control or food safety plan. The conventional culture-dependent methods for detecting foodborne pathogens are based on growth in culture media, followed by isolation, and biochemical and/or serological identification. Most of these methods are powerful and reliable; however, the drawbacks are that they are usually laborious, complex and time-consuming (Jasson et al., 2010). Another problem that arises with culture-dependent detection is that prior knowledge about the pathogen in the sample is needed and that not all pathogens are culturable, including viruses and toxins. The introduction of the molecular diagnostic methods based on the detection of the organism´s nucleic acids (e.g. DNA or RNA), such as PCR, microarray and sequencing have made detection and identification of pathogens faster and with greater specificity and sensitivity (Dwivedi & Jaykus, 2011; Weile & Knabbe, 2009).

3.1 Nucleic acid based diagnostics

Many of the diagnostic methods for detection of foodborne pathogens are based detection of nucleic acids and include different PCR techniques, microarray and sequencing. The detection of microbial nucleic acids for diagnostics depends highly on the separation of nucleic acids from the sample, the quality and amount of the target (Brehm-Stecher et al., 2009).

3.1.1 PCR

PCR has become one of the most important molecular diagnostic methods for detection of foodborne pathogens and is considered to be a valuable alternative to the culture-based detection techniques due to its speed, limit of detection (LOD), sensitivity and specificity (Maurer, 2011; Rodríguez-Lázaro et al., 2013).

PCR was first reported in the mid 1980's (Mullis et al., 1986; Saiki et al., 1985). In PCR, repeated cycles of DNA synthesis are conducted in order to multiply a target DNA sequence. This method can, in theory, detect down to a single target pathogen (Batt, 2007). The PCR reaction comprises of three temperature step. The first step is the denaturation of the double stranded DNA (dsDNA) to form single stranded DNA (ssDNA) as template for the

second step. In the second step, primers anneal to the complementary ssDNA target. After annealing to the target DNA, the DNA polymerase extends the primers by adding one complement nucleotide after another. The extension of the primers creates a DNA strand, complementary to the original DNA target (Figure 1). The three steps (denaturation, annealing and extension) are repeated multiple times in the thermocycler, where each synthesized DNA strand acts as template for the synthesis of new DNA strands (Fairchild et al., 2006; Mullis et al., 1986).

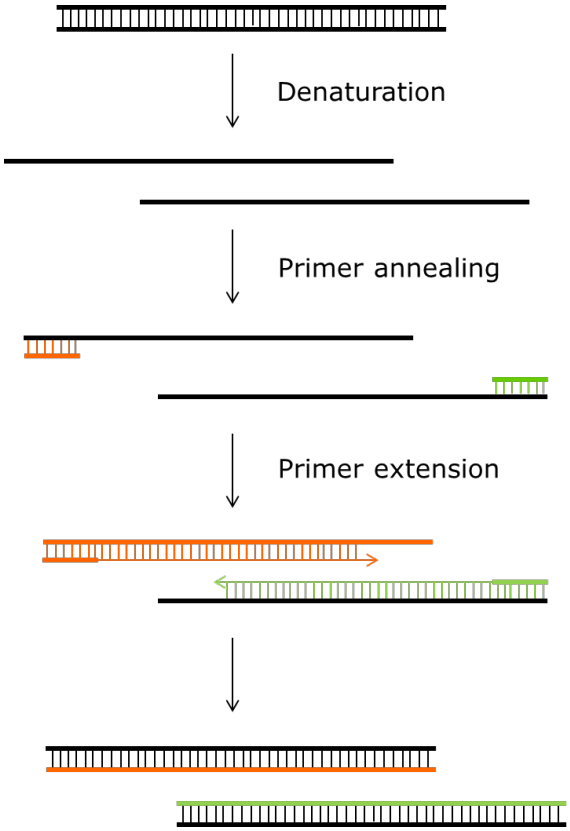

Figure 1 The principle behind PCR. The first step is denaturation, where the dsDNA is separated into ssDNA, where after the primers anneals to the complementary sites on the ssDNA. The primers are extended by the DNA polymerase in order to create copies of the original DNA template.

When appropriate conditions are used, each cycle will generate a doubling of the initial number of targets with each cycle (Erlich, 1989). Conventional PCR works by end-point detection where the produced PCR products (i.e. amplicons) are visualized by gel electrophoresis. The PCR products are loaded on a gel and electrophoresis is performed. The gel is strained with a fluorescent dye e.g. ethidium bromide or SYBR Green I that binds to the PCR products,

which then can be visualized by illumination with an ultraviolet (UV) light source (Mullis et al., 1986; Aaij & Borst, 1972).

Real-time PCR allows for both detection and quantification during the PCR reaction. In real-time PCR is the amplicons and thereby the amplification monitored by incorporation of a fluorescently labeled probe or a DNA-binding dye such as SYBR Green I. There exist a large range of different fluorescence probes (reviewed by Josefsen et al., 2012), however common for all of them are that they produce a change in the fluorescent signal during the PCR amplification. The DNA-binding dyes binds to dsDNA, thereby releasing a fluorescence signal. The increase in fluorescence, whether it is probe or SYBR Green I based assays, is recorded after each replication cycle, and the amplification of the PCR products can thereby be monitored (Mackay, 2004). Computer software associated with the thermocycler records the amount of fluorescence and the amplification cycle at which the fluorescence exceeds a defined threshold level, i.e. the background fluorescence, is known as the threshold cycle (Cq, Cp, or C_T depending on the software used; in this thesis the designation C_T is used), see Figure 2. The higher amount of target DNA present in the sample, the earlier will the fluorescence cross the threshold.

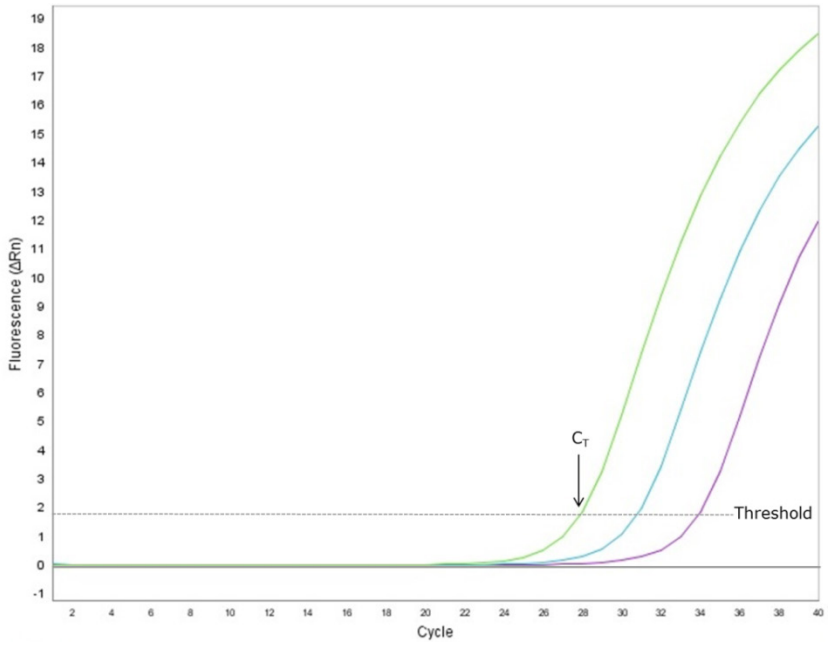

Figure 2 An amplification plot obtained by real-time PCR. The y-axis corresponds to the fluorescence emitted from either the labeled probe or DNA binding dye, which rises in each amplification cycle (x-axis). The C_T is the amplification cycle where the fluorescence exceeds the background fluorescence.

The use of real-time PCR has not only decreased the time to perform PCR detection methods, but also reduced the risk of cross contamination since both the amplification and fluorescent detection occur in the same closed tube (Barken et al., 2007; Valasek & Repa, 2005).

3.1.1.1 Quantification

The use of real-time PCR for detection has the advantage that it is possible to quantify specific genes or parts of the organism's DNA. For quantification, two different approaches can be applied; absolute and relative (Kubista et al., 2006).

The absolute quantification approach is mainly used for specific detection of microorganisms since it can give a precise quantity of the target gene and can be normalized to a unit amount of sample, e.g. volume, nucleic acid content or number of cells (Postollec et al., 2011). The quantification is based on comparison of C_T values with a standard curve of known amounts of the target gene. For that reason, this method is dependent on the quality of the sample (e.g. inhibitors) and the standard curve, which requires similar amplification efficiencies for all samples and standards (Fu et al., 2009).

Relative quantification is mostly used for estimating changes in gene expression, as the setup includes a control, e.g. wildtype or an untreated sample, which all other data are subsequently compared with. The end result of the relative quantification is expressed as a target/reference ratio (Wong & Medrano, 2005). For the relative quantification, normalization is used to correct for sample-to-sample variations. The genes chosen for the normalization must maintain a constant level of expression under the given experimental conditions. For that reason are housekeeping genes or genes such as ribosomal RNA (rRNA) most often used for normalization (Huggett et al., 2005; Schmittgen & Zakrajsek, 2000). However, before conducting gene expression studies, it is necessary to validate the stability of the chosen housekeeping gene under the specific experimental conditions (Radonić et al., 2004).

When using real-time PCR for gene expression studies, an additional step is added to the reaction; the reverse transcription, where the RNA is converted to DNA (Bustin, 2002). The reverse transcription PCR (RT-PCR) can be performed as either a one-step or a two-step reaction. In the one-step reaction the entire reaction from conversion of messenger RNA (mRNA) to complementary DNA (cDNA) to PCR amplification is performed in one

tube, whereas in the two-step reaction, the two steps of RT and PCR amplification occurs in separate tubes (Nolan et al., 2006). Quantitative RT-PCR (qRT-PCR) was applied in Paper I.

3.1.1.2 PCR and food safety

Detection of foodborne pathogens by PCR techniques has the advantage of enabling faster identification, which not only increases the food safety but also the food quality. Furthermore, with qRT-PCR it is possible to study the behavior of pathogens influenced by different environmental conditions by studying the expression of suitable target genes.

Detection

Several studies have been reported describing the development of real-time PCR assays for detection and quantification of particular foodborne pathogens, including *Salmonella* and *Bacillus cereus* (Table 1).

Table 1 Examples of studies with quantification of *Salmonella* and *B. cereus* with real-time PCR

LOD	Food matrix	Enrichment	Reference
B. cereus			
10^3-10^5 CFU/g or 10^1-10^3 CFU/g[A]	Rice	No	Fricker et al., 2007
60 CFU/ml	Liquid egg, infant formula	No	Martínez-Blanch et al., 2009
10^5-10^8 CFU/g[B]	Rice pudding, cereal, carrot puree	No	Wehrle et al., 2010
10^1 CFU/g	Rice pudding, cereal, carrot puree	Yes	Wehrle et al., 2010
Salmonella			
4.4×10^2 CFU/ml	Pork meat	No	Löfström et al., 2011
20 CFU/g	Seafood	No	Kumar et al., 2010
10 cells/10g	Yogurt	No	D'Urso et al., 2009
1 CFU/125 ml	Milk	Yes	Omiccioli et al., 2009
<18 CFU/10 g	Ground beef	Yes	Suo et al., 2010
10^3 CFU/g	Ground beef	No	Wang et al., 2007
10 CFU/g	Ground beef	Yes	Wang et al., 2007

[A] Depending on the assay type
[B] Depending on the matrix and strain

The low detection level of real-time PCR methods gives the possibility to detect pathogens without an enrichment step, leading to shorter identification times. For example, detection of *B. cereus* in gelatine was decreased from two days by the standard culture-based method to two hours using real-time PCR (Reekmans et al., 2009). An advantage with real-time PCR based detection is the possibility for multiplexing, enabling the detection of several pathogens simultaneous. Multiplex real-time PCR assays have e.g. been developed for detection of *E. coli* 0157:H7, *Salmonella* spp. and *Listeria monocytogenes* in milk and meat samples (Omiccioli et al., 2009; Suo et al., 2010). The short time of the real-time PCR based assays enables the possibility for adding a short enrichment step in order to lower the detection level. In a study by Wang et *al*. the detection level for *Salmonella* in ground beef was 10^3 CFU/g without prior enrichment, but including a 10 hour enrichment step, 10 CFU/g could be detected (Wang et al., 2007).

Gene expression measured by qRT-PCR

For *Salmonella* in relation to food safety, qRT-PCR has been used to study e.g. differentiation of dead and alive *Salmonella* cells, impact of growth conditions and attachment (Barak et al., 2005; González-Escalona et al., 2009)(Paper I). In Table 2 is an overview of some published studies.

Table 2 Overview of some published qRT-PCR studies for *Salmonella* in relation to food

Study	Gene(s) investigated	Reference
The change in expression of attachment related genes under different pre-growth conditions	*flhDC, motAB, prgH, fliC, fljB, yhjH*	Paper I
Detection of alive *Salmonella* spp. cells in produce	*invA*	González-Escalona et al., 2009
Detection of *Salmonella* in contaminated minced beef and whole eggs	*sef*A	Szabo & Mackey, 1999
Virulence genes required for attachment to plant tissue	*agfB*	Barak et al., 2005
The role of cellulose and O-antigen capsule in the colonization of plants	*adrA, bcsA*	Barak et al., 2007

In Paper I, qRT-PCR was used to study the impact of different pre-growth conditions on the expression of attachment related genes in five *S*. Typhimurium strains (wildtype, Δ*prg,* Δ*flhDC*, Δ*yhjH* and Δ*fliC*). The gene expression was measured over a three hours period where the cells were grown on a pork meat surface. The gene expression measurements showed that the growth conditions changed the expression of the investigated attachment related genes.

Another application of qRT-PCR is to distinguish between alive and dead cells. PCR alone cannot distinguish between DNA originating from live or dead bacteria. Furthermore can DNA be rather resistance to degradation and be present after the death of the host cell which can lead to false positive results using PCR (Allmann et al., 1995; Josephson et al., 1993; Wolffs et al., 2005). RNA is degraded more rapidly than DNA, which in principle means that the presence of RNA in bacterial cells can serve as an indicator for viability. Several reports have been made on the detection of viable cells from *Salmonella* using RT-PCR (González-Escalona et al., 2009; Szabo & Mackey, 1999).

3.1.2 Microarray

Microarrays represent another useful technique in the diagnostic toolbox for foodborne pathogens. The word "microarray" was introduced for the first time by Schena et *al*. in 1995 and is a further development of Southern blotting (Schena et al., 1995). The basis of microarray technology involves hybridization of a mixture of nucleic acids (targets) with thousands of individual nucleic acids species (probes). The probes are immobilized on the array (spot or feature) at specific positions that can be identified in the later analysis process. The targets are labeled with a fluorescent dye and are deposited as a solution onto the array in order to hybridize to the probes. Each target will be captured by the corresponding probe and the excess of labeled and non-binding targets will be washed away and finally the array is scanned in order to quantify the dye signal for each spot (López-Campos et al., 2012a).

Depending on the technique used for printing the spots, the length of the probes varies. The use of a photolithography approach to synthesize the probes directly on the array usually generates probes of 25mers (Singh-Gasson et al., 1999). Another way to produce the arrays is by a spotter. Here are droplets of DNA oligos, deposited on the surface of the array where after they bind (Lausted et al., 2004). Using a spotter the length of the probes on the

array are normally 25-70mers. The use of longer oligos generally gives higher sensitivity, whereas the shorter ones have a higher selectivity (Bates et al., 2005).

Microarrays can be used to conduct two different types of experiments; gene expression profiling and genotyping. With gene expression profiling, changes in gene expression between two different experimental conditions can be monitored by differences in mRNA levels (based on cDNA). The total mRNA is extracted from the cells from each condition and labeled with different fluorescent dyes, usually red and green dyes. From the fluorescence intensities and the red/green ratio for each spot, the relative expression levels of the genes can be estimated (Debnath et al., 2010; Trevino et al., 2007). For the genotyping microarray the presence of specific DNA sequences of an isolate is analyzed (Pollack et al., 1999). See an illustration of the two approaches in Figure 3.

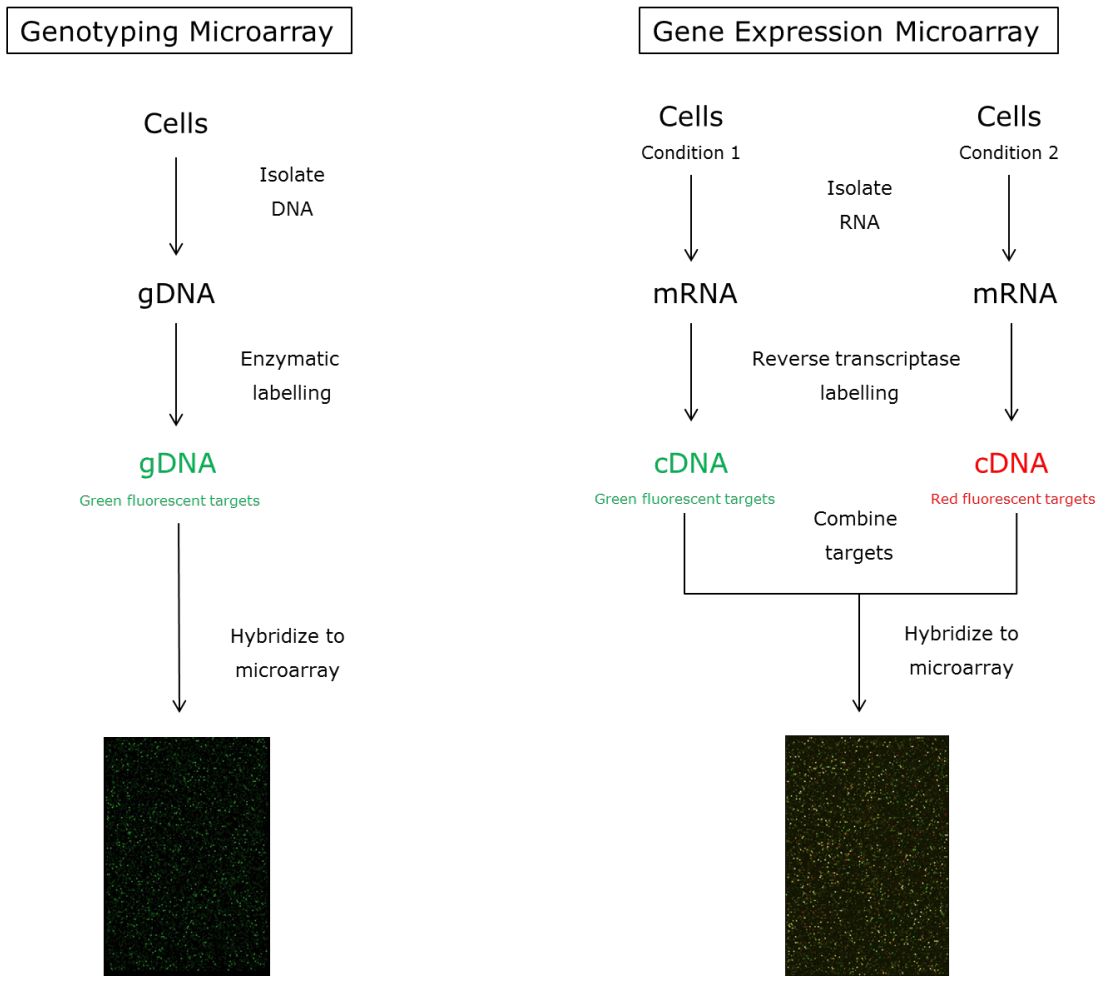

Figure 3 Schematic diagrams of genotyping and gene expression microarray experiments.

Performing genotyping DNA microarray studies can have two purposes: (i) to investigate the difference in gene content between two organisms or (ii) to investigate the gene content of one organism (López-Campos et al., 2012b; Trevino et al., 2007). A genotyping microarray was applied in Paper II and gene expression profiling microarray in Paper III.

3.1.2.1 Microarray and food safety

Genotyping with microarray permits the detection of present pathogens in a sample or to examine the difference in gene content of related strains. For instance, in the study part of this thesis (Paper II) were the genomic DNA compared from 40 *Salmonella* isolates and detected specific patterns of genes present or absent among the isolates covering different serotypes and source of isolation in a slaughterhouse. Difference in the gene content between the same serovars was observed, however it was not possible to identify genes that could be linked to the source of isolation.

Reen et *al*. investigated the genomic diversity among *S. enterica* isolates (Reen et al., 2005). They found that the five recognized *Salmonella* pathogenicity islands were present in all the investigated isolates, and they furthermore identified more than 30 genomic regions that varied in their presence among the isolates. Further, they showed that very little genomic diversity seemed to exist within particular serovars, even though they were widely geographic distributed.

The other use of microarray is for gene expression. It can be used for comparing levels of gene transcription under various growth conditions, chemical treatments or other stress-causing effects associated with food production (López-Campos et al., 2012b). In a study by Wang et *al*. they compared the global gene expression profiles of *S*. Typhimurium over an 8 hour time period for comparing three different growth conditions; swarming, non-swarming and planktonic (Wang et al., 2004). They showed that bacteria grown on the surface of agar had a markedly different physiology compared with those grown planktonic. Furthermore, it was possible to identify putative new genes involved in motility and virulence.

In Paper III, the transcriptional response of *S*. Typhimurium to heat treatment and pH changes was investigated. It was shown that most of the genes up-regulated during the heat shock remained up-regulated 30 minutes after the temperature was set back to normal. In

addition was it found that the cells after heat shock were more resistant to subsequently heat and acid inactivation conditions.

3.1.3 DNA sequencing

The newer developments within molecular diagnostics include DNA sequencing based methods. The conventional DNA sequencing approach was introduced in 1975 by Sanger and is based on the chain-terminator method (Sanger & Coulson, 1975; Sanger et al., 1977). The Sanger sequencing is capable of sequencing up 1 kilobase (kb) of sequence data at a time. The sequencing technology has evolved greatly during the last couple of years. In 2005 the high-throughput sequencing platforms, the so-called next-generation sequencers (NGS), were introduced on the market (Margulies et al., 2005; Shendure et al., 2005). In contrast to Sanger sequencing, the NGS technologies can generate several hundred thousand to tens of millions sequencing reads in parallel (Mardis, 2008a, 2008b; Voelkerding et al., 2009). The drawbacks of the NGS technologies are that they generate shorter reads, i.e. contigs, with lower quality, compared with the Sanger sequencing (Pareek et al., 2011). However, the NGS technologies compensate for this by high sequence coverage at each position (Hui, 2012; Metzker, 2010). Sequencing has several applications for detection and identification, such as targeted sequencing and whole genome sequencing. The focus in the following will be on the targeted sequencing approach; however, whole genome sequencing is described.

Target specific DNA sequencing is used for routine identification of bacteria by utilization of conserved genes (Clarridge, 2004; Doolittle, 1999; Petti et al., 2005). One of these genes is the 16S rRNA gene, which is universally present in all bacteria and is only minimally affected by horizontal gene transfer (i.e. transfer between distantly related organisms (Asai et al., 1999)). Furthermore, the gene contains hypervariable regions flanked by highly conserved stretches, making the these regions suitable for design of universal primers (Baker et al., 2003), see Figure 4 for visualization. These characteristics have made the 16S rRNA gene the most used region for bacterial taxonomy and identification (Doolittle, 1999).

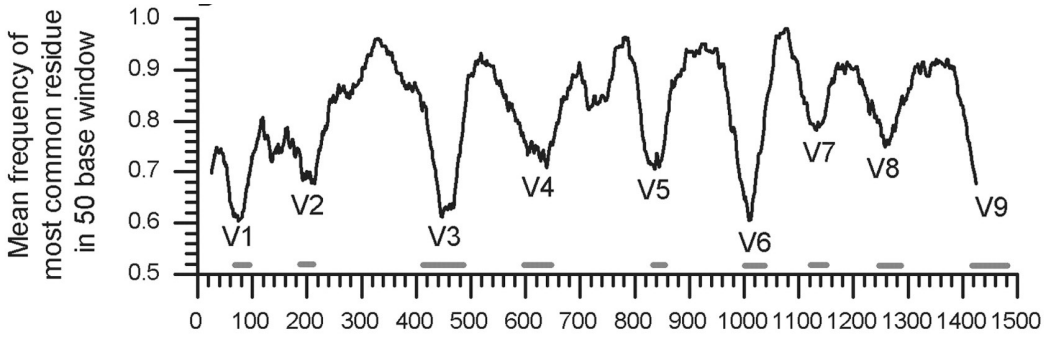

Figure 4 The variable regions within the 16S rRNA gene. The frequency measured of the variability of the most common nucleotide residues at each base position within the 16S rRNA gene. The locations of the hypervariable regions are labeled, with gray bars on the *x* axis defining these regions as V1 to V9 (reprinted with permission from Ashelford et *al*. (Ashelford et al., 2005))

The 16S rRNA gene have nine hypervariable regions (V1-V9), where the regions V2, V4 and V6 provides the lowest error rates for assigning taxonomy (Liu et al., 2008), however the combination of the regions V1-V3 and V7-V9 has been suggested to provide a better characterization (Kumar et al., 2011). For universal distinguishing of all bacteria at the genus level, the regions of V2 and V3 were identified to be the most suitable (Chakravorty et al., 2007). The advantage of using the 16S rRNA gene for characterization and detection is the variety of established searchable 16S rRNA classification databases, such as the Ribosomal Database Project II (RDP-II) (Cole et al., 2007, 2009), and Greengenes (DeSantis et al., 2006). Other genes such as *recA*, *rpoB*, *gyrA* and *gyrB* can also be used as target for sequencing because of their functionally conserved parts with variable regions (Petti, 2007). However, there are some limitations to the use of target specific sequencing, e.g. difficulties in discriminating between many species within the Enterobacteriaceae using the 16S rRNA gene (Chakravorty et al., 2007).

In contrast to targeted sequencing, where only information on the target gene is gained, whole genome sequencing provides all the available genetic information about the isolate. Using whole genome sequencing poses one problem, the assembly of all the generated reads into the final genome sequence. Assembly of whole genomes can be applied in two ways; *de novo* assembly or alignment to a reference sequence (also known as comparative assembly). In the comparative assembly approach, the assembly is guided by alignment to a reference sequence of a closely related organism. However, in order to use comparative assembly, prior knowledge on the genome assembled has to be known. The *de novo* assembly

approach is used when trying to construct the genome of an organism not similar to any previous sequenced organism (Pop, 2009).

3.1.3.1 Metagenomics

One of the ways to use the different sequencing technologies is for metagenomics studies. Metagenomics is defined as the genomic analysis of the entire gene pool of a sample using culture-independent methods (Schloss & Handelsman, 2003). Metagenomics can be used for investigation of all microorganisms present in a particular environment without the bias originating from unculturable or difficult to culture microorganisms.

Sanger sequencing has been used for several metagenomics projects, however due to the general lower cost and higher through-put, a shift to the use of NGS platforms has occurred (Gill et al., 2006; Venter et al., 2004). With high genetic diversity within most microbial communities, obtaining enough sequence depth to identify any gene with sufficient coverage is currently difficult. To overcome this, target specific metagenomics can be a suitable approach to encounter (Suenaga, 2012). With target specific metagenomics, as for target specific DNA sequncing, specific genes of interest are amplified by PCR and the products are then sequenced either by Sanger sequencing or NGS methods (Eckburg et al., 2005; Kalyuzhnaya et al., 2008).

The general approach for target specific metagenomics with Sanger sequencing involves cloning of the PCR product into vectors and thereby creating a metagenomics library, however this can be very laboratory demanding and time-consuming (Riesenfeld et al., 2004). To overcome this, a direct sequencing approach that utilizes multivariate statistical analysis in order to investigate the diversity of mixed community has been reported (Skånseng et al., 2007; Trosvik et al., 2007; Zimonja et al., 2008). The direct sequencing approach was evaluated for detection of *B. cereus* in Paper IV and Paper V.

3.1.3.2 DNA sequencing and food safety

The developments within sequencing technologies have a major impact on food safety. The increased number of genomes available helps improve detection methods such as the possibility for designing more specific primers, enhancing the specificity of the assays. Furthermore,

metagenomics based sequencing can help identify unknown sources of foodborne outbreaks (Kawai et al., 2012; Nakamura et al., 2008).

In Paper IV, two *B. cereus* strains was inoculated in bottled water, from different suppliers, in levels of 10-10^6 CFU/L. Total DNA extraction directly from water and 16S rRNA gene sequencing were used in combination with principle component analysis (PCA) and multi curve resolution (MCR) to study the detection level. The results showed detection at levels of 10^5-10^6 CFU/L, depending of the strain and water supplier. The analysis also revealed that background flora in the bottled water varied between the different water suppliers according to their bottling country.

The method evaluated in Paper IV, was further investigated for the use for detection of *B. cereus* in food and feed samples (Paper V). Detection of *B. cereus* in food and feed samples has some issue compared with the detection in bottled water. The food and feed samples contains much more inhibitors that might interfere in the different steps of the methods, particularly the PCR amplification of the 16S rDNA. The results showed that the detection of *B. cereus* was much more difficult as it was possible to obtain sequence results from fewer samples compared with the results obtained in Paper IV. Despite of the lower quality of the sequence data, there were some indication of distinguish between the two *B. cereus* strains, however every dependent on the matrix.

Whole genome sequencing is being used more frequently for investigations of foodborne outbreaks as it usually can give higher discrimination of isolates (Wilson et al., 2013). Following an outbreak with *S.* Montevideo in peppers for spiced meals in 2009 and 2010 (Lienau et al., 2011), the genomes of 34 highly homogeneous *S.* Montevideo isolates from the outbreak were compared with 24 other *S.* Montevideo genomes from clinical-food matches associated with the same outbreak (Allard et al., 2012). The isolates associated with the outbreak clustered together in a separate group distinct from other closely related *S.* Montevideo isolates not originating from the outbreak. The distance between the outbreak associated and unassociated isolates was clearly distinct, which made it easier to identify isolates linked to the outbreak.

3.2 Extraction of nucleic acids

Common for nucleic acid based diagnostic methods are the initial sample preparation, often starting with the extraction and purification of nucleic acids. Numerous substances originating from food, feed and environmental matrices can inhibit the downstream molecular diagnostic applications. The quality of the purified nucleic acids is critical for the optimal performance of any nucleic acid based method. The presence of cellular or other contaminating materials often disturbs or prevents the downstream applications (Hedman et al., 2013; Rossen et al., 1992; Wilson, 1997). The choice of the right extraction method depends on several parameters including cost, time, downstream application, sample matrix, throughput, quality and yield.

3.2.1 Extraction procedure

The performance of most nucleic acid based diagnostic methods is highly influenced by the quality of the nucleic acids. The required quality of the nucleic acids depends on which method to use, and whether the purpose is detection or quantification

No universal protocol exists for extraction of nucleic acids. There are several different approaches for nucleic acids extraction, however an extraction protocol usually comprises of three steps: cell lysis (chemical, mechanical or enzymatic), removal of cell contaminants and precipitation and purification of the nucleic acids (Tan & Yiap, 2009). The old standard for extraction of high quality DNA/RNA is based with enzymatic lysis of the cell wall and phenol/chloroform extraction, followed by precipitation by ethanol. Today a variety of commercially kits are available for extraction of nucleic acids, usually made for specific types of matrices e.g. blood, soil, culture, food and feed. The commercial kits can be divided into two groups; manual purification and automatic systems. Most of the manual kits are based on solid-phase purification normally performed using a spin column, whereas many of the automatic systems uses magnetic beads to capture the nucleic acids (Berensmeier, 2006).

The release of the nucleic acids is a prerequisite for optimal sensitivity of the assay. Efficient lysis of the bacteria and spores is needed; however to harsh lysis can shatter the integrity of the extracted nucleic acids (Rantakokko-Jalava & Jalava, 2002). Insufficient lysis

can originate from inadequate lysis conditions, inactivation of the enzyme or bad quality of enzymes (Wilson, 1997).

Many kits have been developed and optimized for a limited type of matrices. In Paper V, five different commercial available extraction kits were evaluated on their ability to extract *B. cereus* from four different food and feed samples. The results clearly showed that some extraction kits perform well on multiple sample types whereas for other kits it was found more difficult. This is consistent with other studies showing different efficiency when using the same extraction kit on multiple matrices (Coyne et al., 2004; Elizaquível & Aznar, 2008).

3.2.2 Inhibitors and controls

When using nucleic acid based diagnostic methods, one of the drawbacks is that the methods are sensitive towards inhibitors. Inhibitors have been mentioned to be one of the most important barriers for efficient downstream applications with extracted DNA. Inhibition can occur in several ways; interference with the cell lysis during the extraction, inhibition of enzymes activity and degradation or capture of nucleic acids (Wilson, 1997). The inhibitors can be found in foods, culture media and various chemical compounds originating from either the sample or the extraction kit, such as lipids, proteins, ions, ethanol and isopropanol (Hedman & Rådström, 2013; Rossen et al., 1992).

The different types of inhibitors can interfere with the PCR reaction in different steps of the analysis. From insufficient extraction of the nucleic acids, nucleases can be left in the sample which can degrade the template DNA or RNA (Abbaszadegan et al., 1999; Chandler et al., 1998). Proteases can also be co-extracted with the nucleic acids and have the capability of degrading the DNA polymerase (Powell et al., 1994; Rossen et al., 1992). Many of the inhibitors act either directly or indirectly on the DNA polymerase. Calcium acts by competing with magnesium and thereby reducing the activity of the DNA polymerase (Bickley et al., 1996; Opel et al., 2010). When applying filtration for concentration of pathogens from e.g. water samples, the present inhibitors are likewise co-concentrated, increasing the interference with the PCR reaction (Abbaszadegan et al., 1999; Jiang et al., 2005)(Paper IV).

Since the inhibitors can affect the results of the diagnostic methods, appropriate controls are needed during the analysis in order to verify the obtained results. With inhibition, either at the extraction step or in the detection method, false-negative results can occur. False-negative results can occur from inhibition, insufficient nucleic acids extraction or technical problems originating either from the reagents or the instrument used. Specific positive and negative controls for the quality of the reagents and the function of the instrument are required. In addition, can process controls be required in order to demonstrate that the extraction protocol worked correctly (Girones et al., 2010; Rådström et al., 2004). When using PCR, an internal amplification control (IAC) can be included to avoid false-negative results due to instrument problems, reagents or PCR inhibition (Hoorfar et al., 2004).

3.3 Targets for nucleic acid based diagnostics

For every nucleic acid based method, the target of choice is particular important for the specificity of the assay. Depending on the assay, the ideal target is a gene or non-coding region unique for the organism of interest. However, the gene must not be too specific since it then might fail to detect some strains within one species. The target gene should also be relative stable without high rates of mutation.

One of the most common targets is the genes coding rRNA. The rRNA genes are suitable for molecular diagnostics since they contain regions that are highly conserved between species and regions of higher variability (Gutell et al., 1994; Van de Peer et al., 1996). Depending of the regions within the rRNA genes that are target, different levels of identification can be achieved (Woese, 1987). The 16S rRNA gene is often chosen as target since this gene has defined bacterial phylogenetic relationship, however due to the low mutation rate, discrimination between species in the same genus might not always be possible (Woese, 1987).

The optimal detection target is genes that are completely unique for the pathogen of interest. These can either be virulence, toxin or physiological genes. A common target for detection of *Salmonella* has been the *invA* gene (Galán & Curtiss, 1991; Rahn et al., 1992), however it has been shown that the gene cannot detect all *Salmonella* strains (Malorny

et al., 2003). Another target, the *ttr* locus, has been shown to be suitable for detection of *Salmonella* (Malorny et al., 2004). For *B. cereus*, the toxin gene has been shown to be appropriate target for detection (Ehling-Schulz et al., 2005; Fricker et al., 2007). Chromosomal targets were thought to be useful for specific makers for detection of *B. cereus*, however the selection of a good marker has proven to be difficult due to the high genetic similarity with *B. anthracis* and *B. thuringiensis* (Helgason et al., 2000). An overview of some target genes used for detection of *Bacillus* and *Salmonella* are included in Table 3.

Table 3 Overview of some target genes used for detection of *B. cereus* and *Salmonella*

Gene	Function	Reference
B. cereus		
ces	Cereulide synthetase	Ehling-Schulz et al., 2005
pc-plc	Phosphatidylcholine-specific phospholipase C	Martínez-Blanch et al., 2009
hbl	Haemolysin BL	Wehrle et al., 2009
nhe	Nonhaemolytic enterotoxin	Wehrle et al., 2009
cytK1	Cytotoxin K	Wehrle et al., 2009
Salmonella		
ttrRSBCA	Gene required for the tetrathionate respiration	Malorny et al., 2004
invA	Involved in invasion	Rahn et al., 1992
SipB-SipC	Junction of virulence genes	Ellingson et al., 2004
sefA	Fimbrial subunit	Woodward & Kirwan, 1996

The *ttrC* gene was used as positive control for *Salmonella* in the microarray study in Paper II. The 16S rDNA gene was used for evaluation of detection and distinguishing between two *B. cereus* strains in Paper IV and Paper V.

Chapter 4

Manuscripts

Paper I

Influence of contact time and pre-growth conditions on the attachment of *Salmonella enterica* serovar Typhimurium to pork meat

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Influence of contact time and pre-growth conditions on the attachment of *Salmonella enterica* serovar Typhimurium to pork meat

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Running title: Attachment of *Salmonella* to pork meat

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Keywords: *Salmonella*; attachment; pork meat; pre-growth; gene expression; food safety

Abstract

Contaminated foods from the meat processing industries are a major source of salmonellosis worldwide. In this study, the attachment ability of *Salmonella enterica* serovar Typhimurium 4/74, one wildtype and four knock-out strains (Δ*prg*, Δ*flhDC*, Δ*yhjH* and Δ*fliC*), was investigated on a pork meat surface. Expression of attachment related genes (*flhDC*, *fliC*, *prgH*, *motAB*, *yhjH* and *fljB*) was measured by quantitative reverse-transcriptase PCR. The attachment abilities were calculated for different contact times; 0 or 2 min and 60 min, using cells from planktonic or immobilized growth. Results showed that attachment of wildtype, Δ*prg*, Δ*flhDC* and Δ*yhjH* cells increased with increasing contact time, whereas attachment of Δ*fliC* cells decreased. Planktonic grown Δ*fliC* cells had an almost three-fold increase in detachment probability over time. With a contact time of 2 min, a three-fold decrease in detachment probability was observed between planktonic and immobilized grown Δ*flhDC*, and a two-fold increase for Δ*fliC*. Gene expression studies showed that regulation of attachment related genes changed according to pre-growth conditions. In conclusion, the study indicates that pre-growth conditions and contact times might affect the attachment ability of *Salmonella* on a meat surface, a knowledge that can help designing adequate meat processing operations to increase food safety.

1 Introduction

Severe infections caused by *Salmonella* from contaminated food products pose a threat to human health, and is a major cause of salmonellosis worldwide (EFSA, 2010; Gilliss et al., 2011). One critical source of *Salmonella* in pork originates from cross contamination during the slaughter process (van Hoek et al., 2012). Therefore a better understanding of *Salmonella* behaviour in the food production environments is needed in order to minimize the risk of cross contamination and subsequently the risk of human salmonellosis incidents due to consumption of contaminated food.

Adaptation and persistence of *Salmonella* in food production chains have been suggested to be a result of bacterial attachment and surface colonization (Swanenburg et al., 2001; Vestby et al., 2009) which depends on the formation of biofilm and the flagellar biogenesis plays an important role here (Pratt & Kolter, 1998). Flagellar biogenesis in *Salmonella enterica* serovar Typhimurium (*S*. Typhimurium) involves more than 60 genes (Chilcott & Hughes, 2000; Frye et al., 2006). The master flagellar operon is *flhDC* and proteins encoded in this operon form a complex that directs transcription of other genes required for biosynthesis of the base of the flagellar structure. Some of these proteins are required for transcription of genes which include motor proteins and chemotaxis components, such as *fliC*, *motAB, yhjH* and *fljB* (Frye et al., 2006; Ko & Park, 2000; Wang et al., 2006). The *flhDC* operon also indirectly regulates various non-flagellar genes such as *Salmonella* pathogenicity island 1 (SPI1) genes, e.g. *prgH* (Prüß et al., 2001; Saini et al., 2010; Stafford et al., 2005).

Once cells have attached to a surface, colony formation will occur. There are several studies on the attachment potential of *Salmonella* to different kinds of food and food production materials such as vegetables (Garrood et al., 2004; Patel & Sharma, 2010), beef (Kinsella et al., 2007), stainless steel and polypropylene (Moore et al., 2007). Most studies on the attachment of *Salmonella* to a pork meat surface have been performed using planktonic cell cultures for investigating the effect of different rinsing techniques (Benedict et al., 1990) and tolerance towards disinfectants (Humphrey et al., 1997). However, previous studies have shown that bacteria grow differently when they are immobilized, compared with planktonic growth (Brocklehurst et al., 1995, 1997; Knudsen et al., 2012), which can be important when looking at different food processing environments. In order to mimic immobilized growth in foods, the IFR Gel Cassette System (Brocklehurst et al., 1995) has been developed. The system is also designed to hold a planktonic control.

In the present study, for the first time, the attachment to a pork meat surface of wildtype *S.* Typhimurium 4/74 and four knock-out strains (Δ*prg*, Δ*flhDC*, Δ*yhjH* and Δ*fliC*) was studied after grown either planktonic or immobilized. The attachment ability of the strains was investigated by means of detachment probability and attachment strength using different contact times. In addition, gene expression of selected genes related to attachment, was analysed by quantitative reverse-transcriptase PCR (qRT-PCR) in order investigate changes in expression over time.

2 Materials and Methods

2.1 Bacterial strains and preparation of inocula

The wildtype strain used in this study was *S.* Typhimurium 4/74 (Wray & Sojka, 1978) and it was used for construction of the knock-out strains. Gene deletion of the *prg* and *flhDC* operon and the genes *yhjH* and *fliC* was done as previously described (Datsenko & Wanner, 2000) and moved to a clean background of *S.* Typhimurium 4/74 by P22 transduction, as previously described (Maloy et al., 1996). Primers are listed in Table 1. Bacterial cells were grown in 8 ml of Luria-Bertani (LB) broth (Sigma, Brøndby, Denmark) at 25°C for 24 h. A 100× dilution was made and the suspension was re-incubated at 25°C for 24 h. One mL of inocula was mixed with 5 mL RNA*later* solution (Ambion, Life Technologies, Taastrup, Denmark) and placed at 4°C overnight before storage at -20°C. Inocula for the cassettes was prepared by a 1000× dilution of the cell cultures in Maximum Recovery Diluent (MRD; 1 g peptone and 8.5 g sodium chloride per litre, pH 7.0 ± 0.2), to obtain around 10^6 CFU/ml. Two different media were used for the gel cassettes; LB media (for planktonic growth) and LB with 29.3% pluronic F-127 (Sigma) (for immobilized growth) (Knudsen et al., 2012; Nielsen et al., 2013). Inocula were added to give approximately 10^3 CFU/ml or g media.

2.2 Cassettes

The Gel Cassette System for immobilized growth of bacteria was obtained from IFR Enterprises, Norwich, UK. The gel cassettes were prepared as described previously (Brocklehurst et al., 1995, 1997). An appropriate volume (30 ml) of either LB media or LB media with pluronic, both containing inocula (see "Bacterial strains and preparation of inocula"), was transferred to the cassettes by sterile pipetting. Cassettes were then incubated at 25 ± 0.1 °C for either 16 h (LB) or 18.25 h (LB with pluronic) in order to reach early stationary phase.

2.3 Sterile meat samples

Pork fillet, obtained from local retailer, were chosen as model meat surface. The surfaces of the packages were sprayed with 70% ethanol prior to opening. The individual fillets were scalded with boiling water before use. Slices of meat were cut from the fillet to a thickness of approximately 1 cm under sterile conditions. Samples of meat were punched out from these meat slices with a meat stamp with a diameter of 30 mm and placed on a sterile Petri dish.

2.4 Inocula for meat samples

After incubation of the cassettes (see "Cassettes"), a sample of the gelled medium (~10g) or 10 ml of the LB medium was removed from the cassettes, mixed with 90 ml cooled MRD in sterile Stomacher bags (Seward, VWR, Herlev, Denmark) and blended in a Stomacher 400 (Seward) for 1 min at high speed. Fifty ml of the suspension were centrifuged at 5400×g for 7 min at 10°C and the resulting pellet was dissolved in 6 ml cooled MRD (4°C) to a concentration of approximately 10^8 CFU/ml.

2.5 Detachment

A 250×dilution of the inocula (see "Inocula for meat samples") were made in MRD, 100 µl of the diluted inocula were spread on each of two meat samples and the samples were incubated for 2 and 60 min at 22 ± 1°C. After incubation, meat samples were transferred to 100 ml MRD and shaken for 1 min at 250 rpm. The meat pieces were then transferred to a Xylose Lysine Deoxycholate plate (XLD, Oxoid, Greve, Denmark) with the inoculated surface facing down. The meat piece was left on the plate for 1 min and was then transferred to a new plate. This procedure was repeated for a total of 16 plates. After each move, the liquid remaining on the plate was spread. The plates were incubated at 37°C for 24 h and colonies enumerated. The detachment probability was calculated as described by Garrood et *al*. (Garrood et al., 2004). In short, the $\log_{10}$CFU/plate was plotted against the plate number and the detachment probability was calculated from the slope of the resulting linear relationship.

2.6 Attachment studies

One-hundred µl of inocula (see "Inocula for meat samples") was spread onto the surface of meat samples (~10^7 CFU). After incubation at 22 ± 1°C for 0, 1, 2, 3, 4, 5, 10, 30, 60, 90, 120, 150 and 180 min, pieces of meat in triplicates were transferred to beakers containing 100 ml MRD and shaken for 1 min at 250 rpm. The meat samples were subsequently transferred to 40 ml of MRD in Stomacher bags with filter (Seward classic 400, Seward) and homogenized for 1 min at high speed in a Stomacher 400 (Seward). The meat samples were further massaged by hand through the bag for 20 seconds. Controls (in duplicates) were made by adding 100 µl of MRD to the surface of meat samples and subsequently incubated for 0 and 180 min. Samples of 5 ml were removed from the stomacher bags and mixed with 5 ml RNA*later* solution (Ambion) and incubated at 4°C overnight before storage at -20°C until RNA extraction. In addition, one ml samples removed from the beaker (referred to as loosely attached cells) and the stomacher bag (referred to as strongly attached cells) and serially diluted to 10^{-2}. One-

hundred µl of appropriate dilutions were spread on XLD plates (Oxoid) and incubated at 37°C for 24 h after which the number of CFU were determined and used to calculate the attachment strength. The attachment strength (S_R-value) was calculated as described by Dickson and Koohmaraie (Dickson & Koohmaraie, 1989), using the formula: S_R = (strongly attached cells)/(loosely + strongly attached cells).

2.7 Quantitative RT-PCR

2.7.1 RNA purification

Total RNA was purified from samples stored in RNA*later* (Ambion) using the RNeasy Mini Kit (Qiagen, Copenhagen, Denmark) according to the manufacturer's instructions with minor adjustments; for lysis a 15 mg/ml lysozyme solution with proteinase K (Qiagen) was used, and on-column DNase digestion was performed according to manufacturer's instructions. The quality of the RNA was measured using a NanoDrop (Fisher Scientific, Slangerup, Denmark) with software version 3.7.1.

2.7.2 qRT-PCR

One-hundred ng of total RNA was used for cDNA generation employing random priming with the SuperScript Vilo cDNA synthesis kit (Invitrogen, Life Technologies). The resulting cDNA was diluted 1:1 in TE buffer (pH 8.0, Fluka). RNA samples were checked for genomic DNA contamination by omitting the reverse transcriptase. Quantitative RT-PCR was performed on a StepOne Plus thermocycler (Applied Biosystems, Life Technologies) using the Power SYBR green PCR master mix (Applied Biosystems). For each experiment a standard curve was prepared from a serial dilution of a known concentration of cDNA. Each reaction comprised 2 µl template, 10 µl 2×Power SYBR Green PCR Master Mix, 0.4 µl (10 µM) of each primer and 8.2 µl double distilled H_2O. Primers are listed in Table 1. The samples were subjected to an initial denaturation step of 95°C/10 min, followed by 40 cycles of 95°C/15s and 60°C/60s. A melting curve analysis was performed at the end of the PCR run and consisted of an initial step of 95°C/15s, followed by 60°C/60s and then increasing the temperature stepwise by 0.3°C every 15 s up to 95°C. Each run included a negative control for each primer set.

2.7.3 Data analysis

Real-time PCR data was analysed with the StepOne Software v. 2.0 (Applied Biosystems). The reference gene guanlyate kinase (*gmk*), having stable transcription (data not shown), was chosen as an internal control to normalize initial RNA concentrations. Relative expression of

the target genes (*flhDC*, *fliC*, *prgH*, *motAB*, *yhjH* and *fljB*) in the knock-out strains, normalized to *gmk* and relative to the expression of the wildtype, was calculated for each sample using the relative standard curve method (Applied Biosystems, 1997). The calculations were done in MS Excel (Microsoft® Office Excel® 2007).

2.8 Statistical analysis

Detachment probabilities and attachment strength results were calculated as mean ± standard deviation (SD) for two independent biological replicates. For the gene expressions, results were calculated as mean ± SD for two different PCR reactions representing independent biological replicates. For the detachment probabilities, analysis of variance (ANOVA) was used to estimate the effects of contact time, pre-growth condition, strain and their interaction. Prior to statistical analysis, the normal distribution of the data was confirmed by a qq-plot. The analysis was carried out in two steps. Initially a model including the main effects of the variables time, strain and pre-growth condition was estimated. Secondly, significant variables were kept in the model, and the effects of two-terms interactions between the variables were added to the model. The model was reduced backwards, eliminating non-significant interaction terms. A p-value of < 0.05 was considered statistically significant. The R software (v. 2.15) was used for the ANOVA analysis (R Core Team, 2012).

3 Results and discussion

Most studies on the attachment of *S.* Typhimurium has been conducted on vegetables and using planktonic cultures. However, when the bacteria are present in food, including pork meat, the cells are often immobilized to the surface (Brocklehurst, 2003). In the present study, it has been investigated how different *Salmonella* strains, carrying deletions of genes involved in attachment and virulence, attach and subsequently detach from a pork surface after various contact times and pre-growth conditions, i.e. immobilized and planktonic. Gene candidates for the knock-out were chosen from genes having the greatest difference in expression, in a wildtype strain, between the two pre-growth conditions (data not shown).

3.1 Liquid contamination constitutes to higher risk of stronger attachment to pork meat

The effect of the pre-growth conditions, i.e. planktonic or immobilized, and contact time (0 or 2 min and 60 min) on the surface of pork meat was investigated by determination of attachment strength and detachment probability. The attachment strength was calculated as the portion of strongly attached cells out of the total number of cells (Dickson & Koohmaraie, 1989). The detachment probability was determined from the slope of the linear relationship between the number of detaching CFU's and the corresponding plate number (Garrood et al., 2004).

An accordance between the attachment strength and the detachment probabilities for all the strains investigated was observed, i.e. when the attachment strength is high, the detachment probability is low (Table 2). For the wildtype, Δ*yhjH* and Δ*fliC* strains, the attachment was stronger for both contact times when grown planktonic compared with immobilized growth. The opposite was seen for Δ*prg* and Δ*flhDC* mutant cells for which the strongest attachment was seen for immobilized pre-growth conditions for both contract times.

The wildtype and the Δ*yhjH* strains had similar levels of attachment abilities for both pre-growth conditions and both contact times (Table 2). Though the Δ*yhjH* mutant strain was not significantly different from the wildtype strain ($P > 0.05$) it had however a slightly weaker attachment for both planktonic and immobilized pre-growth compared. These similar attachment abilities might indicate that attachment genes in the wildtype and Δ*yhjH* strains might already be down-regulated when applied to the meat surface, resulting in a weaker attachment due to a slower start of expression, particularly seen for wildtype cells after immobilized pre-growth. Findings of Wang et *al*. are in accordance with this (Wang et al., 2004). They report that transcription of genes, involved in flagellum biosynthesis, was down regulat-

ed after 4 h, when *Salmonella* was grown on 0.6% agar. In the current study, cells have been immobilized for 18.25 h before contact with the meat surface. The long immobilization time could indicate that attachment genes already are down-regulated when the cells are applied to the meat surface and therefore need more time to adjust compared with planktonic grown cells.

When Δ*fliC* cells were grown immobilized before contact with the meat, the ability of these to attach did not change with contact time and the attachment strength were three-times lower compared the planktonic grown Δ*fliC* cells (Table 2). Studies by Knudsen et *al*. (Knudsen et al., 2012) report that immobilized growth of *S*. Typhimurium resulted in down-regulation of flagella genes. Furthermore, they observed low levels of FliC when cells were entering the stationary phase, comparable with cells used in this study, which could explain the weaker attachment of Δ*fliC* cells to the meat surface. However, since FliC is not present, the flagella might not be functional and this might explain the unaltered attachment strength seen over time for these cells after immobilized growth.

The two strains, Δ*flhDC* and Δ*prg*, demonstrated a tendency of decreasing detachment probability for both pre-growth conditions when contact time was increased, having the highest probability of detachment for planktonic growth compared with immobilized growth. This finding for Δ*flhDC* correlates well with previous studies in which the *flhDC* operon was found to be activator of the flagellum biosynthesis pathway which includes up-regulation of the *prg*, *fliC* and *yhjH* genes (Frye et al., 2006). For Δ*prg*, a significant increase in attachment strength was seen when grown immobilized compared with planktonic at 60 min ($P < 0.05$). This is supported by the observation that the detachment probability for immobilized growth of Δ*prg* and Δ*flhDC* cells was lower compared with planktonic growth. Increased contact time to 60 min, resulted in only a slightly stronger attachment for planktonic Δ*prg* cells.

In general, the attachment strength of cells to a surface of pork meat was found to increase with increasing contact time, except for Δ*fliC* cells. This tendency was independent of whether cells were initial grown as planktonic or immobilized cells (Table 2). These observations are comparable to studies performed using other bacteria and matrices. For example the detachment probability of *Listeria monocytogenes* from potato was found to decrease during the first two min and then remained constant up to 60 min (Garrood et al., 2004). In another experiment the probability of detachment of *Campylobacter jejuni* from

stainless steel was found to decrease when the contact time was increased (Nguyen et al., 2010).

No significant differences in the detachment probabilities for Δ*prg*, Δ*fliC*, Δ*yhjH* and Δ*flhDC* cells were seen compared with the wildtype strain after immobilized growth ($P > 0.05$). This might indicate that other genes affecting the attachment, not investigated in this study, such as *ycgR* and *csgD* (Jonas et al., 2007; Ryjenkov et al., 2006), have changed regulation during the immobilization in compensation for loss of the *prg, fliC, yhjH* or *flhDC* genes, respectively.

A recent study (Knudsen et al., 2012) has shown that immobilized growth of *S.* Typhimurium differs from growth in biofilm or as swarming cells, which are the two growth conditions that normally are investigated and compared. However, the immobilized growth, as mediated in gel cassettes, has been found to mimic bacterial growth in a structured food matrix best (Brocklehurst et al., 1997). In this study, an increase in contact time of the cells to a meat surface resulted in stronger attachment and this tendency seems to be more pronounced for planktonic cultures compared with immobilized pre-growth. This suggests that a liquid contamination might constitute to a higher occurrence of bacterial attachment to a meat surface. However, if including other conditions, such as temperature or chemical composition, this may not be valid. Conditions that would reduce the expression of the *prg* and *flhDC* genes in immobilized cultures could contribute to a stronger attachment compared with the corresponding planktonic cells. Immobilized growth seemed to have the highest impact on reducing the attachment strength of the Δ*fliC* strain.

An ANOVA analysis revealed that pre-growth conditions of the strains had a significant effect ($P < 0.05$) on the detachment probability of the investigated strains, whereas type of strain did not have a significant effect on the detachment. However, interaction between type of strain and pre-growth condition had an effect on detachment probability ($P < 0.05$). All knock-out strains had a negative relationship with the wildtype strain, when grown planktonic, revealing that the these mutant strains detach easily from the meat surface after planktonic growth compared with the wildtype strain, and attach stronger than the wildtype after immobilized growth.

3.2 Pre-growth conditions change the expression of attachment related genes

Relative expression of the *flhDC*, *fljB*, *motAB*, *prgH*, *yhjH* and *fliC* genes in either immobilized or planktonic grown cultures of the five *Salmonella* strains (wildtype, Δ*prg,* Δ*flhDC*, Δ*yhjH* and Δ*fliC*) was measured after 0, 5, 10, 30, 60, 120 and 180 min of incubation on the pork meat surface using qRT-PCR. The housekeeping gene *gmk* was used for normalization (Botteldoorn et al., 2006) and the relative expression was measured as N-fold difference of gene expression in the knock-out strain relative to gene expression in the wildtype strain (Figs 1 and 2).

In general, the relative gene expression was shown to be down-regulated in all knock-out strains compared with the wildtype. Particularly in Δ*flhDC* cells expression of all investigated genes (*flhDC*, *fliC*, *prgH*, *motAB*, *yhjH* and *fljB*) was down-regulated after planktonic growth compared with the wildtype strain (Fig 1C). This might explain the lower attachment ability observed for the Δ*flhDC* strain after planktonic growth as the synthesis of flagellar might not be occurring optimally in these cells. For Δ*flhDC* cells grown immobilized an up-regulation over time was observed for the *motAB* operon (Fig 1D). The up-regulation of *motAB* indicates enhancement of the motility of the cells (Cogan et al., 2004) which are consistent with the weaker attachment seen for Δ*flhDC* cells compared with the wildtype strain (Table 2). When Δ*prg* and Δ*fliC* mutant strains were grown immobilized before being applied to the pork meat surface, an up-regulation of *yhjH* and *motAB* was observed (Fig 1B and Fig 2D). Up-regulation of *yhjH* and *motAB* enhances the motility of the cells (Ko & Park, 2000; Simm et al., 2004) which would give less attachment of the cells to the meat surface. The up-regulation of these genes for the Δ*fliC* strain is consistent with the weak attachment ability compared with the wildtype strain after immobilized growth (Table 2). For Δ*prg* cells after planktonic growth, an up-regulation in expression of *yhjH* was seen until 30 min, and then decreases compared with the wildtype strain (Fig 1A). The effect of the up-regulation of *yhjH* in the Δ*prg* cells is seen in the low attachment strength of the Δ*prg* strain after planktonic growth compared with the wildtype strain (Table 2).

Δ*fliC* and Δ*yhjH* mutant strains displayed the lowest effect on gene expression after immobilized growth (Fig 2B and Fig 2D). The reduced gene expression of *fljB* (Fig 2D) also indicates that cells have not entered a phase variation to express FliC as a compensation for the knock-out of the *fliC* gene. For planktonic grown Δ*fliC* cells, measurements of attachment strength and detachment probability indicate that the ability of these cells to attach de-

clines over time, probably due to lack of the flagella. The gene expression pattern shows that an up-regulation of *fljB* gene expression is seen for Δ*fliC* cells compared with the wildtype after 60 min (Fig 2C), indicating that a phase variation is occurring in contrast to that seen for the immobilized growth. For Δ*fliC*, no apparent difference in expression was observed for the remaining genes investigated compared with the wildtype strain after planktonic growth (Fig 2C). A slight up-regulation of *fliC*, *motAB, fljB* and *flhDC* was observed in Δ*yhjH* cells after planktonic growth (Fig 2A).

4 Conclusion

In conclusion, the findings of the changed ability of the knock-out strains to attach to a surface of pork meat using different pre-growth conditions and contact times, can be used for further investigation of other factors that reduce the expression of attachment related genes. The analysis of the attachment abilities of the strains investigated clearly indicates that growth conditions have the highest impact. Furthermore, the contact time influences that attachment of planktonic bacteria which potentially can increase the probability of a large number of *Salmonella* cells being strongly attached to a food product. This may hamper subsequently decontamination steps and increase the risk of cross contamination.

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Table 1 List of primers used in the present study[1]

Target gene	Primer name	DNA sequence (5'→3')
Primers for knock-out mutants		
yhjH	yhjhredf	GAGTCCGGACAGTCACACTCCCATTAACAGGACAACTGAG**GTGTAGGCTGGAGCTGCTTC**[2]
	yhjhredr	CGCGCCTCGTAATACCACGTATTACGGGAACAGTCTGGCG**CATATGAATATCCTCCTTAG**
flhDC	flhcredf	TGCTCAATGAAGTGGACGATACGGCGCGTAAGAAAAGGGC**GTGTAGGCTGGAGCTGCTTC**
	flhcredr	TGACTTACCGCTGCTGGAGTGTTTGTCCACACCGTTTCGG**CATATGAATATCCTCCTTAG**
	flhdredf	GTGCGGCTACGTCGCACAAAAATAAAGTTGGTTATTCTGG**GTGTAGGCTGGAGCTGCTTC**
	flhdredr	ATATCGCGAGCTTCCTGAACAATGCTTTTTTCACTCATTA**CATATGAATATCCTCCTTAG**
fliC	flicredf	AGCCCAATAACATCAAGTTGTAATTGATAAGGAAAAGATC**GTGTAGGCTGGAGCTGCTTC**
	flicredr	CCTTGATTGTGTACCACGTGTCGGTGAATCAATCGCCGGA**CATATGAATATCCTCCTTAG**
prg	prghredf	CTGCTGCTATCGAGAACGACAGACATCGCTAACAGTATAT**GTGTAGGCTGGAGCTGCTTC**
	prghredr	ACCAAGGTGTTGCCATAATGACTTCCTTATTTACGTTAAAC**ATATGAATATCCTCCTTAG**
Primers for qRT-PCR		
fliC	fliC sense	GCAATCGTATCGGCATATCC
	fliC antisense	GCAGATCAACTCTCAGACCCTG
flhD	flhD sense	CACTTCATTGAGCAGACGCG
	flhD antisense	CCCGTTTGACTCAGGATTCG
yhjH	yhjH sense	CAGGATGGGTGACGACAGTGAG
	yhjH antisense	CGCTATTGGCTGCAATGCGAG
motAB	motAB sense	GGCCATCAGGCGATAGAGCAAC
	motAB antisense	GCACGATGAAAGCTATCCCG

prgH	prgH sense	GTCGCATCCGTATCCACCTG
	prgH antisense	GCGCTCACTGCTTCAGGTCAAC
fljB	fljB sense	GTTCAGTGAGTCCAGACCCAG
	fljB antisense	GCAGGACAACACCCTGACCATC
gmk	gmk forward	TTGGCAGGGAGGCGTTT
	gmk reverse	GCGCGAAGTGCCGTAGTAAT

[1]All primers were designed in the present study with the exception of the *gmk* primers which are from Botteldoorn et *al*. (Botteldoorn et al., 2006)

[2]The bold indicates the original primers from Datsenko and Wanner (Datsenko & Wanner, 2000)

Table 2 Attachment strength and detachment probabilities of *Salmonella* strains, grown under different conditions, on pork meat. Significantly different values in columns are indicated by upper case letters, whereas significantly different values in rows are indicated by lower case letters.

Pre-growth condition	**Strain**	**Attachment Strength (S_R)[1]**		**Detachment probability (x)[2]**	
		0 min	60 min	2 min	60 min
Planktonic	4/74	0.570^{A}	0.810^{CD}	0.067	0.067
		(± 0.061)[3]	(± 0.043)	(± 0.020)	(± 0.019)
	4/74::Δ*prg*	0.201^{AB}	0.279^{DG}	0.098	0.082
		(± 0.103)	(± 0.039)	(± 0.038)	(± 0.009)
	4/74::Δ*yhjH*	0.385	0.546	0.063	0.064
		(± 0.023)	(± 0.008)	(± 0.000)	(± 0.020)
	4/74::Δ*fliC*	0.654^{B}	0.594	0.048	0.122
		(± 0.164)	(± 0.116)	(± 0.028)	(± 0.064)
	4/74::Δ*flhDC*	0.399	0.483^{C}	0.164	0.104
		(± 0.129)	(± 0.000)	(± 0.084)	(± 0.012)
Immobilized	4/74	0.419	0.667^{E}	0.079	0.070
		(± 0.054)	(± 0.079)	(± 0.012)	(± 0.003)
	4/74::Δ*prg*	0.361^{a}	0.730^{aG}	0.084	0.061
		(± 0.095)	(± 0.074)	(± 0.030)	(± 0.006)
	4/74::Δ*yhjH*	0.286	0.504	0.070	0.066
		(± 0.025)	(± 0.084)	(± 0.010)	(± 0.001)
	4/74::Δ*fliC*	0.201^{b}	0.202^{bEF}	0.073	0.089
		(± 0.077)	(± 0.134)	(± 0.025)	(± 0.005)
	4/74::Δ*flhDC*	0.439	0.519^{F}	0.058	0.046
		(± 0.039)	(± 0.009)	(± 0.000)	(± 0.009)

[1] Attachment strength defined as S_R = (strongly attached cells)/(loosely + strongly attached cells), estimated according to Dickson and Koohmaraie (Dickson & Koohmaraie, 1989)

[2] Probability of detachment of a bacterium from a meat surface during a single blotting event, estimated according to Garrood et *al*. (Garrood et al., 2004)

[3] Mean values from two independent replicate experiments ± standard deviation

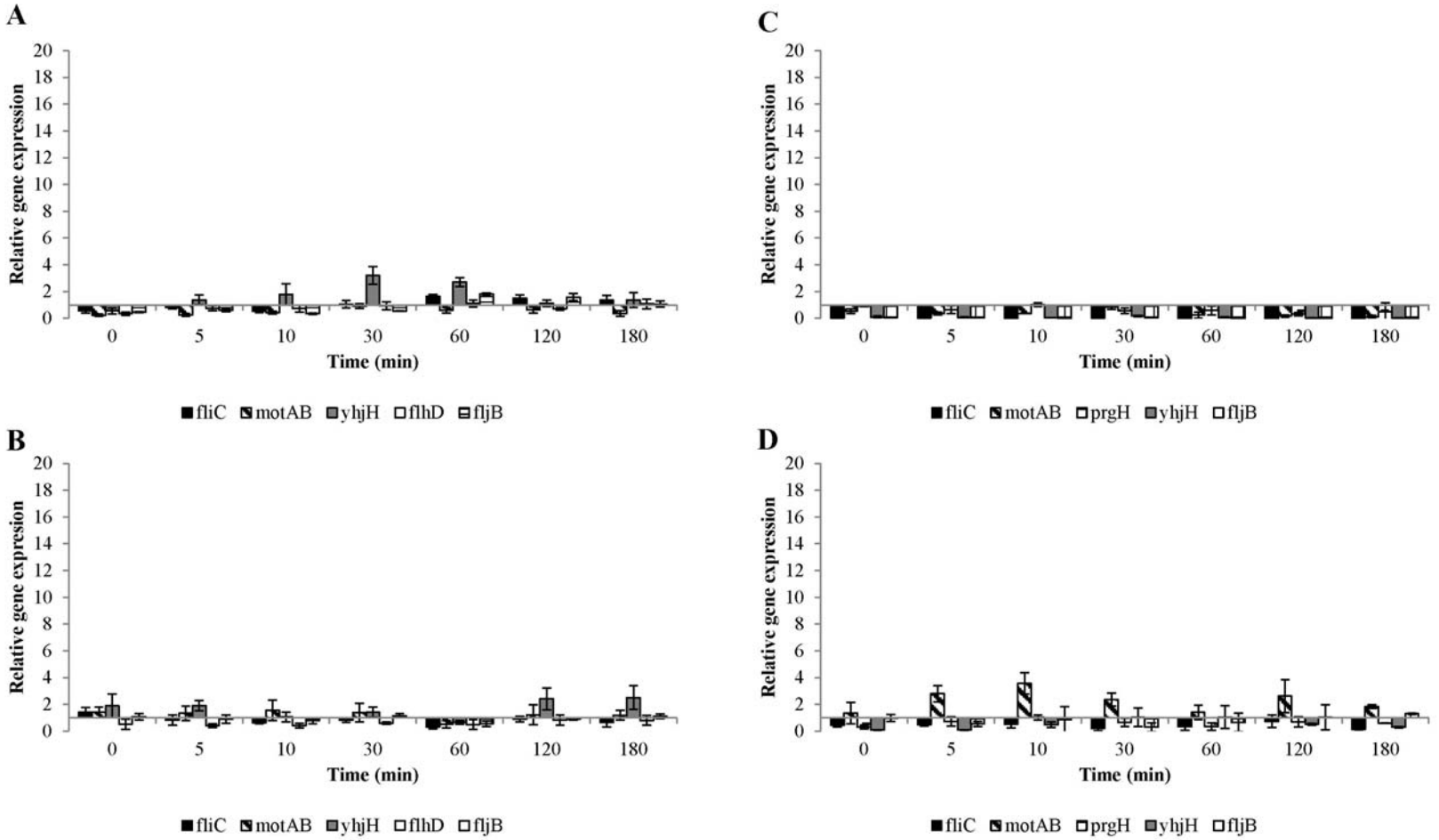

Figure 1 Relative expression of genes relevant for attachment of *S*. Typhimurium strains. Relative expression of genes in (A) Δ*prg* cells after planktonic growth, (B) Δ*prg* cells after immobilized growth, (C) Δ*flhDC* cells after planktonic growth and (D) Δ*flhDC* cells after immobilized growth. *gmk* was used as endogenous control for data normalization. Values below 1 denote a decrease in expression for the knock-out strain relative to the wildtype strain, while values above 1 denote an increase in expression. Bars indicate standard deviation of two independent replicated experiments.

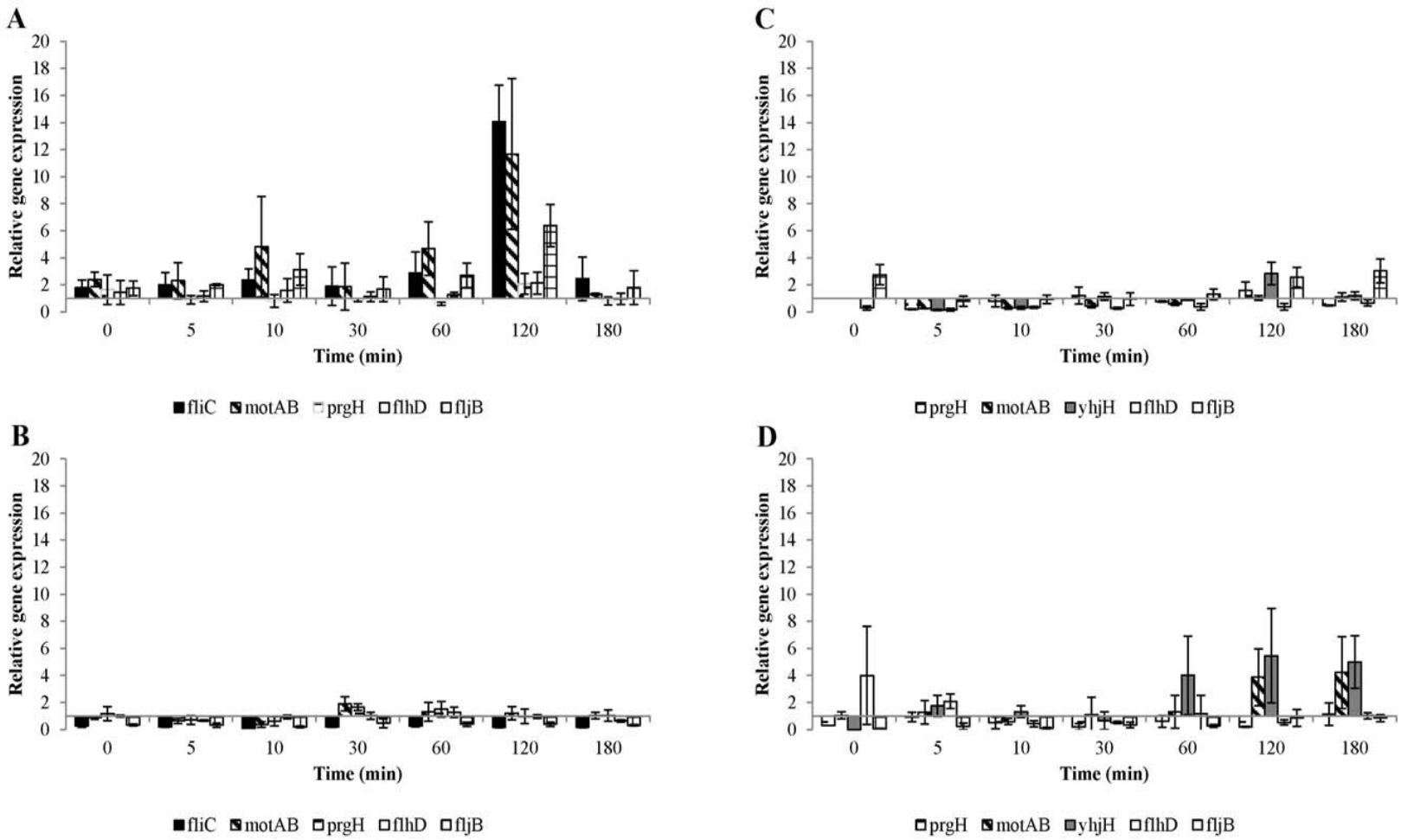

Figure 2 Relative expression of genes relevant for attachment of *S*. Typhimurium strains. Relative expression of genes in (A) Δ*yhjH* cells after planktonic growth, (B) Δ*yhjH* cells after immobilized growth, (C) Δ*fliC* cells after planktonic growth and (D) Δ*fliC* cells after immobilized growth. *gmk* was used as the endogenous control for data normalization. Values below 1 denote a decrease in expression for the knock-out strain relative to the wildtype strain, while values above 1 denote an increase in expression. Bars indicate standard deviation of two independent replicated experiments.

Paper II

Gene content and attachment ability of *Salmonella* isolated from a pork slaughter-line

Paper in preparation

(Page numbers are relative to paper)

Gene content and attachment ability of *Salmonella* isolated from a pork slaughter-line

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Running title: Characterization of *Salmonella* strains isolated from slaughterhouse

Keywords: *Salmonella*, slaughterhouse, detachment, pre-growth conditions, gene content

Abstract

Salmonella is one of the leading causes of zoonotic food-borne disease worldwide. Pork is recognized as an important source of foodborne *Salmonella*. *Salmonella* can enter the slaughterhouse with the pigs or cross contaminate through persistent flora. Therefore is a better understanding of the behavior of *Salmonella* in pork production environments needed in order to minimize risk of cross contamination and the subsequently risk of human salmonellosis incidents due to consumption of contaminated food. The objective of this study was to investigate the difference in genomic content and ability of attachment to a pork meat surface of 40 *Salmonella* strains isolated from a pork slaughter-line. The investigated serovars included monophasic *Salmonella enterica* ser. Typhimurium (*S.* 4,5,12:i:-), *S.* Typhimurium, *S.* Derby , *S.* Bredeney, *S.* Brandenburg, *S.* Infantis and *S.* Rissen. The analysis of genomic content was conducted by DNA microarray. A collection of the isolates was further investigated with regards to their ability to detach from a pork meat surface after different pre-growth conditions. DNA microarray clustered the majority of the isolates according to their serovars. Difference in gene content between the isolates from different sampling points at the slaughter-line was not observed. For all the isolates investigated, cells from immobilized pre-growth had the highest detachment probability, e.g. were easiest to remove from the meat surface and thereby given higher risk of cross contamination. A *S.* Rissen isolated from slaughterhouse equipment had the highest detachment probability after immobilized growth of all the isolates giving this isolate the highest chance for causing problems in the slaughter-line. This isolate was shown by microarray analysis to lack the two fimbriae genes *safC* and *lpfD*, important for adhesion and biofilm formation, which might explain the low attachment ability to the pork meat surface. In conclusion, knowledge about the influence of pre-growth conditions and genes specific for the source of isolation in the food production line can help in the development of more optimized processing environments, which eventually can decrease cross contamination or persistence of *Salmonella*, and thereby increase food safety.

1 Introduction

Salmonella is one the most common bacterial pathogens and the causative agent of salmonellosis. In various European countries, many cases of salmonellosis have been connected with the consumption of pork and pork products (EFSA, 2013). One of the critical sources of *Salmonella* in pork is cross contamination during the slaughter process from carrier pigs (Alban & Stärk, 2005; Baptista et al., 2010; Berends et al., 1997), but also *Salmonella* persisting in the slaughterhouse (house flora) contributes to the spread (Baptista et al., 2010; Hald et al., 2003; Visscher et al., 2011). Therefore is a better understanding of the behavior of *Salmonella* in pork production environments needed in order to minimize the risk of cross contamination and subsequently human salmonellosis incidents due to consumption of contaminated food.

Adaptation and persistence of *Salmonella* in food production chains have been suggested to be a result of bacterial attachment and surface colonization (Swanenburg et al., 2001; Vestby et al., 2009). Studies have shown that surface adhesion of *Salmonella* in the production chain is strain dependent and may be influenced by differences in the surface structure (Chia et al., 2009). The colonization of *Salmonella* on food processing equipment poses a major problem for the food industry as this can serve as a reservoir for cross contamination. Cross contamination occurs when cells detach from biofilms, e.g. when food passes over the contaminated surface. Previous studies have shown that bacteria grow differently when they are immobilized, compared with planktonic growth (Brocklehurst et al., 1995, 1997; Knudsen et al., 2012). It has further been reported that immobilized grown bacteria, such as *Salmonella*, are more resistant to treatments with disinfectants, organic acids and heat compared with their planktonic counterparts which impacts the decontamination in food processing equipment (Gawande & Bhagwat, 2002; Morild et al., 2011; Nielsen et al., 2013; Scher et al., 2005; Wong et al., 2010). A previous study has shown that the pre-growth conditions, planktonic or immobilized, of *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) influence the ability of *Salmonella* to attach on a pork meat surface (Hansen et al., 2013).

Salmonella contain various fimbrial genes that encode surface hair-like structures involved in the primary surface attachment (Fernández & Berenguer, 2000). Different serotypes harbor different sub-sets of these genes (Porwollik et al., 2004). The presence or even absence of certain fimbrial genes might be assigned to different environments or selection pressures. The use of microarray technology makes is possible to screen many *Salmonella* isolates to investigate their gene repertoire and biological properties (Huehn & Malorny,

2009; Malorny et al., 2007; Scaria et al., 2008). A microarray has been developed that can be used for molecular characterization and typing of *Salmonella* isolates and it contains different genetic markers including antibiotic resistance, pathogenicity, fimbriae, phage-associated genes, flagella and lipopolysaccharides (Huehn et al., 2009).

The objectives of this study was to characterize 40 *Salmonella* isolates from a pork slaughter-line (van Hoek et al., 2012), see Table 1, using the DNA microarray described by Huehn et *al*. (2009) in order to identify specific genes related to the source of isolation. In addition, a selection of the isolates was investigated with regards to their ability to detach from a pork meat surface after different pre-growth conditions to see if this impacts their ability to cause cross contamination. The *Salmonella* isolates included strains of the serotypes monophasic *S.* Typhimurium (*S*. 4,5,12:i:-), *S.* Typhimurium, *S*. Derby, *S.* Bredeney, *S*. Brandenburg, *S*. Infantis and *S*. Rissen.

2 Material and methods

2.1 Bacterial strains

Forty *Salmonella* isolates originating from a Dutch slaughterhouse (van Hoek et al., 2012) were used in this study, representing seven different serotypes, i.e. monophasic *S.* Typhimurium, *S.* Typhimurium, *S.* Derby, *S.* Bredeney, *S.* Brandenburg, *S.* Infantis and *S.* Rissen. The different isolates were used for DNA microarray analysis (all 40 isolates) and for detachment studies (ten isolates), see Table 1.

2.2 Detachment studies

2.2.1 Cassettes

Bacterial cells (see Table 1) were grown in 8 ml of Luria-Bertani (LB) broth (Sigma, Brøndby, Denmark) at 25°C for 24 h. A 100× dilution was made and the suspension was re-incubated at 25°C for 24 h. Inocula for the cassettes were prepared by a 1000× dilution of the cell cultures in Maximum Recovery Diluent (MRD; 1 g peptone and 8.5 g sodium chloride per litre, pH 7.0 ± 0.2), to obtain around 10^6 CFU/ml. Two different media were used for the gel cassettes; LB media (for planktonic growth) and LB with 29.3% pluronic F-127 (Sigma) (for immobilized growth). Inocula were added to give 10^3 CFU/ml or g media. The Gel Cassette System for immobilized growth of bacteria was obtained from IFR Enterprises, Norwich, UK. The gel cassettes were prepared as described previously (Brocklehurst et al., 1995, 1997). An appropriate volume (30 ml) of LB media or LB media with pluronic, both containing inocula, was transferred to the cassettes by sterile pipetting. Cassettes were then incubated at 25 ± 0.1 °C for either 16 h (LB) or 18.25 h (LB with pluronic) in order to reach early stationary phase.

2.2.2 Sterile meat samples

Pork fillet, obtained from local retailer, were chosen as model surface. The surfaces of the packages were sprayed with 70% ethanol prior to opening. The individual fillets were scalded with boiling water before use. Slices of meat were cut from the fillet to a thickness of approximately 1 cm under sterile conditions. Samples of meat were punched out from these meat slices with a meat stamp with a diameter of 30 mm and placed on a sterile Petri dish.

2.2.3 Inocula for meat samples

After incubation of the cassettes (see "Cassettes"), a sample of the gelled medium (~10g) or 10 ml of the LB medium was removed from the cassettes, mixed with 90 ml cooled MRD in sterile Stomacher bags (Seward, VWR, Herlev, Denmark) and blended in a Stomacher 400 (Seward) for 1 min at high speed. Fifty ml of the suspension were centrifuged at 5,400×g for 7 min at 10°C and the resulting pellet was dissolved in 6 ml cooled MRD (4°C) to a concentration of approximately 10^8 CFU/ml.

2.2.4 Detachment

A 250×dilution of the inocula (see "Inocula for meat samples") were made in MRD, 100 µl of the diluted inocula were spread on each of two meat samples and the samples were incubated for 2 min at 22 ± 1°C. After incubation, meat samples were transferred to 100 ml MRD and shaken for 1 min at 250 rpm. The meat pieces were then transferred to a Xylose Lysine Deoxycholate plate (XLD, Oxoid, Greve, Denmark) with the inoculated surface facing down. The meat piece was left on the plate for 1 min and was then transferred to a new plate. This procedure was repeated for a total of 16 plates. After each move, the liquid remaining on the plate was spread. The plates were incubated at 37 °C for 24 h and colonies enumerated. The detachment rate was calculated as described by Garrood et *al*. (Garrood et al., 2004). In short, the $\log_{10}$CFU/plate was plotted against the plate number and the detachment rate calculated from the slope of the resulting linear relationship.

2.2.5 Statistical analysis

Detachment probabilities were calculated as mean ± standard deviation (SD) for two independent biological replicates. For the detachment probabilities, analysis of variance (ANOVA) was used to estimate the effects of pre-growth condition, strain, origin and their interaction. Prior the statistically analysis, the normal distribution of the data was confirmed by a qq-plot. The analysis was carried out in two steps. Initially a model including the main effects of the variables origin, strain, source and pre-growth state was estimated. Secondly, significant variables were kept in the model, and the effects of two-terms interactions between the variables were added to the model. The model was reduced backwards, eliminating non-significant interaction terms. A p-value of < 0.05 was considered statistically significant. The R software (v2.15) (R Core Team, 2012) was used for the ANOVA analysis.

2.3 Microarray analysis

2.3.1 DNA extraction

Strains were incubated on Tryptic Soy Blood Agar (TSA, Oxoid) plates at 37 °C overnight, followed by a single colony inoculation in 25 ml Buffered Peptone Water (BPW, Oxoid) and incubation at 37 °C overnight. Subsequently, 1 ml of inoculated BPW was spun down for 7 min at 7,000×g, and the supernatant was discarded. DNA was extracted using DNeasy Blood & Tissue Kit with Proteinase K and RNase A (Qiagen, Copenhagen, Denmark) according to the manufacturer's instructions. DNA concentration and purity was measured using NanoDrop (Fisher Scientific, Slangerup, Denmark) with software version 3.7.1.

2.3.2 Fluorescence labeling and purification of DNA

Purified DNA was labeled with Agilent Genomic DNA enzymatic Labeling Kit (Agilent Technologies, Santa Clara, USA) according to the manual supplied.

2.3.3 Microarray slide design

The DNA microarray used in this study was previously described (Huehn et al., 2009) and contains a set of 281 gene-specific oligonucleotides. The probes were added to an 8x16K DNA microarray and were ordered from Agilent Technologies (Agilent Technologies).

2.3.4 Hybridization, washing and microarray scanning

Hybridization and washing was performed according to the protocol for the Agilent Genomic DNA enzymatic Labeling Kit. Hybridized spots were visualized in a GenePix 4200 two-color laser scanner (Axon, Foster City, USA), according to the manual supplied. The GPR files containing signal intensities for the DNA/probe hybridizations were collected.

2.3.5 Data analysis

The analysis of the microarray data were conducted in R (v2.15) (R Core Team, 2012), using different available packages as described below.

2.3.5.1 Normalization of data

The GPR files were imported into R using the "read.maimages" function in the "limma" package (Smyth, 2005). Ninety seven quality assurance probes produced by Agilent, termed "DarkCorner", "NA", "NC1", "NC2" and "SM_", were removed and quantile normalization was subsequently performed between microarrays using the "normalizeBetweenArrays" function in the "limma" package (Smyth, 2005) in order to reduce the systematic variation. Median intensities across the replicate probes for each gene were calculated, and normalized

against the *ttrC* gene (Malorny et al., 2004). Following the intensity normalization, intensity ratios below and above an intensity threshold of 0.25 (Huehn et al., 2009) were converted to binary numbers, with 0 indicating an absent gene, and 1 indicating a present gene.

2.3.5.2 Hierarchical clustering

For hierarchical clustering was the function “hclust” in the “stats” package of R used and the algorithm adopted was that of Ward (Ward, 1963). Visualization of hierarchical clustering was done by the “plclust” function in the “stats” package of R. Heat maps was constructed using the function “heatmap” in the “stats” package of R.

3 Results and discussion

3.1 DNA microarray studies

All 40 *Salmonella* isolates were investigated by DNA microarray analysis. The isolates were analyzed for the presence and absence of 281 genes covering marker groups of genes related to antimicrobial resistance (49 probes), fimbriae (22 probes), metabolism (20 probes), mobility (57 probes), pathogenicity (87 probes), phages (ten probes) and serotyping (33 probes) (Huehn et al., 2009). In most microarray analyses, it is common to select subsets of the data prior to analysis in order to reduce noise or to be able to perform particular downstream analysis. Different subset selections were included in this study: (i) attachment related genes and (ii) source related genes.

3.1.1 Gene content of the isolates

The binarized data were used for hierarchical clustering analysis in order to see the grouping of the isolates by serotype. The cluster dendrogram (Figure 1) is based on similarities between the isolates in their gene content i.e. which genes that are present and absent. The dendrogram splits into two branches with primarily *S.* Typhimurium and monophasic *S.* Typhimurium in one group and *S.* Derby, *S.* Rissen, *S.* Infantis, *S.* Bredeney and *S.* Brandenburg in the other. A few *S.* Derby are grouping together with the *S.* Typhimurium group and some *S.* Typhimurium is grouping with the *S.* Derby. The overall grouping is in accordance with the one by Litrup et *al*., where they used the same microarray design (Litrup et al., 2010). In conclusion, grouping is based on the serotype and not the source of the isolates.

Attachment of *Salmonella* is related to pathogenicity, fimbria and mobility (Foley & Lynne, 2008; Wang et al., 2006). The probes related to fimbria, mobility and pathogenicity were extracted and a heat map was constructed, see Figure 2. Most of the *S.* Typhimurium isolates are located to the right, whereas the *S.* Derby together with *S.* Brandenburg, *S.* Bredeney, *S.* Infantis and *S.* Rissen are located to the left. There seems not to be any grouping according to the source of isolation, however in accordance with the hierarchical clustering in Figure 1. The lack of grouping of the data by the source of isolation could be caused by the fact that the original purpose and therefore also the design of the microarray, was for serotyping and the virulence potential of *Salmonella* strains (Huehn et al., 2009; Malorny et al., 2007). Other genes, not included on the microarray, could be of more interest. The use of whole-genome microarray instead would capture more variation of the gene content and enable tracking of the sources. Another reason for the poor separation of the sources could be the

unequal distribution of source and serotypes leading to wrong conclusions based on the source specific genes.

3.1.2 Source related genes

As the use of attachment related genes did not give a clear split of the isolates into the different source, extraction of source specific and absent genes was conducted from the microarray data. The genes conserved in all serotypes of a source were extracted giving four genes from exsanguination, one gene from carcass splitter, one gene from meat inspection and none from belly opener (Table 2). The genes cover pathogenicity, metabolism, mobility, and phages. The exsanguination source has the majority of the present genes which is caused by the fact that this source is overrepresented in the data set. One of the genes unique for the exsanguination source is the serotyping gene *wzyB* which is associated with serogroup B (Malorny et al., 2007). Of the *Salmonella* isolates investigated, only *S.* Infantis and *S.* Rissen does not belong to this serogroup (Grimont & Weill, 2007), and since these have not been isolated from the exsanguination source, the presence of the *wzyB* is a consequence of the missing serotypes for this source. No unique present genes were observed for the belly opener, which might be due to the low number of isolates from this source. Additionally, the genes absent in all serotypes of a source were extracted (Table 2). A lot of resistance related genes are observed among the absent genes, which probably results from the fact that the strains do not exhibit the resistance. The serotyping related genes were removed before further analysis.

Pathogenicity and mobility genes dominate the source specific and absent genes, which are in accordance with the literature since these are involved in the attachment ability of *Salmonella* (Barak et al., 2005). The collection of absent and present genes was used to investigate any similarities between the different sources (Figure 3). There is no consensus in the grouping by the source of isolation; however the grouping seems to be directed by the serotype of the isolates. The clustering of the isolates seems to resemble the hierarchical clustering using the entire data set, indicating that it might be serotype specific genes that influence where the isolates are located. The carcass splitter is only associated with *S.* Derby and *S.* Rissen, whereas *S.* Typhimurium is the only one isolated from the belly opener (van Hoek et al., 2012). For better investigation of source specific genes, more isolates for each isolation source should be included.

3.2 Detachment studies

The effect of different pre-growth conditions (planktonic or immobilized) on cells detaching from the surface of pork fillet was determined for ten *Salmonella* isolates by a blotting series, see Table 3. Detachment probability was represented by a straight line plot of which the slope (detachment probability, x) and standard deviation was determined.

For all serotypes, the general tendency is that the planktonic growth yields lower detachment probabilities compared with the immobilized growth, independent of the source. The *S*. Derby originating from the meat inspection has a slightly lower detachment probability for immobilized growth compared to the planktonic grown cells, but it is not significantly different ($P > 0.05$). This indicates that the immobilized grown cells have the easiest to be removed from the meat surface and thereby given higher risk of cross contamination in the slaughterhouse environment, consistent with the previous study by Hansen et *al*. (Hansen et al., 2013). Another study by Knudsen et *al*. revealed that immobilized *S*. Typhimurium did not produce flagellar and the cells were less invasive (Knudsen et al., 2012) which could explain the lower attachment to the meat surface. However, to be able to draw adequate conclusions, this has to be investigated further.

All the isolates showed similar detachment probabilities for immobilized and for planktonic growth, independent of the serotype. An ANOVA analysis revealed that pre-growth conditions of the isolates had a significant effect on the detachment probability ($P < 0.05$), whereas the serotype of the isolates does not have a significant effect. The source of the isolates did not affect the detachment probability which is consistent with the study by Solomon et *al*. were they found that biofilm formation is independent of the source of isolation (Solomon et al., 2005). Studies by Chia et *al.* and Oliveira et *al.* found that *Salmonella* adhesion on different surfaces used in kitchens and poultry processing plants was strain dependent (Chia et al., 2009; Oliveira et al., 2006). The lack of significant difference between the serotypes in this study on the detachment from a meat surface could be a result of difference in the surface properties of the surfaces used in the two studies, i.e. meat versus plastic and rubber. Studies have shown that the surface influences the attachment ability (Dickson & Koohmaraie, 1989; Jonas et al., 2007; Tresse et al., 2006). Due to the no-equal distribution of serotypes and sources (i.e. some serotypes and sources have only one or few representatives) it is not possible to provide reliable conclusions of the impact of these.

S. Derby, *S.* Rissen and *S.* Typhimurium are the only three serotypes isolated from the carcass, both exterior and interior, that are found on the robots after slaughtering, i.e. carcass splitter and belly opener (van Hoek et al., 2012) indicating that these have higher risk of causing cross contamination. The *S.* Rissen strain isolated from the carcass splitter displays the highest difference in the detachment probability between the two pre-growth conditions and it further has the highest value compared with the other serotypes for the immobilized pre-growth. This indicates that *S.* Rissen after immobilized growth has the easiest to detach from the meat surface, leading to higher risk of cross contamination in the slaughter-line. In the study by van Hoek et *al.* (van Hoek et al., 2012), a recurring contamination of *S.* Rissen was found on one of the carcass splitters and furthermore was *S.* Rissen the second most frequently isolated serotype from the carcasses in the end of the slaughter-line.

From the microarray data of the ten *Salmonella* isolates used in the detachment study, the genes present or absent in all isolates was omitted, and a heat map was constructed on the rest of the genes (Figure 4). The *S.* Rissen lacks the two fimbriae genes *safC* and *lpfD* which are involved in the adhesion to the intestinal epithelium (Weening et al., 2005). The LpfD protein has been shown to be important for long-term intestinal persistence of *S.* Typhimurium in genetically resistant mice (Weening et al., 2005) and it is further crucial for biofilm formation on intestinal tissue of chickens (Ledeboer et al., 2006). The lack of the two genes could result in lower adhesion potential, explaining the low attachment observed after immobilized growth. However, the attachment potential of other *S.* Rissen should be investigated.

The detachment probability of the *S.* Derby isolated from belly opener shows the highest values for both planktonic and immobilized growth (Table 3) compared with the other *S.* Derby isolates investigated. As only one *S.* Derby isolates originates from the belly opener could indicate that this isolates has some properties different from the other *S.* Derby. The three *S.* Derby isolates originating from the belly opener, exsanguination and carcass splitter has the same patterns of present and absent genes associated with fimbriae and pathogenicity genes. The *S.* Derby originating from meat inspection differs from the other *S.* Derby isolates used in the detachment study. First of all, it has a different pattern of detachment probabilities as it has a slightly higher detachment probability for planktonic growth compared with the immobilized growth (Table 3), which is the opposite of what is seen for the other isolates investigated. Comparing with the other *S.* Derby isolates, the isolate from the meat inspection has the pathogenicity associated gene *cdtB,* which is absent in the other *S.* Derby. The *cdtB*

gene plays a role in invasion and survival of *Salmonella* in host environments (Haghjoo & Galán, 2004; Skyberg et al., 2006), which could explain the higher attachment ability of this isolate.

The environmental conditions during the slaughtering process might have an influence on the expression of certain genes, thereby influencing the attachment ability of the isolates. The isolates might have been exposed to conditions such as altered pH or temperature under the slaughter process which not have been the case during the experimental setup in this study. Previous studies have shown that *S.* Typhimurium exposed to high temperatures was accompanied by increased tolerance to heat and acid inactivation conditions (Pin et al., 2012). This might have influenced some of the isolates making them more resistant and thereby surviving longer in the slaughter-line.

The current design of the microarray seems not to be adequate for the use for tracking the source of *Salmonella* isolates in the slaughter-line and other genes should be investigated. Using a whole genome microarray or whole genome sequencing could identify more gene differences between the isolates with respect to the source of isolation. In addition, for gaining more insight into the attachment ability of the isolates, conducting experiments to see whether deletion of the genes found to differ between the isolates affects the attachment ability of the isolates.

3.3 Conclusion

Based on the microarray data, 26 genes were identified to be either present or absent in all serotypes of a source. The genes were related to pathogenicity and mobility, which are important for the attachment potential of *Salmonella*. For all serotypes, cells after immobilized growth had the highest detachment probability e.g. were easiest to remove from the meat surface and thereby give a higher risk of cross contamination. The *S.* Rissen isolate had the highest detachment probability after immobilized growth of all the isolates giving this isolate the highest risk for causing problems in the slaughter-line. The *S.* Rissen lacks the *lpfD* gene that is important for adhesion and biofilm formation, which might explain the low attachment ability to the pork meat surface.

In conclusion, knowledge about the influence of pre-growth conditions and genes specific for the source of isolation in the food production line can help with the development of more optimized processing environments, which eventually can decrease cross contamination or persistence of *Salmonella*, and thereby increase food safety.

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Table 1 List of *Salmonella* isolates used in the study and their origin[A]

Source of isolation	Serovar	Microarray study	Detachment study
Exsanguination	Monophasic *S.* Typhimurium	x	x
Exsanguination	Monophasic *S.* Typhimurium	x	
Meat inspection	Monophasic *S.* Typhimurium	x	x
Meat inspection	*S.* Brandenburg	x	
Exsanguination	*S.* Bredeney	x	
Meat inspection	*S.* Derby	x	x
Meat inspection	*S.* Derby	x	
Exsanguination	*S.* Derby	x	
Carcass splitter	*S.* Derby	x	
Exsanguination	*S.* Derby	x	
Carcass splitter	*S.* Derby	x	
Exsanguination	*S.* Derby	x	
Exsanguination	*S.* Derby	x	
Exsanguination	*S.* Derby	x	x
Exsanguination	*S.* Derby	x	
Belly opener	*S.* Derby	x	x
Carcass splitter	*S.* Derby	x	
Carcass splitter	*S.* Derby	x	
Carcass splitter	*S.* Derby	x	x
Exsanguination	*S.* Infantis	x	
Carcass splitter	*S.* Rissen	x	
Carcass splitter	*S.* Rissen	x	x
Exsanguination	*S.* Typhimurium	x	
Exsanguination	*S.* Typhimurium	x	
Meat inspection	*S.* Typhimurium	x	
Exsanguination	*S.* Typhimurium	x	
Exsanguination	*S.* Typhimurium	x	
Meat inspection	*S.* Typhimurium	x	x
Belly opener	*S.* Typhimurium	x	x
Exsanguination	*S.* Typhimurium	x	

Exsanguination	*S.* Typhimurium	x	
Exsanguination	*S.* Typhimurium	x	
Exsanguination	*S.* Typhimurium	x	
Exsanguination	*S.* Typhimurium	x	
Exsanguination	*S.* Typhimurium	x	
Exsanguination	*S.* Typhimurium	x	
Belly opener	*S.* Typhimurium	x	
Exsanguination	*S.* Typhimurium	x	x
Meat inspection	*S.* Typhimurium	x	
Exsanguination	*S.* Typhimurium	x	

[A] All strains were isolated form a Dutch slaughterhouse in a sampling described by van Hoek et al (2012).

Table 2 Extracted genes present or absent in all serotypes of one source

Source of isolation	Gene	Marker group
	Present genes	
Carcass splitter	*glxK*	Metabolism
Exsanguination	*STM1896*	Metabolism
	SB10	Phages
	wzy_B	Serotyping
	tnpAIS20	Mobility
Meat inspection	*orgA*	Pathogenicity
	Absent genes	
Belly opener	*sopB*	Pathogenicity
	STM2701	Pathogenicity
	STY4625	Phages
	STY3676	Phages
	tet(C)	Resistance
	repC_R64	Mobility
	tcfA	Fimbrial
	fliC_b	Serotyping
	wbaA_C1	Serotyping
Carcass splitter	*tnpR_IS10*	Mobility
	tet(B)	Resistance
	sul2	Resistance
	gogB	Pathogenicity
	int1	Resistance
	strB	Resistance
	qacED1	Resistance
	repC_DT193	Mobility
	ISCR3	Mobility
	sul1	Resistance
	rck	Pathogenicity
	spvR	Pathogenicity
Meat inspection	*oafA*	Metabolism
	rep_W	Mobility

Table 3 Detachment probabilities of ten *Salmonella* isolates, grown under two different conditions, on pork meat.

		Detachment probability (x)[A]	
		Pre-growth state	
***Salmonella* strain**	**Source of isolation**	**Planktonic**	**Immobilized**
Monophasic Typhimurium	Exsanguination	0.072 ± 0.021[B]	0.094 ± 0.015
	Meat inspection	0.084 ± 0.028	0.102 ± 0.038
Typhimurium	Exsanguination	0.089 ± 0.003	0.113 ± 0.052
	Meat inspection	0.087 ± 0.007	0.097 ± 0.005
	Belly opener	0.058 ± 0.002	0.070 ± 0.016
Derby	Exsanguination	0.075 ± 0.012	0.081 ± 0.027
	Meat inspection	0.073 ± 0.024	0.067 ± 0.016
	Belly opener	0.079 ± 0.007	0.088 ± 0.012
	Carcass splitter	0.067 ± 0.02	0.080 ± 0.029
Rissen	Carcass splitter	0.078 ± 0.018	0.115 ± 0.016

[A] Probability of detachment of a bacterium from a meat surface during a single blotting event, estimated according to Garrood et *al*. (2004)

[B] Mean values from two independent replicate experiments ± standard deviation

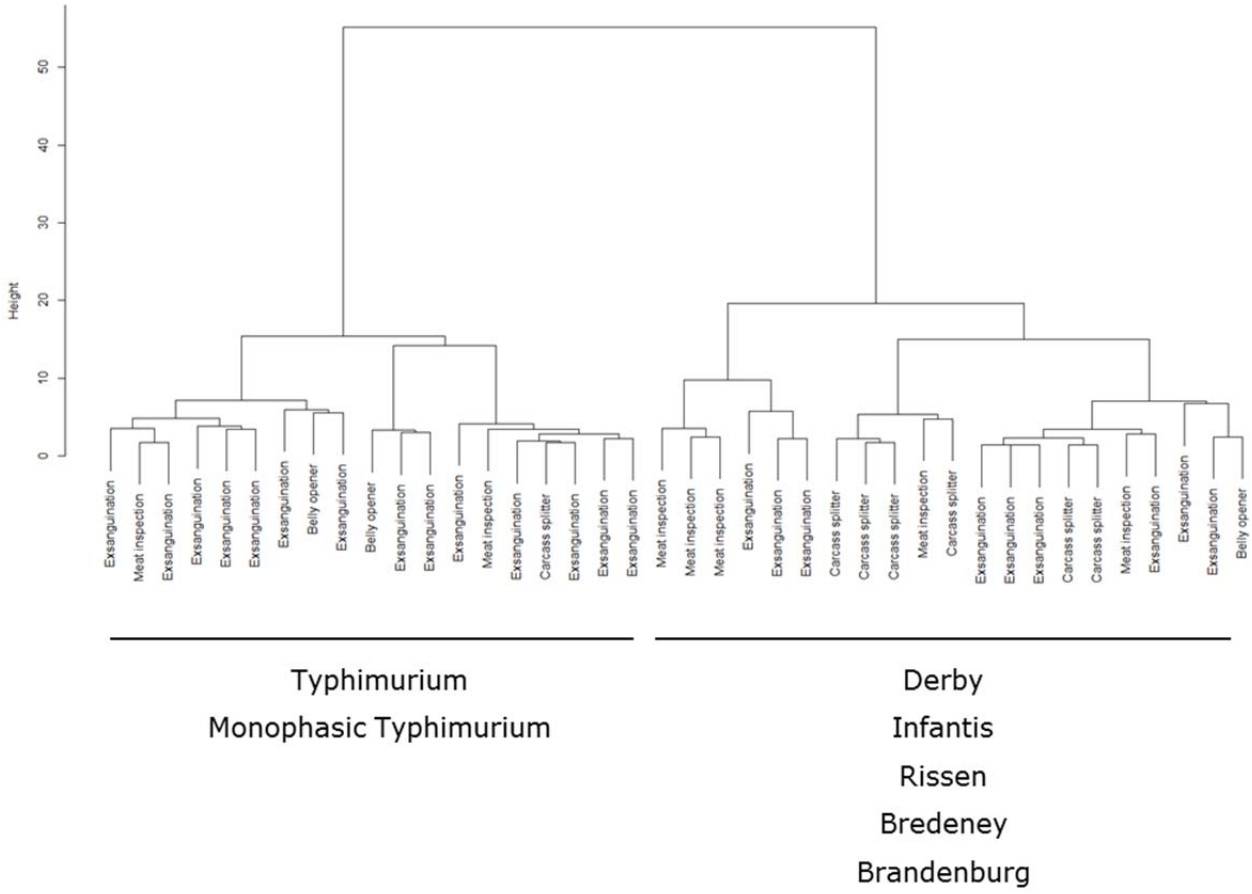

Figure 1 Hierarchical clustering dendrogram calculated on the basis of the microarray data.

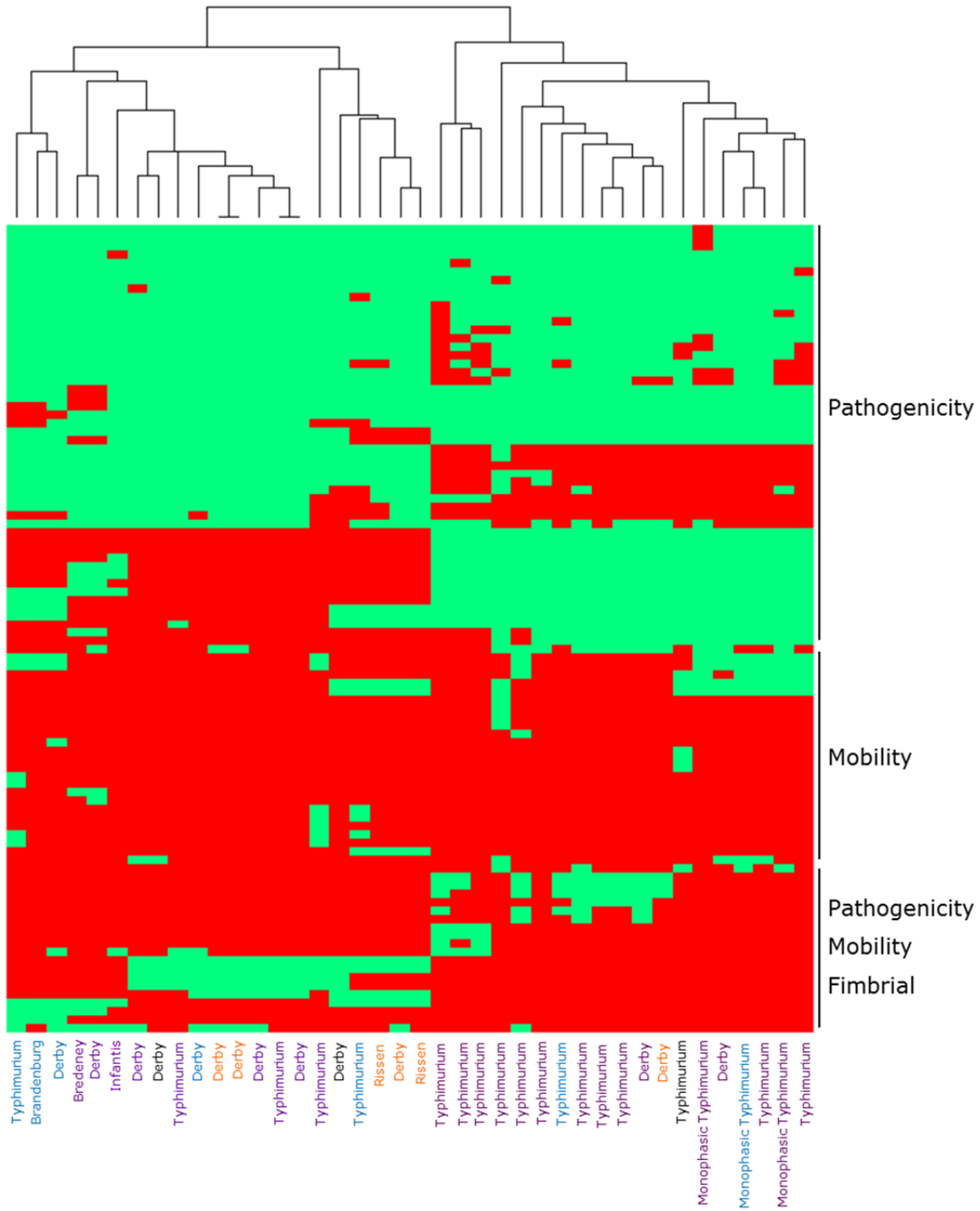

Figure 2 Heat map of attachment related genes. The figure includes genes related to mobility, pathogenicity and fimbriae, excluding the genes present and absent in all the *Salmonella* isolates. Green indicates a present gene, whereas red indicates an absent gene. The hierarchical clustering in the top indicates the genetic relationship between the isolates. On the right side is the marker groups indicated. The isolates in the bottom are coloured according to their isolation source with exsanguination, belly opener, meat inspection and carcass splitter.

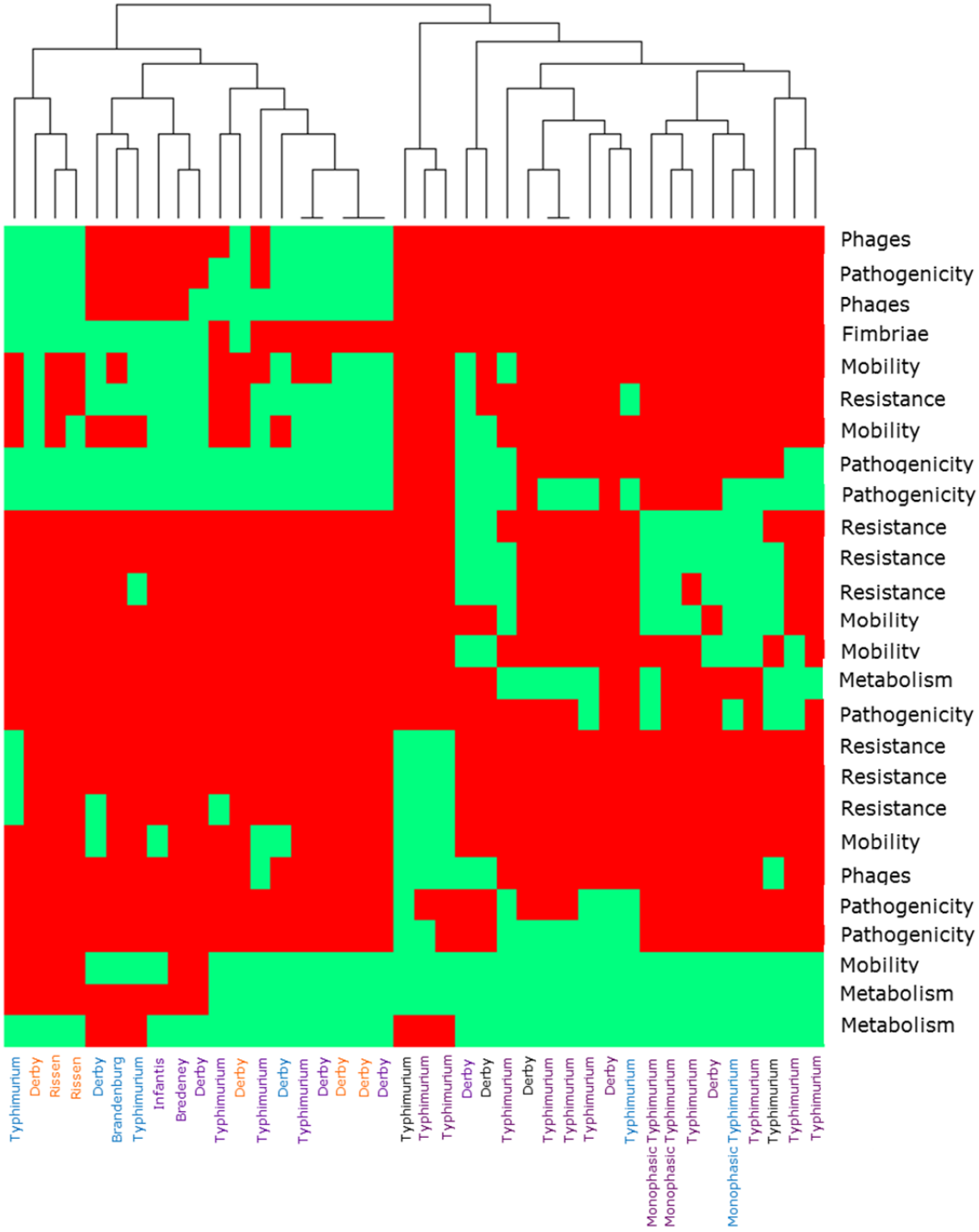

Figure 3 Heat map of source related genes. Heat map of the extracted genes present or absent in all *Salmonella* serotypes of a particular source. Green indicates a present gene, whereas red indicates an absent gene. The hierarchical clustering in the top indicates the genetic relationship between the isolates. On the right side is the marker groups indicated. The isolates in the bottom are coloured according to their isolation source with exsanguination, belly opener, meat inspection and carcass splitter.

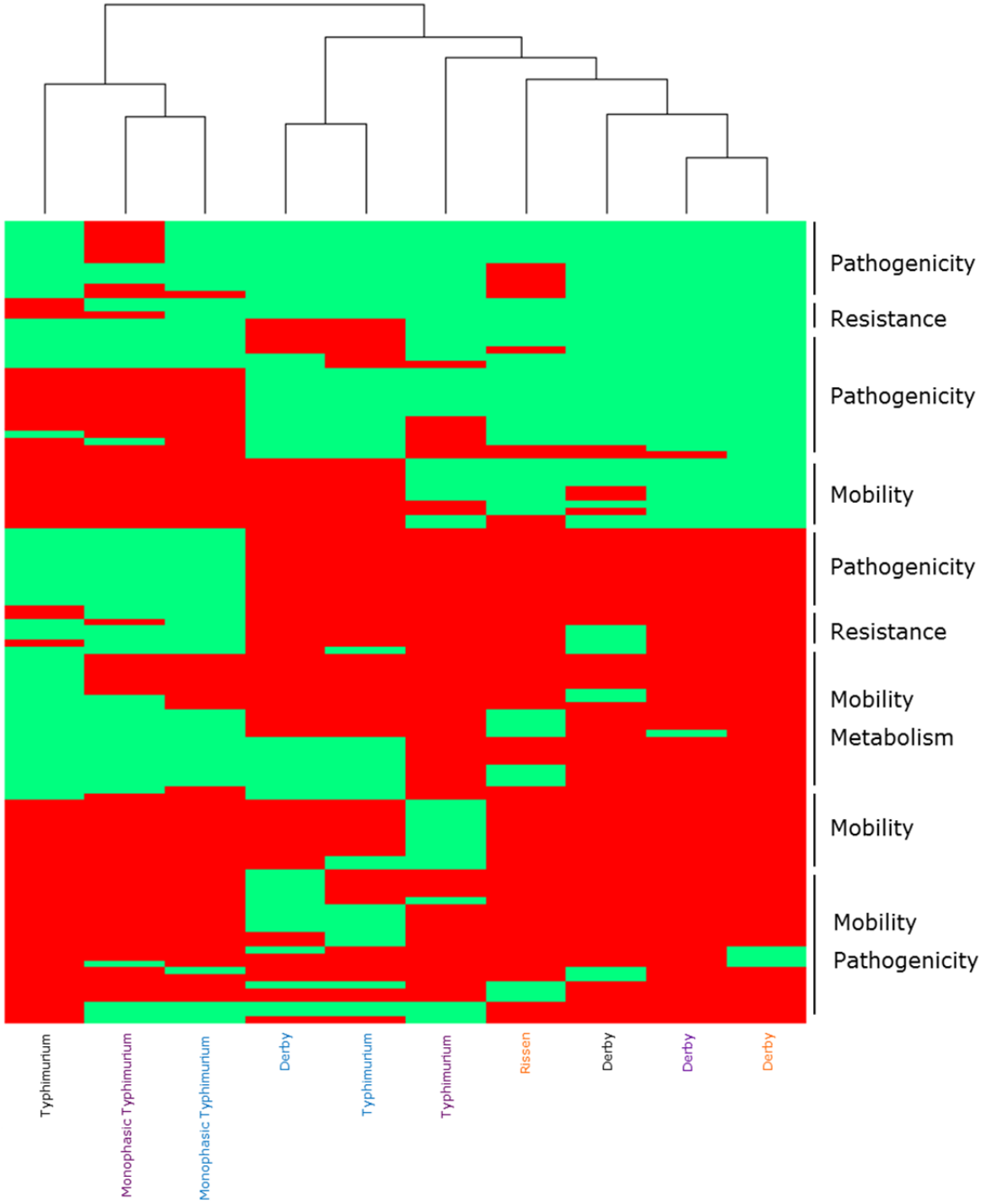

Figure 4 Heat map of gene content. The heat map of the gene content of the ten *Salmonella* isolates used in the detachment study excluded the genes present and absent in all the isolates. Green indicates a present gene, whereas red indicates an absent gene. The hierarchical clustering in the top indicates the genetic relationship between the isolates. On the right side is the marker groups indicated. The isolates in the bottom are coloured according to their isolation source with exsanguination, belly opener, meat inspection and carcass splitter.

Paper III

The Transcriptional Heat Shock Response of *Salmonella* Typhimurium Shows Hysteresis and Heated Cells Show Increased Resistance to Heat and Acid Stress

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The Transcriptional Heat Shock Response of *Salmonella* Typhimurium Shows Hysteresis and Heated Cells Show Increased Resistance to Heat and Acid Stress

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Abstract

We investigated if the transcriptional response of *Salmonella* Typhimurium to temperature and acid variations was hysteretic, i.e. whether the transcriptional regulation caused by environmental stimuli showed memory and remained after the stimuli ceased. The transcriptional activity of non-replicating stationary phase cells of *S.* Typhimurium caused by the exposure to 45°C and to pH 5 for 30 min was monitored by microarray hybridizations at the end of the treatment period as well as immediately and 30 minutes after conditions were set back to their initial values, 25°C and pH 7. One hundred and two out of 120 up-regulated genes during the heat shock remained up-regulated 30 minutes after the temperature was set back to 25°C, while only 86 out of 293 down regulated genes remained down regulated 30 minutes after the heat shock ceased. Thus, the majority of the induced genes exhibited hysteresis, i.e., they remained up-regulated after the environmental stress ceased. At 25°C the transcriptional regulation of genes encoding for heat shock proteins was determined by the previous environment. Gene networks constructed with up-regulated genes were significantly more modular than those of down-regulated genes, implying that down-regulation was significantly less synchronized than up-regulation. The hysteretic transcriptional response to heat shock was accompanied by higher resistance to inactivation at 50°C as well as cross-resistance to inactivation at pH 3; however, growth rates and lag times at 43°C and at pH 4.5 were not affected. The exposure to pH 5 only caused up-regulation of 12 genes and this response was neither hysteretic nor accompanied of increased resistance to inactivation conditions. Cellular memory at the transcriptional level may represent a mechanism of adaptation to the environment and a deterministic source of variability in gene regulation.

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Introduction

Natural environments are spatially and temporally complex. Bacteria interact with the environment responding to changes and changing the environment in return. Many studies on bacterial responses to environmental conditions focus on the quantitative analysis of growth, survival or inactivation of the population [1]. The investigation of the molecular response to the environmental conditions pursues a better understanding of the system bacteria-environment.

Hysteresis refers to a process by which a bistable system exhibits memory. Such systems switch between two distinct stable steady states, and switching from one state to the other happens when a stimulus exceeds a threshold. Once switched, the system remains at that steady state until the stimulus decreases to a level below the original switching level. In between these two switching stimulus levels the state of the system depends on the previous history [2]. Hysteresis has for example been described in the expression of components of the lactose utilization network of *Escherichia coli* [3]. In the absence of glucose, the *lac* operon is uninduced at low concentrations (<3 mM) of the inducer thio-methylgalactoside (TMG), and fully induced at high TMG concentrations (>30 mM). Between these switching thresholds, the response of the system is hysteretic: TMG levels must exceed 30 mM to turn on initially uninduced cells but must drop below 3 mM to turn off initially induced cells [3]. Thus, the pattern of lactose consumption adopted by bacteria is environmentally controlled and the key determinant is the direction of change of the environmental inducer [4].

The aim of the current study was to characterize the transcriptional responses of *Salmonella enterica* serotype Typhimurium (*S.* Typhimurium) to heat treatment and pH changes, specifically if these responses were hysteretic. We quantified the transcriptional response of stationary non-proliferating cells of *S.* Typhimurium to an increase of temperature from 25 to 45°C and a decrease of pH from 7 to 5. The conditions were maintained for 30 minutes before resetting the original values, and samples were taken at the end of the environmental stress, immediately after resetting the initial conditions and again 30 minutes later. Network science was applied to identify the metabolic pathways and

functional categories in which the differentially transcribed genes were involved and confirmed the hysteretic behaviour of the transcriptional activity. In addition, the resistance to inactivating conditions of 50°C and pH 3 and the adaptation response to extreme growth environments at 43°C and pH 4.5 were monitored during the exposure to the stressing condition and immediately and 30 minutes after resetting the initial conditions.

Results

Acidification Affected Transcription of Few Genes, While a Large Number of Genes were Affected by Heat Shock

Only 12 genes were detected as up-regulated in a culture maintained for 30 minutes at pH 5 when compared with a control culture maintained at pH 7. Immediately after re-establishing the original pH value of 7 in the acidified culture, 19 genes were up-regulated and 1 gene was down-regulated. Thirty minutes after re-establishing the original conditions, 23 genes were up-regulated in the culture previously exposed to acidic conditions (Fig. 1). In contrast with these results, the increase of temperature from 25 to 45°C affected transcription of a much larger number of genes. The transcription of 293 genes was repressed while 120 genes were induced in the culture maintained at 45°C for 30 minutes when compared with control cultures at 25°C. Immediately after the heat shock ceased, the number of up-regulated genes increased considerably to 470 while the number of down-regulated genes decreased to 113. Thirty minutes after the heat condition was removed, there were still a large number of induced genes, 214, and a smaller number of down-regulated genes, 127, in the culture previously exposed to high temperature (Fig. 1). Thus, during the heat shock, most of affected genes showed repressed transcription while when the temperature was reset to the initial value, transcription was mostly induced with respect to control cultures.

The few up-regulated genes during the acid shock were associated with general metabolic and cellular functions (Fig. S1). In the acid stress experiment, the transcription of three genes associated to adaptation processes to atypical conditions *ibpB*, *pspB* and *pspC* was induced but only 30 minutes after the acid shock ceased. The induction of the phage shock protein operon, *pspABCE*, has been observed in response to a variety of stressful conditions [5], while the small heat-shock protein, IbpB, has been reported to stabilize stress-denatured proteins in *E. coli* [6].

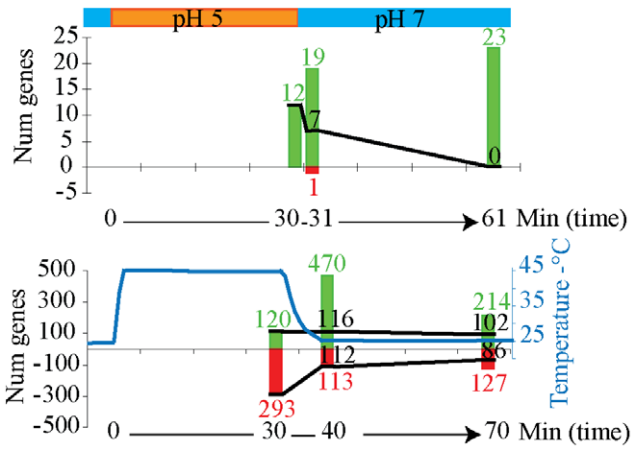

Figure 1. Number of genes up- (green) and down- (red) regulated in *S.* Typhimurium under acid and heat stressing conditions and immediately and 30 min after removing the stressing conditions. Few genes were affected by acid stress (A) while transcription of a large number of genes was altered under heat stress (B). Majority of up-regulated genes under heat stress remained up-regulated 30 minutes after stress condition ceased. Black numbers and solid lines show up- and down- regulated genes maintained throughout the experiment.
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The heat shock in particular caused the induction of genes encoding for chaperones, including heat shock proteins, and plasmid genes and the repression of genes involved in pathogenesis, energy production and motility. Some general pathways and functions were detected as both induced and repressed during all sampling times throughout the heat shock experiment (Fig. S2). However the majority of specific cellular functions or pathways included in these general categories had a consistent response being either up- or down-regulated throughout the experiment (Fig. S3).

Most of Induced Genes in Response to Heat Stress Exhibited Hysteresis and Remained Induced after Resetting the Initial Conditions

From the 120 genes up-regulated in cultures exposed to 45°C for 30 minutes, 102 genes were still up-regulated 30 minutes after resetting the temperature to 25°C with respect to control cultures (Fig. 1). Conversely, none of the 12 genes up-regulated during the acidic shock was detected 30 minutes after the acidic condition was removed (Fig. 1).

Thus, while the response to acid stress was very modest, heat shock caused a major alteration on gene transcription and this transcriptional response exhibited hysteresis. The majority of genes induced when cells were exposed at 45°C remained induced 30 minutes after the temperature was reset at 25°C. Among those genes, there were ten genes encoding for heat shock proteins as well as numerous genes encoding for other products involved in protein stabilization and DNA repair (Fig. 2). A significant number of genes encoded in the three plasmids of *S.* Typhimurium strain 4/74 also showed a hysteretic response (Fig. 2). Down-regulated genes throughout the experiment were associated with non-stress specific responses such as motility, pathogenesis and energy production (Fig. 2, Fig. S3).

Down-regulation of Genes was Significantly Less Organized According to Cellular Functions or Metabolic Pathways than Transcriptional Induction during and after the Heat Shock

We constructed a genome scale bi-partite network for the genome and plasmids of *S.* Typhimurium SL1344 as previously described for *E. coli* K 12 [7]. The network was bipartite and thus edges connected two sets of nodes. Genes constituted one of these sets of nodes while the other set of nodes included metabolic pathways and cellular functions. Information was collected from public available resources and databases specified in the Material and Methods section. The bipartite sub-networks corresponding to genes up- and down-regulated during and after the heat shock were extracted from the genome scale network in order to study if network properties were affected by the environmental stresses.

The genome scale network was structured in modules or communities of nodes more connected to the nodes belonging to the same module than to other external nodes. To quantify this organization in communities the modularity value, Q [8], was calculated. The value of Q varies between 0 to a maximum value of 1. In practice it is found that a value above about 0.3 is a good indicator of significant community structure in a network [8]. The value of Q was 0.68 for our genome scale network. The modular or compartmentalized pattern of the

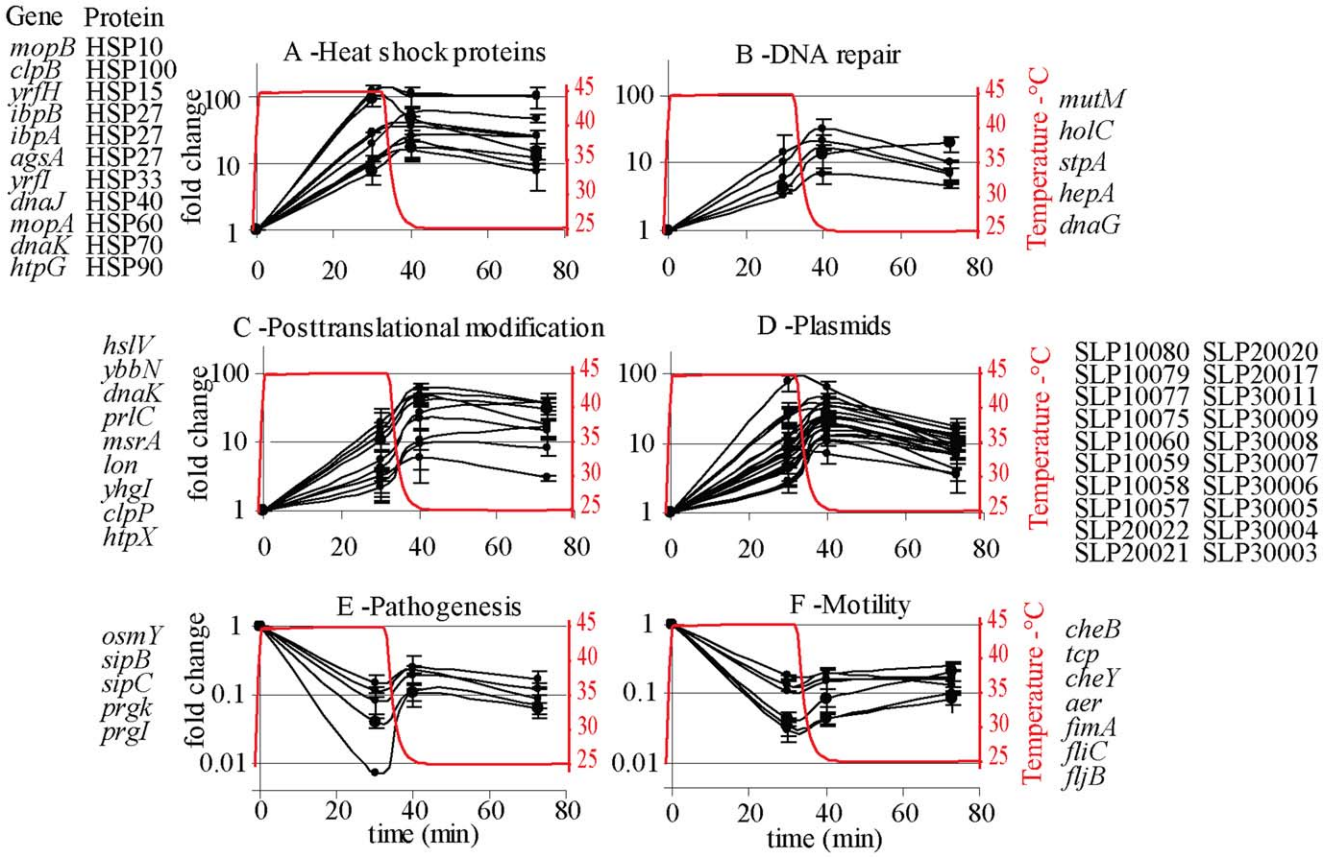


Figure 2. Fold change of transcript levels of genes during heat stress and immediately and 30 minutes after temperature was set back at 25°C that exhibited hysteresis and encoded for heat shock proteins (A), for DNA repair (B) for products involved in protein stabilization (C) or were encoded in plasmids (D). Down-regulated genes throughout the experiment were associated with non stress specific response such as pathogenesis (E) and motility (F). Genes represented in each plot showed a similar tendency; they are listed by the side of each plot but not differentially represented.
doi:10.1371/journal.pone.0051196.g002

genome network can be understood as a strategy to increase network stability, since this property retains the impact of perturbations within a single module and minimizes their effect on other metabolic pathways or cellular functions [9]. Modularity was estimated on the sub-networks from the genome scale network corresponding to genes differentially expressed during and after the heat treatment (Table 1). To assess the significance of the differences in the modularity of the sub-networks of the genes up- and down-regulated during and after the heat shock, we compared these results with the modularity measured in 10 random networks, with the same number of genes as the networks of up- and down-regulated genes but randomly selected from the genome scale network. Data in Table 1 shows that the value of the modularity coefficient for the networks of up-regulated genes during and after the heat treatment were between 0.75 and 0.8. These modularity values were slightly higher although not significantly different from those measured in the correspondent randomly generated networks. Fig. 3 shows the modular organization of the networks of genes differentially transcribed during the heat shock. Plasmid genes formed 3 disconnected modules that increased the modularity coefficient of the networks of induced genes (Fig. 3). Removing the modules formed by plasmid genes, the modularity coefficient of the networks of induced genes decreased slightly, taking values between 0.73 and 0.77 which are in the range of those of the random networks. Plasmid genes were not connected to other functions because the knowledge on their function is limited. Plasmid genes are known to be involved in intra-macrophage survival of *Salmonella*, antibiotic resistance and increased resistance to phage infection but their annotation is not public yet [10].

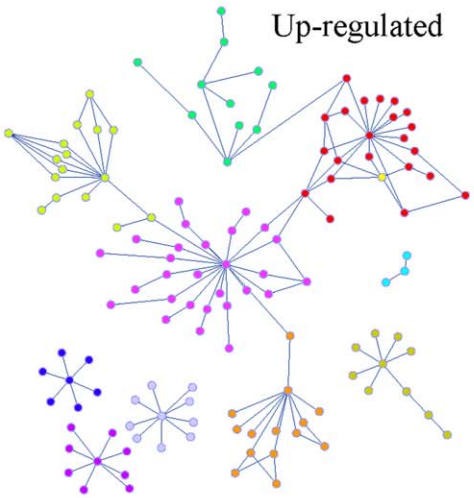
Therefore the modular structure observed in the networks of up-regulated genes during and after the heat shock was the same as observed in the random networks derived from the genome scale network reflecting its organization in metabolic pathways and cellular functions. However, modularity analysis of the networks of down-regulated genes during and after the heat shock revealed that the level of organization of repressed genes in functional modules was smaller than expected. Networks of repressed genes showed modularity coefficients in between 0.62 and 0.64 while randomly extracted networks had significantly greater Q values of 0.74–0.81 (Table 1).

Hysteretic Transcriptional Response to Heat Shock is Accompanied by Increased Resistance to Inactivation Conditions of 50°C and pH 3

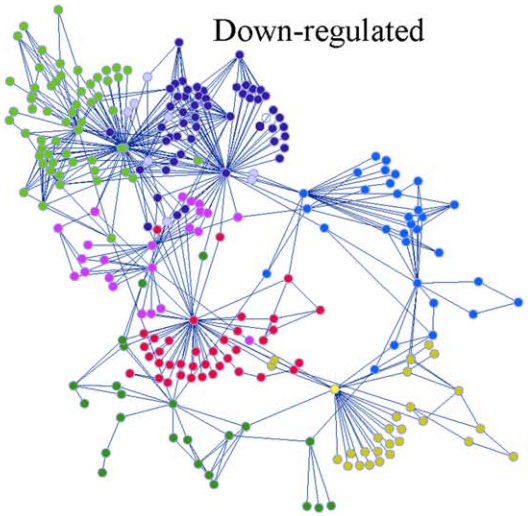
The stressing conditions, 45°C or pH 5 for 30 minutes, were selected by choosing extreme environmental conditions that did not cause inactivation while challenging *S.* Typhimurium. To study if these stressing treatments had an effect on the ability of *Salmonella* to grow in extreme conditions, we followed the

During heat treatment

Up-regulated

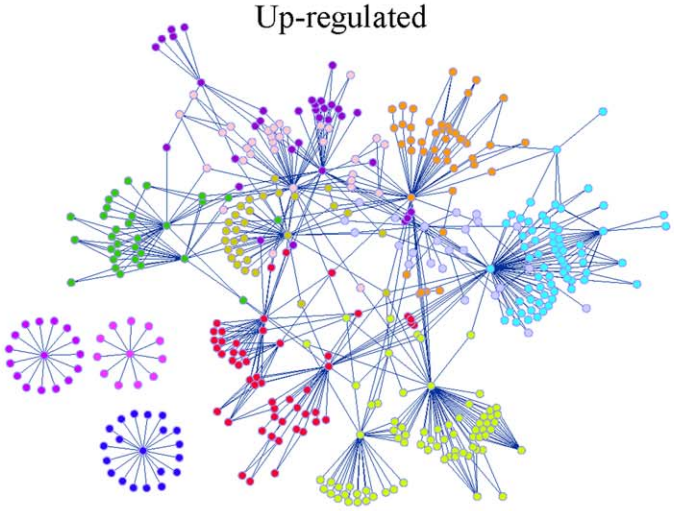


Down-regulated

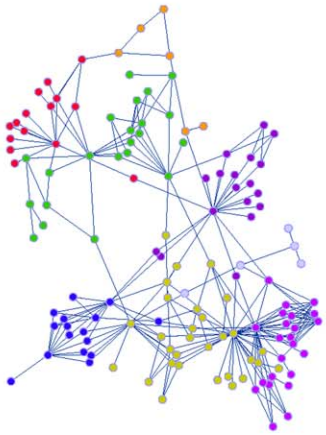


Immediatly after heat treatment ceased

Up-regulated

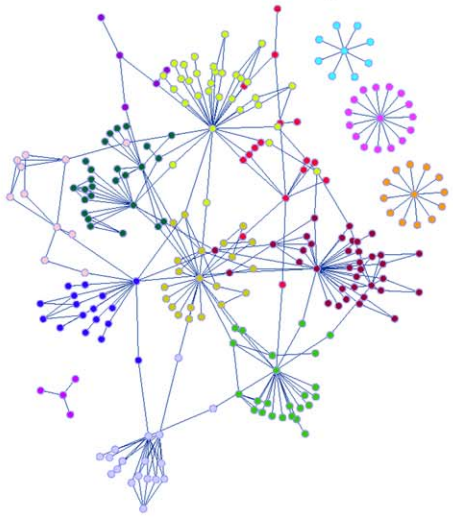


Down-regulated



Thirty min after heat treatment ceased

Up-regulated



Down-regulated

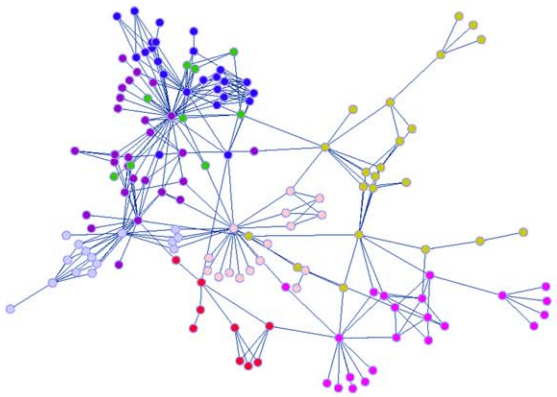


Figure 3. Network representation of genes up- and down-regulated during and after the heat shock. Modularity of networks of down-regulated genes was significantly lower than that of up-regulated networks. The induction of genes belonging to a metabolic pathway or cellular function was more synchronized than their down-regulation because of the hysteretic transcriptional response or persistence of the induction of the majority of genes induced by the heat shock once the temperature is set back to 25°C. Different colours represent different modules in each network.
doi:10.1371/journal.pone.0051196.g003

kinetics of the stressed populations at 43°C and at pH 4.5. On the other hand, to investigate if the exposure to stress conditions affected the resistance of the population to inactivation conditions, the kinetics of the stressed populations were measured at 50°C and at pH 3.

Cultures exposed at 45°C for 30 minutes exhibited an increased resistance to inactivation temperatures of 50°C as well as cross-resistance to inactivation by acidic conditions at pH 3 showing significantly greater D values than non-heated control cultures (Fig. 4). The D-values measured in cells exposed at 45°C for 30 minutes were not significantly different from those measured immediately and 30 minutes after the heat shock was removed (Fig. 4). Thus, the increased resistance to heat and acid inactivation conditions in cells exposed at 45°C for 30 minutes persisted for 30 minutes after the temperature was reset to 25°C. However, cultures exposed at pH 5 for 30 minutes did not show increased resistance to either heat or acid inactivation conditions when compared with control cultures kept at pH 7 (Fig. 4).

The growth parameters, duration of the lag phase and maximum specific growth rate, measured at either 43°C or at pH 4.5 were not affected by the previous exposure of the cells to 45°C or to pH 5 (Table 2).

Discussion

The transcripts of the heat or acid shocked *S.* Typhimurium cells were compared with those of an untreated control kept at 25°C and pH 7 during the experimental course. We conducted growth curves to check that the populations used in the experiments were in early stationary phase after 16 hours at 25°C (data not shown), and thus mostly comprising non-proliferating cells although eventually some bacterial cells could divide or die. We targeted the use of non proliferating populations in order to avoid the interference of division cycle genes in the transcriptional response and the generation of new bacterial cells, which may not preserve the cellular memory of their ancestors. The bacterial concentration measured at early stationary phase was not affected during or after the application of the stressing conditions (Fig. S4).

We found a hysteretic transcriptional response to heat shock accompanied by an increased resistance to heat and acid inactivation conditions. The results supports previous observations that the resistance of *S.* Typhimurium to heating at 55°C was enhanced by exposing cells to a previous heat shock at 48°C and this thermotolerance was accompanied by increased synthesis of heat shock proteins. When cells were shifted from 48 to 37°C, thermotolerance was lost with a variable rate of decay within the first hour after the temperature shift while the synthesis of proteins persisted for longer time [11]. Thus, hysteretic behaviour is detectable at both transcription and translation and may be one of the reasons for the expression of apparently unneeded proteins that reduce growth rate of cells and is known as protein cost or burden [12]. We observed that the transcriptional response of *S.* Typhimurium to the acidification of the medium was not hysteretic and did not last after the acidic condition was removed. The decrease of pH from 7 to 5 did not seem to be a challenging stress condition for *S.* Typhimurium because it caused a very modest and unspecific transcriptional response and was not associated with either an increase of the resistance to inactivation conditions or with adaptation to extreme growth conditions. It seems that there was a lack of specific transcriptional response in *S.* Typhimurium exposed to pH 5. The few differentially transcribed genes detected at pH 5 may reflect the high variation intrinsic to gene transcription in bacteria. More than 60 genes have been reported to exhibit between 2–5 folds difference in the expression ratios when the strains being compared were grown in identical conditions but in different batches [13]. We think that one of the reasons for the different responses of *S* Typhimurium to the different environmental stresses may be that while a pH value of 5 does not challenge *Salmonella* spp., 45°C is a more stringent condition close to the limiting growth temperature. *Salmonella* spp. is able to grow at pH values below 4, while growth is not detected above 48°C. Moreover, culturing *Salmonella* to stationary phase in media containing

Table 1. Modularity quantification in sub-networks extracted from the *Salmonella* genome scale network containing those genes differentially expressed during and after the heat treatment and comparison with random extracted sub-networks with the same number of genes.

	Num genes & Num other nodes		Modularity coefficient (Q)[1]			
	Up-regulated	Down-regulated	Up-regulated	Random networks	Down-regulated	Random networks
End of heat treatment (45°C-30 min)	93 & 38	211 & 53	0.81	0.79±0.042[2]	0.64*	0.74±0.034
Immediately after heat treatment ceases	373 & 64	94 & 41	0.76	0.71±0.037	0.63*	0.82±0.040
Thirty minutes after heat treatment ceases	187 & 61	103 & 43	0.80	0.78±0.041	0.62*	0.81±0.044

[1]Modularity coefficient from 0 to 1 (maximum modularity).
[2]Mean value and standard deviation of the modularity coefficient of 10 random networks.
*Significant: Smaller than the mean value minus 3 times the standard deviation of random networks.
doi:10.1371/journal.pone.0051196.t001

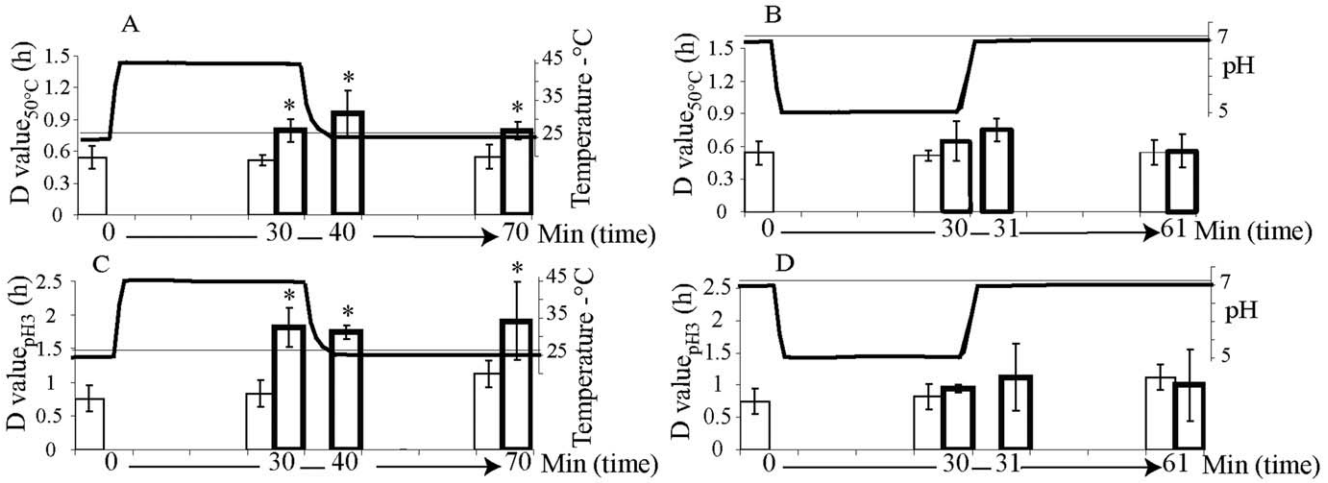


Figure 4. Increased resistance to heat and acid inactivation conditions in cultures of *S.* Typhimurium previously exposed to heat stressing conditions holds 30 min after resetting the initial conditions. Columns represent *D* values, time required for a decimal reduction of the population at the inactivating conditions, while lines represent the previous temperature or pH profile for each culture. Thick strokes are used for results at stressing profiles, 45°C or pH 5, and thin strokes for control experiments at 25°C and pH 7. D values at 50°C (A) and pH 3 (C) were significantly greater in cultures exposed to heat shock, 45°C for 30 min (thick strokes), and immediately and 30 min after the temperature was set back at 25°C than D values of control cultures at 25°C (thin strokes). However D values at 50°C (B) and pH 3 (D) of cultures previously exposed at pH 5 (thick strokes) were not significantly different from D values of control cultures at pH 7 (thin strokes). Stars indicate those cultures exposed to stressing conditions with D values significantly different from correspondent controls.
doi:10.1371/journal.pone.0051196.g004

glucose has been reported to induce acid tolerance response [14]. Cultures in stationary phase might have an enhanced transcription of genes involved in acid tolerance response, which could contribute to explain the lack of differences between the transcriptional response in control and in acid stressed populations.

A wide range of stabilities has been observed for individual mRNAs of *E. coli*, although approximately 50% of all mRNAs had half-lives shorter than 3 minutes and all of them shorter than 15 minutes [15]. Similar results have been observed for *Staphylococcus aureus* in stationary phase, although during exponential growth the percentage of mRNAs with a half-life shorter than 2.5 minutes increased to ca. 85% [16]. An increase of the turnover time of mRNAs has been reported under heat shock with the extended half-life of some transcripts being longer than 30 minutes [17,18]. One of these studies reported the effects of heat shock not on few transcripts but on the global response on RNA half-lives. During heat shock approximately 60% of log-phase transcripts of *S. aureus* had a longer half-life than 5 minutes, but only 7.1% of them were stable for 30 minutes under heat stress [17]. The increase of the half-life of mRNAs in response to heat shock is not likely to be the explanation for

Table 2. Duration of the lag phase and maximum specific growth rate at 43°C and pH 4.5 of *S.* Typhimurium previously exposed to heat and acid shock.

	43°C		pH 4.5	
Cell history	**lag (h)**	**μ (h^{-1})**	**lag (h)**	**μ (h^{-1})**
End of heat shock (45°C-30 min)	2.24±0.864*	0.911±0.136	20.5±0.636	0.444±0.0745
Immediately after heat shock and resetting temperature at 25°C	2.35±0.856	1.01±0.142	21.6±0.675	0.627±0.0804
Thirty minutes after heat shock and resetting temperature at 25°C	2.37±0.817	1.09±0.0378	22.1±0.169	0.687±0.0242
Control (25°C-pH 7-0 min)	2.56±0.878	1.12±0.152	22.1±0.0834	0.713±0.0605
Control (25°C-pH 7-30 min)	2.67±0.922	1.01±0.079	21.4±0.0476	0.57±0.0331
Control (25°C-pH 7-60 min)	1.9±0.00354	0.982±0.237	21.6±0.124	0.598±0.00135
End of acid shock (pH 5-30 min)	2.58±0.921	1.17±0.106	21.7±0.397	0.636±0.0472
Immediately after acid shock and resetting pH value at 7	2.57±0.889	1.09±0.183	21.6±0.122	0.591±0.0402
Thirty minutes after acid shock and resetting pH value at 7	2.4±0.992	0.951±0.309	21.8±0.389	0.617±0.0694

*mean±standard deviation of 3 independent replicated experiments.
doi:10.1371/journal.pone.0051196.t002

the post heat shock detection of transcripts that we are reporting, and there are basic differences between those studies and our results. The extension of half-life is reported during the heat shock and it may be attributable to a failure in RNA degradation [18], while we are reporting that genes remained induced 30 min after the heat shock ceased. In addition, while during the heat treatment only 7% of genes were found to have a half life longer than 30 min [17], in our work we are reporting that 96% of the genes induced during the heat shock were detected immediately when the temperature was reset to 25°C and 85% of them were still induced 30 minutes later. Thus our results seem to be due to an hysteretic behaviour of transcription according to the cell pre-history rather than to the extension of the half-life of the transcripts. However, the switching levels for the induction/repression of heat shock genes cannot be determined from our data and it is unknown how this hysteretic response is affected by cell division, or by other environmental stimuli impacting on transcription.

The modularity of the structure of the genome scale network derives from the network construction itself and means that in general genes can be grouped in functional modules according to the cellular functions and/or metabolic pathways in which the proteins they encode are involved. Modularity was estimated on the sub-networks from the genome scale network corresponding to genes differentially expressed during and after the heat treatment. The obtained modularity values were different from the value exhibited by the genome network; however, this is expected in sub-networks of smaller dimension extracted from a primary larger network. Therefore, to assess the significance of the differences in the modularity of the sub-networks of the genes up and down-regulated during the heat shock, we compared their modularity values with those measured in 10 random networks. The random networks used for comparison had the same number of genes as the networks of up- or down-regulated genes; the genes were randomly selected from the genome scale network. The modularity values of the up-regulated network were not significantly different from those measured in the correspondent randomly generated networks. Therefore the modular structure observed in the networks of up-regulated genes during and after the heat shock was the was not significantly different from that observed in the random networks derived from the genome scale network, reflecting its organization in metabolic pathways and cellular functions. However, modularity analysis of the networks of down-regulated genes during and after the heat shock revealed that the level of organization of repressed genes in functional modules was smaller than expected.

Repressed genes showed a lower level of organization in metabolic pathways and functional categories than expected while induced genes maintained the level of modularity expected in sub-networks derived from the genome scale network. Gene induction, during and after the heat shock, exhibited hysteresis and was organized in metabolic pathways and functional categories. On the other hand, although a large number of genes (293) were repressed during the heat shock only one third of them remained repressed immediately and 30 minutes after the heat shock ceased and the networks of these repressed genes in metabolic and functional modules were significantly less organized in metabolic pathways and functional categories. Possibly gene repression is less synchronised than gene induction as a result of the hysteretic behaviour of induction. If genes remained induced once stimuli cease, gene repression which is the complementary event to induction has to be affected.

The hysteretic behaviour of gene transcription may mechanistically be explained by a dynamic switching threshold that changes according to the state of the gene. The concentration of inducer needed to initiate transcription of uninduced genes may be higher than that needed to maintain transcription of induced genes which may be coupled with metabolic reaction rates. Bistability can arise from substrate inhibition or product activation in metabolic pathways [19]. It is also possible that the switching threshold is not affected but the signal of the inducer is amplified by the induced gene in a positive feedback mechanism. In fact, all known bistable signalling systems contain a "positive" circuit such as the double-negative feedback in the artificial genetic circuit for *E. coli* [20], the positive feedback loop in the genetic network described for *Saccharomyces cerevisiae* [21] or in the transcriptional network of *Bacillus subtilis* leading to biofilm formation, sporulation and the generation of multiple distinct phenotypes within an homogeneous population [22]. However, further studies are needed to explain the exact mechanism behind the hysteretic responses we have observed in the current study.

Monitoring transcription at genome scale by microarray hybridization is usually associated with very high variability between replicates. In the experiments carried out in this work we detected 1931 genes significantly differentially regulated at least in one sample but only 856 (44%) were detected in replicated samples from independent cultures (data not shown). We estimated the number of false detected differentially transcribed genes due to experimental error based on hybridizations of identical samples. We detected only 60 genes with variable transcriptional results in 6 hybridizations of identical samples to slides with printed products for all genes in the genome (>4000). Thus, experimental error cannot be the cause of 1075 false detections. Recent studies have focussed on investigating stochastic fluctuations in gene expression [23,24] describing intrinsic noise resulting from stochasticity in the biochemical reactions taking place at the gene level and extrinsic noise originated from fluctuations in other cellular components involved in gene expression. The extrinsic component of the noise is dominant with a major contribution to the variability of gene expression [24] and being propagated in the gene network to affect expression fluctuation of its downstream genes [25]. These studies highlight the importance of stochastic fluctuations on the variability of gene regulatory networks. In addition, our results suggest that the hysteretic response associated to the history of cells may also contribute to explain this large variability of gene regulation. We have observed that 102 genes associated with response to heat shock may be either induced or repressed at 25°C, depending on the previous culture conditions. Regulation of some genes may be unexpected as a response to current conditions but explicable and determined by past environments. In addition, due to the network structure of gene regulation, the variable expression associated with the hysteretic response can be expected to be amplified when affecting the regulation of downstream genes contributing substantially to the large variability of gene expression.

We demonstrated that the hysteretic transcriptional response to the exposure to 45°C for 30 minutes was accompanied with increased resistance to heat and cross-resistance to acid inactivation conditions even 30 minutes after the stress has ceased. Thus, hysteresis may explain other cross-protection mechanisms against environmental stresses such as the reported higher thermotolerance of acid-adapted *Salmonella* cells [26]. This phenomenon could also explain the dependence of the duration of the bacterial lag phase on the previous growth conditions [27,28]. Some studies have reported that non-replicating bacteria also remember previous environmental conditions and this cell memory seems to be associated to exposure time. *E. coli* starved for long periods in stationary phase maintained anaerobic metabolism, typical of

stationary phase, during the lag period when inoculated in fresh media while young stationary cells switched immediately to aerobic respiration [7]. In another study, *Listeria monocytogenes* was incubated at no-growth a_w values (0.90) for days and it was observed that the longer the pre-incubation period, the faster the initiation of the subsequent growth at also low but growth permitting a_w values [29]. The lack of an adaptation response to extreme growth conditions involving the lag phase and/or growth rate in our experiments was most likely due to the short duration, 30 minutes, of the previous exposure to the stressing conditions.

In food and animal feed chains, producers are responsible for the safety of their products. Thus tracing the origin of accidental or deliberate microbial contamination of feed and food is essential to establish corrective actions that prevent this contamination [30]. Hysteretic responses to environmental conditions and stresses associated with food production and process could be investigated to infer the time and point of contamination throughout the food chain. The detection of proteins and/or transcripts associated with past environments might represent a rapid inferring system for the reconstruction of the contamination scenario.

Methods

Bacterial Strain, Environmental Culture Conditions and Sample Preparation

Salmonella enterica subsp. enterica serovar Typhimurium strain 4/74 which is the parental strain of the *hisG* mutant SL1344 [31] was subcultured twice in tryptone soy broth (TSB, Oxoid, Basingstoke, UK) and incubated at 25°C for 24 hours. before being inoculated in 200 ml of TSB and incubated at 25°C for 16 hours to early stationary phase. This culture was divided in two parts; one untreated control culture kept at 25°C and pH 7 while the other part of the culture underwent stressing conditions either at pH 5 or at 45°C.

The temperature shift was carried out by moving the culture from a 25°C water bath to one at 45°C. Once the culture reached 45°C, it was left there for 30 minutes before being moved back to 25°C. The change of temperature with time was measured by a thermocouple applied to a replicate bottle of uninoculated medium. For the pH stress condition, the pH was lowered to pH 5 with hydrochloride acid and after 30 minutes changed back with sodium hydroxide to the original pH. Solutions to adjust the pH of the media were highly concentrated to avoid the dilution of the population and the possibility of growth initiation. Samples from the untreated control populations were processed at the same sampling times as the stressed cultures.

RNA samples for gene expression analysis and culture samples for growth and heating experiments were obtained before applying the stress, after 30 minutes with stress, immediately after the removal of stress and 30 minutes after the removal of stress. At each sampling time, 10 ml culture was harvest by adding 10 ml Ambion RNA*later* Tissue Collection solution (Life Technologies, Taastrup, Denmark) and placed at 4°C overnight and stored at −20°C until RNA extraction. Samples of all cultures were used straight away for growth and inactivation experiments.

Three independent biological replicates for both pH and temperature stress were run.

Growth and Inactivation Experiments

One ml of each sample was inoculated in 100 ml of TSB to reach a concentration of ca. 10^7 cfu/ml. For extreme growth conditions experiments, cultures were incubated either at 43°C or at pH 4.5. Growth was monitored by optical density (OD) at 600 nm. For inactivation experiments, cultures were incubated at either 50°C or pH 3 and bacterial concentration measured by plate counts on tryptone soy agar (TSA, Oxoid).

Estimation of Growth and Inactivation Parameters and Statistical Analysis

The duration of the lag period and the maximum specific growth rate were estimated by fitting the model of Baranyi and Roberts [32] to the growth curves recorded as natural logarithm of OD measurements *vs* time while D-values, exposure time required for a decimal reduction of the population at constant inactivating temperatures, were estimated from the log linear inactivation curves of cfu/ml *vs* time.

A one way ANOVA model with one factor was fitted to the ranked growth and inactivation parameters. The factor of the model was the history of the cells previous to exposure to inactivating conditions or extreme growth environments and included 6 levels. Three levels referred to stressed cells and they were "end of stress" and "immediately" and "30 minutes" after initial conditions were reset. The other 3 levels refereed to control cultures maintained for "0", "30" and "60" minutes at 25°C and pH 7.

Orthogonal contrasts were set up to investigate if growth an inactivation parameters of control cultures were significantly different from those of stressed cultures as well as the significance of the effect of the time spent at control conditions.

DNA Microarray Hybridizations

Total RNA was purified from the RNA*later* solution using the RNeasy Mini Kit (Qiagen, Copenhagen, Denmark) according to the manufacturer's instructions ("RNeasy Mini Protocol for Isolation of Total RNA from Bacteria") with minor adjustments; for lysis, a 15 mg/ml lysozyme solution with proteinase K was used and on-column DNase digestion was performed. The quality of the RNA was checked using the NanoDrop (Fisher Scientific, Slangerup, Denmark). Labeled cDNA was synthesized from total RNA using the FairPlay III Microarray Labeling Kit (Agilent Technologies, Hoersholm, Denmark) according to manufacturer's instructions without the Spike-in step. cDNA from the untreated cultures was labeled with Cy5 and cDNA from the stressed culture was labeled with Cy3. The labeled cDNA from the two cultures were mixed together and competitively hybridized on an 8×15 K Agilent microarray slide constructed for *Salmonella* Typhimurium strain SL1344 (deposited with GEO database ref. number: GPL15227) at 65°C for 17 hours, washed and scanned according to the "Two-Color Microarray-Based Prokaryote Analysis Protocol" (Agilent Technologies). The scanning was done using an Axon GenePix 4200A Microarray scanner (Axon, Foster City, CA) and the feature intensities were quantified using GenePix Pro 6.1 software (Axon, Foster City, CA).

Microarray Data Analysis

Data normalization and analysis was carried out using the program ArrayLeaRNA (freely available at http://www.ifr.ac.uk/safety/ArrayLeaRNA/) which implements a Bayesian inference method based on the variability of the hybridization to internal controls probes in each array and operon predictions if available [33]. Genes detected as up- (down-) regulated at least in two of the three replicated samples were considered as differentially expressed for a given sampling time. In addition, genes up- (down-) regulated in only one replicate of a given sampling time were considered as differentially expressed if detected in at least two of the three replicates of the other two sampling times (Tables S1 and S2).

Microarray datasets have been deposited with GEO database (series accession number: GSE37636).

Network Analysis

A bi-partite network was constructed for the genome and plasmids of *S.* Typhimurium SL1344 as previously described for *E. coli* K 12 [7]. Edges connected two sets of nodes. Genes constituted one of these sets of nodes. The genome composition was obtained from the Genome Project NCBI database [34]. The other set of nodes included metabolic pathways and cellular functions, according to the KEGG database [35], the CMR-TIGR database [36] and the COGs (Clusters of Orthologous Groups of proteins) functional categories obtained from the Genome Project NCBI database [34]. The number of nodes was 5153, from which 4717 were genes and the remaining 436 nodes represented metabolic pathways and cellular functions. There were 11626 edges between these two sets of nodes.

The genome scale network was used to extract the bipartite networks corresponding to genes up- and down-regulated during and after the heat shock. Only functional categories and metabolic pathways connected to a significant number of genes up or down-regulated were included in these networks. The significance was statistically evaluated assuming that number of genes up(down)-regulated belonging to a metabolic pathway or cell functional category follows the commonly assumed hypergeometric distribution as previously described [7].

For networks representation we used the program Cytoscape [37]. Networks modularity was estimated with the program implementing the fast modularity maximization algorithm [8].

Supporting Information

Figure S1 Metabolic pathways and cellular functions associated with those genes up-regulated during acid shock, immediately after acid shock ceased and 30 minutes after acid shock ceased. Columns had positive values if functions were up-regulated and negative if down-regulated.
(TIF)

Figure S2 Main metabolic pathways and general cellular roles with a significant ($p<0.1$) proportion of genes up- or down-regulated during heat stress (During), immediately (After) and 30 minutes (Later) after heat stress ceased. Columns had positive values if functions were up-regulated and negative if down-regulated.
(TIF)

Figure S3 Specific metabolic pathways and cellular sub-roles with a significant ($p<0.1$) proportion of genes up- or down-regulated during heat stress (During), immediately (After) and 30 minutes (Later) after heat stress ceased. Columns had positive values if functions were up-regulated and negative if down-regulated.
(TIF)

Figure S4 Bacterial concentration (closed symbols) during and after the exposure to stressing conditions: A) heat shock at 45°C 30 minutes and B) acidification of the medium at pH 5 for 30 minutes. Concentrations were also measured immediately after the cease of the stressing conditions and 30 minutes after resetting the original conditions as well as in untreated control populations maintained at 25 °C and pH 7 during the experimental course (C). Significant variation of the bacterial concentration was not detected in any population under any condition.
(TIF)

Table S1 Number of replicated samples in which genes were up- or down- regulated because of heat stress (45°C).
(PDF)

Table S2 Number of replicated samples in which genes were up- or down- regulated because of acid stress (pH 5) and comparison with results of expression under heat stress in Table S1.
(PDF)

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Author Contributions

Conceived and designed the experiments: CP CL HA JEO. Performed the experiments: TH MM-C RdJ JTR. Analyzed the data: CP. Contributed reagents/materials/analysis tools: CP CL HA JEO. Wrote the paper: CP JEO.

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Paper IV

Evaluation of direct 16S rDNA sequencing as a metagenomics based approach for screening of bacteria in bottled water

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Evaluation of direct 16S rDNA sequencing as a metagenomics based approach for screening of bacteria in bottled water

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Abstract

Deliberate or accidental contamination of food, feed and water supplies pose a threat to human health worldwide. The needs for rapid and sensitive detection techniques that are in contrast to the current labor-intensive and time-consuming culture-based methods are therefore highly desirable. In addition to species specific assays, such as PCR, there is a need for generic methods to screen for unknown pathogenic microorganisms in samples. This work presents a metagenomics based direct sequencing approach for detection of unknown microorganisms, using *Bacillus cereus* (as a model organism for *B. anthracis*) in bottled water as an example. Total DNA extraction and 16S rDNA gene sequencing were used in combination with principle component analysis and multi curve resolution to study detection level and possibility for identification. Results showed a detection level of 10^5-10^6 CFU/L. Using this method it was possible to separate two *B. cereus* strains by the principle component plot, despite of the close sequence resemblance. A linear correlation between the artificial contamination level and the relative amount of the *Bacillus* artificial contaminant in the metagenome was observed and a relative amount value above 0.5 confirmed presence of *Bacillus*. The analysis also revealed that background flora in the bottled water varied between the different water types that were included in the study. This method has the potential to be adapted to other biological matrices and bacterial pathogens for fast screening of unknown bacterial threats in outbreak situations.

Introduction

Some biological agents or toxins can potentially be used as very dangerous weapons in acts of bioterrorism (bioterror organisms). Fast detection methods are highly desirable in order for the identification of the agent to be possible before it has caused too much harm (1). In an outbreak situation, whether it is caused by accident or a deliberate act, the conventional culture-based methods for identifying pathogenic bacteria are laborious and time-consuming. When considering a deliberate contamination, the circumstances of the threat might be unclear. With unknown or multiple agents present at the same time, a rapid answer is of central importance. A problem that arises when analyzing bioterror organisms from the Bacteria domain, including *Bacillus*, is that there often exist other closely related harmless organisms that have large sequence regions in common with these (2,3). The bioterror agent *B. anthracis*, which is classified as a critical biological agent of category A (4), are closely related to *B. cereus* and *B. thuringiensis* due to their high degree of DNA sequence similarity (5,6). This is one of the major hurdles when designing a rapid and reliable detection method. It is very important not to declare a bioterror threat in public before it is absolutely assured since a delayed or even false positive results have enormous economic consequences (7–9).

The culture-based approaches for detection usually require some prior knowledge of the microbial content of the sample. Molecular methods like PCR, which often is used as the detection method, can provide fast identification (10,11). However, these assays are usually optimized for detection of a single organism or a very limited number of pathogens. If the cause of the contamination is unclear or even unknown, a large number of assays and runs are needed to determine the actual species, which can be very laboratory demanding and time consuming. Variant of the targets might not be detectable if changes in the nucleotide sequence have occurred in primer and/or probe binding site. Furthermore, as a significant proportion of microorganisms are unculturable they cannot be detected using these techniques (12,13). For the unknown threat, a culture independent technique where you screen for the presence of any targets would simplify the analysis considerably. A powerful technique for resolving samples with unknown composition is metagenomics. In this approach, the entire microbial gene pool of e.g. food, feed, water or environmental samples is examined. In contrast to tools like PCR and microarray where specific biomarkers are searched for, the metagenomics-based approach identifies those biomarkers that are actually present in the sample and it is subsequently possible to match them to the microflora that is present (14). There are

two basic approaches that are employed when metagenomics is used to define the content of microbial communities: (i) whole genome based and (ii) target specific approaches (reviewed by Karlsson et al., this Issue and Suenaga 2012 (15)). The whole genome based approach gives more detailed information, but it is technically more difficult to perform, both concerning need for specific equipment, as well as computer power and storage of the huge amounts of generated data. In the target specific approach, a smaller fraction of the genome is amplified and sequenced. This gives rise to less complicated data that is easier and faster to process, but the information on the content of the microflora is limited to the targets used. A marker commonly used is the 16S rDNA gene, which is universally conserved, and also has a high degree of diversity across bacterial species (16,17). One method that has been used for target specific metagenomics is the direct sequencing approach where the entire microflora is sequenced without prior cultivation (18,19).

In this study, the direct 16S rDNA targeted sequencing approach (18,19) has been evaluated as a diagnostic tool for detection of *B. cereus* artificially inoculated in bottled water without prior cultivation. For an overview of the experimental design, see Fig.1. *B. cereus* was used as a model organism for *B. anthracis*, the causative agent of anthrax. Two different *B. cereus* isolates were used, the F2085/98 and NVH0597-99, previously found to have a close resemblance to *B. anthracis* (20). Spectra generated from the sequencing of the samples were compared using multivariate statistical methods, including principle component analysis (PCA) and multivariate curve resolution (MCR).

Materials and methods

Bottled mineral water

Four brands of noncarbonated natural mineral water originating from two countries were analyzed. These waters were contained in 1.5 L or 2 L polyethylene terephthalate (PET) bottles and were purchased from local retail stores in Denmark and Sweden. Three of the waters were produced in Denmark (A, B and D) and the last one in Norway (C). The bottles were stored at room temperature (20-25°C) prior to analysis.

Bacterial strains and inoculation of water samples

The strains used in this study are the two *B. cereus* strains, F2085/98 and NVH0597-99 (20) . Cells were grown in 8 ml of Luria-Bertani (LB) broth (Sigma, Brøndby, Denmark) at 37°C for 18 h. A 10×dilution series in 0.9% NaCl were made and the dilutions from 10^{-4} to 10^{-8} were plated in duplicates on Plate Count Agar (PCA, Oxoid, Greve, Denmark) and incubated overnight at 37°C. The weighted average of the Colony Forming Units (CFU) was calculated. The mineral waters (A-D, samples of 1.5 L) were inoculated in with one of the two *B. cereus* strains in the levels 10, 10^2, 10^3, 10^4, 10^5 and 10^6 CFU/L and all samples were performed in duplicates.

Sample concentration and DNA extraction

After *B. cereus* inoculation, each 1.5 L water sample was filtered using a Nalgene filtration assembly (Sigma-Aldrich, Brøndby, Denmark) through a 0.45 µm, 47 mm Whatman® Membrane Filter (GE Healthcare, Brøndby, Denmark). After filtration, the filter paper was transferred to a tube and 2 ml Phosphate Buffered Saline (PBS, Sigma, pH 7.4) + 1% Tween® 20 (Sigma) was added. The tubes were vortexed for 3×10 seconds and the liquid was transferred to an Eppendorf tube and centrifuged for 10 min at 16,300×g. The supernatant was removed and the resulting cell pellet was stored at -20°C until DNA purification.

DNA was purified from the cell pellet using the DNeasy Blood & Tissue Kit (Qiagen, Copenhagen, Denmark) with a modified Gram-positive protocol for the lysis step; lysis was done at 37°C for 60 min and an RNase A digestion step was included. The DNA was stored at -20°C until further analysis.

Direct sequencing and data processing

DNA from the water samples was amplified with universal 16S rDNA primers (21) and PCR was thereafter performed according to Skånseng et al. 2007 (18). The PCR products were purified before sequencing, using 0.4 µl ExoSap-IT (USB Corp., Cleveland, Ohio) to 5µl of PCR product. A universally conserved primer (22) was used for sequencing. The sequencing procedure was performed according to Skånseng et al. 2007 (18).

The direct sequencing spectra were aligned, trimmed and processed with the use of MATLAB (MathWorks, Natick, MA) as described by Zimonja et al. 2008 (19). The generated data were imported into R v2.15.1 (23) for PCA (24) and Unscrambler software v9.6 (CAMO Software) for MCR analysis (25).

Results

Classification of the total bacterial flora of the water samples

The direct sequencing approach of a universally conserved 16S rDNA gene region (26) was used for classification of the total bacterial flora in the *B. cereus* artificial contaminated water samples. Out of the 80 samples sequenced, spectra were obtained from 68 samples (data not shown), which was further analyzed.

The mixed spectra were first transformed into frequencies of pentamers for determination of the phylogenetic differences between the samples and thereafter PCA was performed (27). PCA is used to reduce the dimensionality of data with many variables, while retaining most of the variation in the data set (28). PCA was chosen to describe the phylogenetic relations instead of the tree-based system to avoid DNA sequence alignment. The resulting PCA plot is visualized in Fig. 2. The two first principle components (PCs) explained 72% of the total variance in the data set, with PC1 representing 60% (Fig.2). Adding further PCs did not notably increase the percentage of variance explained. The samples in the lower left corner of the PCA plot were selected and the corresponding sequences were searched in the Ribosomal Database Project (RDP) database (29). This search was done in order to be able to identify which organisms that were represented in the different samples. The microflora in these samples was identified as belonging to the order of Bacillales (Fig.2). PC1 separated the samples where *Bacillus* was detected from those where it was not detected. Furthermore, the two *B. cereus* isolates (F2085/98 and NVH0597-99) were separated by PC1, with samples inoculated with *B. cereus* NVH0597-99 in *Bacillus* group 1 and samples inoculated with *B. cereus* F2085-98 in *Bacillus* group 2 (Fig. 2).

Samples from the right part of the PCA plot were searched in RDP, and it was found that the flora of these samples was dominated by the orders Burkholderiales and Rhodocyclases, depending of the water supplier. The water supplier A, B and D were associated with Burkholderiales, whereas water type C was associated with Rhodocyclases (Fig.2).

Detection level of *B. cereus* in water samples

MCR was used for determination of the detection level. MCR is a method that estimates concentrations of contributing components when no prior information is available about the nature and composition of the mixtures analyzed (25). One of the components from the MCR analysis, component 2, was found to explain the artificial contamination level of *B. cereus*.

The relative amount of the component 2 is shown in Table 1. Those samples with the highest relative values of component 2 (> 0.5), was confirmed as *Bacillus* by search in RDP and BLAST (29,30). The interpretation is that artificial contamination with 10^5 and 10^6 CFU/L gives detection of *Bacillus*. It was furthermore seen that contamination with *B. cereus* NVH0597-99 gave higher relative amount values compared with F2085/98 at the same contamination level, and the tendency was that with higher contamination level, the difference between the two strains increased (data not shown). For both *B. cereus* isolates it was possible to detect 10^5 CFU/L when inoculated in water from supplier C, whereas for the water supplier A and B, the detection was at 10^6 CFU/L. For the water supplier D, it was not possible to detect *B. cereus* in any of the samples.

Discussion

In this study, a metagenomics based direct sequencing approach (26) for detection has been evaluated on artificial *B. cereus* contaminated water samples. Using this method for metagenomics based analysis generates low amount of data which makes the data handling and data analysis easier, compared with other metagenomics based methods since assembly of sequences is not necessary.

The sequence similarity between *B. cereus* and *B. anthracis* is very high (5,6), which makes it difficult distinguish between them based on sequence analysis. Furthermore, studies have shown that certain isolates of *B. cereus*, such as the two strains used in this study, demonstrate the same growth pattern, germination and biochemical characteristics as *B. anthracis* (20). From the PCA plot (Figure 2), the two strains of *B. cereus* seem to be separated by the PC1, even though they have highly similar DNA sequences. The separation is most likely not caused by the difference in water type since the samples found positive for *Bacillus* originates from three different water suppliers (A, B and C). The separation of the two *B. cereus* isolates by the PCA plot point to the possibility of distinguishing between *B. anthracis* and other related species like *B. cereus* and *B. thuringiensis* which normally pose a problem (5,6). Further studies are needed using *B. anthracis* to confirm this.

High-resolution melting curve analysis (HRM) on the amplified 16S rDNA genes could be an alternative to the sequencing step, also offering the possibility for quantification (31). However, in order to use HRM on any potential bacterial pathogen in a sample, a complete database of melting points characteristics for all bacterial pathogens has to be established. Furthermore, it could be difficult to differentiate closely related species, such as *B. cereus*, *B. thuringiensis* and *B. anthracis* solely based on the melting point characteristics. A study by Klaschik et *al*. used HRM on the 16S rDNA gene to differentiate between 17 bacteria (32). The melting curve analysis was able to classify most of the bacteria on species level, apart from *Staphylococcus aureus* and *S. epidermidis*. The two staphylococci could only be classified on species level.

From the MCR analysis (Table 1), it was found that the detection level of the *B. cereus* strains was around 10^5 -10^6 CFU/L, depending on the water supplier, with water supplier D having the highest detection level. The relative amount values from water supplier D were lower compared with the other supplier at same artificial inoculation concentration (data not shown). This could indicate that there is a difference in the composition of the water, of

either the bacterial or chemical composition that might interfere with the filtration, DNA extraction or the PCR amplification. The pH of the different water varies from between pH 7 and 7.6 (values as stated on the bottles) which could affect the membrane filters. It has previously been demonstrated that changes in pH and salt concentrations can change the properties of the filter (33). The water from the different suppliers contains various amounts of Mg^{2+} and Ca^{2+}. Excess of Mg^{2+} is known to inhibit the PCR reaction (34) and the water supplier A, B and D has more than ten times as much Mg^{2+} as supplier C. High concentrations of Ca^{2+} can lead to a competitive binding by the DNA polymerase instead of Mg^{2+}, making it inaccessible for the DNA polymerase and thereby decreasing the activity of the polymerase (35). The higher concentrations of Mg^{2+} and Ca^{2+} for the water supplier A, B and D could explain the lower detection level compared with supplier C. Furthermore, the filtration of the water, before the DNA extraction, results in a concentration of any inhibitors present in the samples (36). In a further development of this method it can be recommended to include a DNA extraction control to check for PCR inhibition. Those samples positive for *Bacillus* were also the ones having the highest relative amount value (> 0.5) and this was only observed for samples with inoculation levels of 5 and 6 log CFU/L. Large standard deviations were noted for the low inoculation levels, indicating that for these samples the identification is less confident. Due to the short analysis time of this method, it is possible to include a short enrichment step in order to improve the detection level.

A better detection of *B. cereus* NVH0597-99 was seen from the MCR and PCA analysis (Figure 2 and Table 1, respectively), together with the sequence search in BLAST and RDP. Furthermore, a slightly higher number of samples with *B. cereus* NVH0597-99 were regarded as positive for *Bacillus* at a contamination level of 10^5 CFU/L compared with samples inoculated with *B. cereus* F2085/98. The difference in detection level of the two strains could be a result of differences in the DNA extraction efficiency or variations in 16S rDNA copy number, which has been shown to affect the amplification of 16S rDNA genes from mixed samples (37). Generally, levels below 10^3 CFU *B. cereus* per g or ml of foods are considered to be safe (38). It has been shown that samples implicated in outbreaks of *B. cereus* contain $>10^3$ CFU/ml or g food (39), which would then be detectable using this method The lethal dose of *B. anthracis* in humans resulting from consumption of contaminated food is not known since data from both outbreak investigations and animal models show a huge variation in the LD values (reviewed by Erickson & Kornacki 2003(40)). However, the detection level obtained in this study is in the same range as for other published methods (41,42).

Another shortcoming with the described method is that it might be unable to detect contaminations from spores. The spores from both *B. cereus* and *B. anthracis* are more resistant compared with vegetative cells (43). The DNA extraction method applied in this study might not be sufficient in releasing DNA from the spores, thus enabling amplification of the 16S rDNA gene which would give false negative result. To solve this, the lysis step during the DNA extraction could be extended for sufficient release of DNA from spores. Another limitation of the method, that needs to be further investigated, is the possibility for the method to distinguish *B. anthracis* from *B. cereus* and *B. thuringiensis*. The 16S rRNA gene sequences of these species have been shown to have high levels of sequence similarity (5,44).

The direct sequencing method revealed a distinct difference in the composition of the background flora between the four different water suppliers. The background flora of the water was mainly dominated by the orders Burkholderiales and Rhodocyclases, which previously has been associated with bottled water (45). Interesting, the composition of the background flora seemed to be dependent on the country of the origin of the water supplier (Denmark or Norway). Further analysis has to be conducted to see if the differences are caused by variation in storage time and/or location prior to the purchase. Studies have shown that location, temperature and storage time has a profound effect on the variation of the bacterial flora (46–48). The results, however, implies that the method might be used for quality insurance to check the authenticity of bottled water but further analysis with a larger sample set has to be conducted in order to confirm these findings.

In conclusion, the direct sequencing method evaluated in this study has the potential to give fast identification of unknown biological threats in an outbreak situation, enabling quick answers to the public and thereby minimizing the economic consequences and further spread. The method presented here can be used for acquiring knowledge about the variation of the normal background flora in a given matrix, which can be useful for detection and identification of any changes in the composition of the bacterial flora. Knowing the normal bacterial flora in a given matrix, e.g. food or feed, a single sample can be used as an indicator for any abnormal microflora present, whether it is deliberate or accidental contamination. However, in order for the method to be applied to other kinds of biological matrices, additional optimization and verification are needed.

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Table 1 Results from the multivariate curve resolution (MCR) analysis for different contamination levels of *Bacillus cereus* for the investigated water suppliers A-D.

***B. cereus* inoculation level (log CFU/L)**	***B. cereus* strain inoculated**	**Number of samples for each inoculation level[A]**	**Relative amount of total flora[B]**	**Main order(s) identified by the RDP database[C]**
1	F2085/98	10 (9)	0.04 ± 0.03	Burkholderiales
	NVH0597-99		0.02 ± 0.03	Burkholderiales
2	F2085/98	12 (10)	0.04 ± 0.08	Burkholderiales Rhodocyclases
	NVH0597-99		0.05 ± 0.07	Burkholderiales Rhodocyclases
3	F2085/98	12 (6)	0.05 ± 0.01	Burkholderiales
	NVH0597-99		0.05 ± 0.02	Burkholderiales
4	F2085/98	17 (15)	0.06 ± 0.08	Burkholderiales Rhodocyclases
	NVH0597-99		0.18 ± 0.13	Burkholderiales Rhodocyclases
5	F2085/98	16 (16)	0.18 ± 0.19	Burkholderiales Rhodocyclases Bacillales
	NVH0597-99		0.66 ± 0.25	Burkholderiales Rhodocyclases Bacillales
6	F2085/98	13 (12)	0.48 ± 0.09	Bacillales Burkholderiales
	NVH0597-99		0.99 ± 0.01	Bacillales Burkholderiales

[A] The total number of samples for each inoculation level and the number in parenthesis is the number of samples that gave a sequencing result

[B] Mean relative amount ± standard deviation

[C] Order listed with the most frequent first

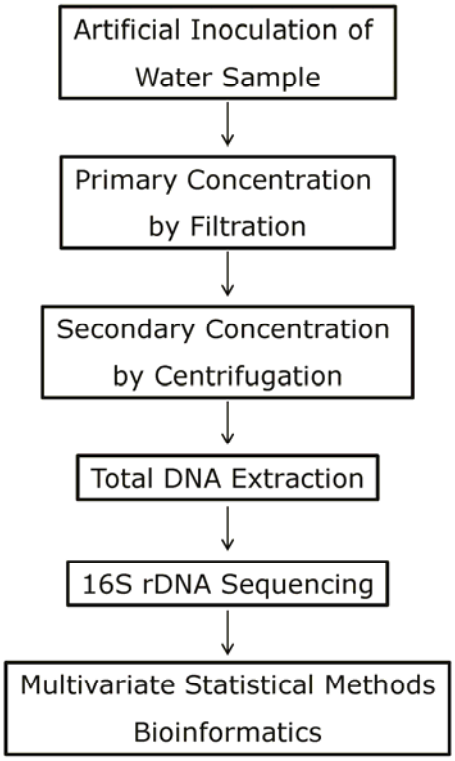

Figure 1 Schematic overview of the direct sequencing approach using the marker 16S rDNA.

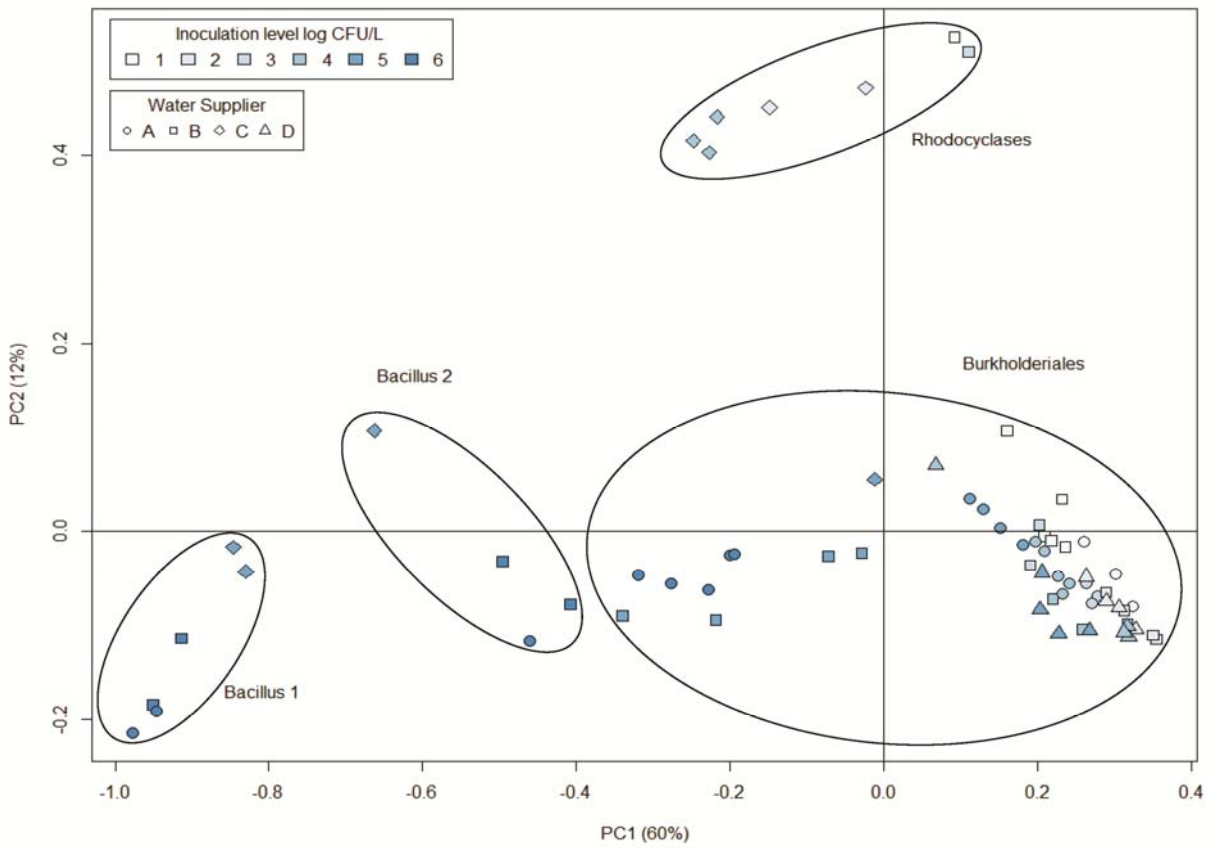

Figure 2 Principle component analysis (PCA) score plot. The principle component 1 (PC1) was plotted against PC2. The colouring of samples represents the level of *B. cereus* inoculation in the water and symbols the water supplier. The percentage shown on each axis represents the portion of variance explained by that principal component (PC). Circles around data points represent the four main groups that were visually identified from the PCA plot with the main order/genus that was identified in each group written next to the circle. Bacillus 1 and Bacillus 2 are two separate groups where Bacillales was identified as the most frequently order found in these samples.

Paper V

Evaluation of a direct 16S rDNA sequencing approach for screening of *Bacillus cereus* in feed and food

Paper in preparation

(Page numbers are relative to paper)

Evaluation of a direct 16S rDNA sequencing approach for screening of *Bacillus cereus* in feed and food

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Running title: *Bacillus* detection in food and feed

Keywords: *Bacillus cereus*, principal component analysis, feed and food, direct detection

Abstract

Deliberate or accidental contamination of food, feed and water supplies pose a threat to human health worldwide. There is need for generic detection methods in contrast the current labor-intensive and time-consuming culture-based methods which usually are specific for a limited number of species. This works evaluates a metagenomics based direct sequencing approach for detection of *Bacillus cereus* in food and feed. Different DNA extraction kits were evaluated for the ability to extract *B. cereus* from inoculated food and feed samples. One kit, the MasterPure DNA Purification Kit, was found to be suitable. Total DNA extraction and 16S rDNA gene sequencing were used in combination with principle component analysis to study detection level and possibility for identification. The PCA could not clearly distinguish between the two *B. cereus* strains and based on the sequences it was only possible to classify five samples as belonging to the class of Bacilli. The results show that the method could not provide reliable conclusions on the ability to distinguish or detect *B. cereus* in the food and feed samples and for the method to be useful in detection of bacteria in food and feed samples, additional optimization and verification are needed.

1 Introduction

The food and feed chains are both recognized as targets for accidental and deliberate contamination with biological agents or toxins, which can have widespread economic and social consequences (Knutsson et al., 2011). In an outbreak situation, whether it is caused by accident or a deliberate act, the conventional culture-based methods for identifying pathogenic bacteria are to laborious and time-consuming. Fast and accurate detection methods for the identification of biological agents are for that reason very desirable. However, when considering a deliberate contamination, the circumstances and identity of the threat might be unclear. A problem that arises when analyzing bioterror organisms from the Bacteria domain, which includes the species *Bacillus*, is that there often exist other closely related harmless organisms with high sequence similarity (Kim et al., 2005; Kolstø et al., 2009). The bioterror agent *B. anthracis*, which is classified as a critical biological agent of category A (Khan et al., 2000), have a high degree of sequence similarity to *B. cereus* and *B. thuringiensis* which makes the distinction between them very difficult (Helgason et al., 2000; Rasko et al., 2005).

The use of nucleic acid based methods for detection, in particular PCR techniques, allows faster detection with greater specificity and sensitivity (Dwivedi & Jaykus, 2011; Weile & Knabbe, 2009). The detection of microbial nucleic acids for diagnostics depends highly on the separation of the nucleic acids from the sample. The quality of extracted nucleic acids from the samples is crucial for optimal performance of the nucleic acids based detection methods. Nevertheless, numerous substances from food and feed samples can inhibit downstream molecular diagnostic applications, such as PCR (Hedman & Rådström, 2013; Wilson, 1997). Removal of inhibitors that can interfere with the detection are particular important when sensitivity is critical. Inhibitors originating from food and feed include e.g. fats, proteins and particles (Rossen et al., 1992). Besides the removal of inhibitors, the release of nucleic acids from the pathogen is essential. Efficient lysis of the bacteria and spores is needed; however to harsh lysis can shatter the integrity of the extracted nucleic acids (Rantakokko-Jalava & Jalava, 2002). Consequently, the sample processing and extraction of nucleic acids plays a crucial role to obtain an optimal detection method. A variety of studies have compared commercial kits for extraction of nucleic acids from a number of biological matrices (Dauphin et al., 2009, 2010; Rantakokko-Jalava & Jalava, 2002; Rose et al., 2011). The optimal extraction method for one type of matrix might not be suitable for another.

However, one problem arises when using nucleic acid based methods. Many of the assays designed for detection are usually optimized for a single organism or a very limited

number of similar pathogens. This makes the methods suitable for screening of particular pathogens but when the identity of the pathogen causing an outbreak is unknown it gets more complicated. A powerful technique for investigating samples of unknown composition is metagenomics (Handelsman, 2004). With metagenomics, the entire microbial gene pool of e.g. environmental, food, feed or water samples is examined. A commonly used marker for metagenomics is the 16S ribosomal DNA (rDNA) gene, which is universally conserved and also has a high degree of diversity across bacterial species (Clarridge, 2004; Petti et al., 2005). A metagenomics-based direct sequencing method (Skånseng et al., 2007; Zimonja et al., 2008) has previous been evaluated on detection in bottled water samples inoculated with *B. cereus* and it was found that the method could distinguish between different strains of *B. cereus* and also between the water types tested based on the composition if the microbial background flora (Hansen et al., 2013) .

In this study, the direct sequencing approach (Hansen et al., 2013; Skånseng et al., 2007; Zimonja et al., 2008) was evaluated as a diagnostic tool for detection of *B. cereus* artificially inoculated in ten types of food and feed samples without prior cultivation. *B. cereus* was used as a model organism for *B. anthracis*, the causative agent of anthrax. Two different *B. cereus* strains were used, the F2085/9885/98 and NVH0597-99, previously found to have a close resemblance to *B. anthracis* (Fricker et al., 2011). Spectra generated from the sequencing of the samples were compared using principle component analysis (PCA). To further evaluate the method, samples inoculated with different ratios of *B. cereus* and *B. thuringiensis* were included in the analysis together with samples inoculated *Salmonella enterica* serovar Typhimurium.

2 Material and methods

2.1 Food and feed samples

Ten types of feed and food samples were used in this study (Wielinga et al., 2011). The samples consists of corn gluten meal (Belgium), whey powder (Belgium), wheat flour (Belgium), soybean flour (Belgium), heat-treated corn grain (Belgium), WPC35 (Ireland), whey powder (Ireland) together with low, medium and high heat skim milk powders (SMP, Ireland).

2.2 Bacterial strains

The strains used in this study are the two *B. cereus* strains, F2085/98 and NVH0597-99 (Fricker et al., 2011). Cells were grown in 8 ml of Luria-Bertani (LB) broth (Sigma, Brøndby, Denmark) at 37°C for 18 h. A 10×dilution series in 0.9% NaCl were made and the dilutions from 10^{-4} to 10^{-8} were plated in duplicates on Plate Count Agar (PCA, Oxoid, Greve, Denmark) and incubated over night at 37°C. The weighted average of the Colony Forming Units (CFU) was calculated. For further evaluation, strains of *B. thuringiensis* and *Salmonella* were used as well. *B. thuringiensis* subsp. *kurstaki* was cultured from the biological insecticide Di-Pel® WP (Valent BioSciences, the Netherlands) in LB broth (Sigma) for 18 h at 37°C. *S.* Typhimurium 4/74 (Wray & Sojka, 1978) was grown in 8 ml LB broth (Sigma) at 37°C for 18 h.

2.3 Evaluation of DNA extraction

2.3.1 DNA extraction

Five commercial extraction kits were evaluated in this study; PrepSEQ Rapid Spin Sample Preparation Kit (Applied Biosystems, Life Technologies, Taastrup, Denmark), prepGEM Bacteria (ZyGEM, VWR, Herlev, Denmark), PowerPlant Pro DNA Isolation Kit (Mo Bio Laboratories, Inc., Copenhagen Biotech Supply, Brønshøj, Denmark), PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Copenhagen Biotech Supply) and MasterPure DNA Purification Kit (epicenter, VWR). The kits use different methods to extract, recover DNA or total nucleic acids and to remove inhibitors from the samples.

The PrepSEQ Rapid Spin Sample Preparation Kit (Applied Biosystems, Life Technologies) is a column based pre-filtration technique. The standard protocol with proteinase K was used to perform the DNA extractions. The DNA was dissolved in 100 µl dH_2O.

The prepGEM Bacteria (ZyGEM, VWR) utilizes enzymatic extraction system. The protocols “DNA Extraction Using prepGEM Bacteria” and “Development Recommenda-

tions for a method to extract DNA from stool using prepGEM" were used to perform the DNA extractions. The final volume was 100 µl.

The PowerPlant Pro DNA Isolation Kit (Mo Bio Laboratories, Copenhagen Biotech Supply) combines bead beating and silica gel spin column technologies. The standard protocol was used to perform the DNA extractions with bead beating for 3 min at 1800 rpm. The DNA was eluted in 100 µl elution buffer supplied with the kit.

The PowerSoil DNA isolation kit (Mo Bio Laboratories, Copenhagen Biotech Supply) combines bead beating and silica gel spin column technologies. The standard protocol was used for the DNA extraction. The DNA was eluted in 100 µl elution buffer supplied with the kit.

The MasterPure DNA Purification Kit (epicenter, VWR) utilizes a precipitation methodology. The protocol "Lysis of Tissue Samples" was used to perform the DNA extractions. DNA pellet was dissolved in 100 µl TE buffer supplied with the kit.

The manufacturer's protocols were followed for each kit with one additional pre-treatment step prior the extraction. To compare the five extraction kits for their ability to isolate DNA from *B. cereus*, extractions were performed in duplicates on samples inoculated with 10^4 and 10^6 CFU/100 mg with either of the two *Bacillus* strains in the four matrices; soybean flour, SMP low heat, wheat flour and corn gluten meal (Wielinga et al., 2011). For the extractions using the kits PrepSEQ and MasterPure, 400-600 µl LB were added to the inoculated samples and mixed thoroughly. The samples were centrifuged at 200×g for 60 seconds and the supernatant was transferred to a new tube and used for the DNA extraction. For the extraction kits PowerPlant Pro and PowerSoil, the inoculated samples were added directly to the provided bead tubes, whereas for the prepGEM the inoculated samples were added the wash buffer supplied with the kit.

2.3.2 Evaluation of extraction by PCR

To evaluate the performance of the different extraction kits, PCR were performed on the extracted DNA. The DNA was amplified with universal 16S rDNA primers (Nadkarni et al., 2002) and PCR was thereafter performed according to Skånseng et *al.* (Skånseng et al., 2007) using 3 µl template. Visualization of PCR products were done by gel electrophoresis using a 1% agarose gel.

For performance of the different DNA extraction kits, Kruskal-Wallis test was used to estimate the effects of the matrices and extraction protocol. The R software (v. 2.15)

was used for the analysis (R Core Team, 2012). A p-value of < 0.05 was considered statistically significant.

2.4 DNA extraction from food and feed

2.4.1 Inoculation of samples

The ten feed and food samples were inoculated with one of the two *B. cereus* strains in the levels 10, 10^2, 10^3, 10^4, 10^5 and 10^6 CFU/100 mg, all in duplicates. In addition, *B. thuringiensis* was inoculated in whey powder (Belgium) in the levels 10, 10^2, 10^3, 10^4, 10^5 and 10^6 CFU/100 mg. Furthermore, whey powder (Belgium) was inoculated in the levels 10, 10^2, 10^3, 10^4, 10^5 and 10^6 CFU/100 mg with *B. thuringiensis* and *B. cereus* NVH0597-99 in the ratios 1:1 and 1:3, respectively. *S.* Typhimurium was inoculated in heat-treated corn grain and WPC 35 UF in the levels 10, 10^2, 10^3, 10^4, 10^5 and 10^6 CFU/100 mg. For overview of all samples see supplement Table S1. After inoculation, 400-600 µl LB was added to each of the samples and samples were mixed thoroughly. The samples were centrifuged at 200×g for 60 seconds and the supernatant was transferred to a new tube.

2.4.2 DNA extraction

The MasterPure DNA Purification Kit (epicenter) showed the highest efficiency and was selected for the further DNA extractions. DNA was purified from the supernatant and the final volume was 100 µl. The DNA was stored at -20°C until further analysis.

2.5 Direct sequencing and data processing

DNA from the samples was amplified with universal 16S rDNA primers (Nadkarni et al., 2002) and PCR was thereafter performed according to Skånseng et *al.* (Skånseng et al., 2007). The universally conserved primer U515F (Baker et al., 2003) was used for sequencing. The sequencing procedure was performed according to Skånseng et al. (Skånseng et al., 2007).

Sequences extracted from the direct sequencing spectra were aligned, trimmed and processed with BioEdit v7.1.11 (Hall, 1999). The generated data were imported into R v2.15 (R Core Team, 2012) and analyzed by PCA (Wold et al., 1987).

For length of the sequencing products, analysis of variance (ANOVA) was used to estimate the effects of the matrices and strains. Prior to the statistical analysis, the normal distribution of the data was confirmed by a qq-plot. The R software (v. 2.15) was used for the

ANOVA analysis (R Core Team, 2012). A p-value of < 0.05 was considered statistically significant.

3 Results and discussion

In this study, a metagenomics based direct sequencing approach for detection of unknown bacteria was evaluated on food and feed model samples artificial inoculated with *B. cereus*. The method has previous been evaluated on bottled water (Hansen et al., 2013). Developing the method for food and feed samples requires higher demands for pre-processing steps before the actual detection. This involves primarily finding a suitable DNA extraction method from the given matrices.

3.1 Comparison of extraction kits

Five different DNA extraction kits were evaluated on their ability to extract *B. cereus* DNA from four inoculated food and feed matrices without any optimization. The choice of DNA extraction kits were selected on prior experience, the price per extraction and processing time. The choice of the samples for the evaluation of extraction kit was based on the study by Wielinga et *al*. (Wielinga et al., 2011). These four matrices, i.e. corn gluten meal, wheat flour, soybean flour and SMP low heat, were the ones where the lowest DNA recovery was achieved. The performance of the different extraction kits was evaluated by PCR and visualized by gel electrophoresis, see Table 1. Of the five extraction kits evaluated, the MasterPure was the most efficient to extract *B. cereus* DNA from the inoculated samples as a PCR amplicon was observed for all matrices at both inoculation levels (Table 1). The PowerSoil kit performed the worst, as no PCR amplification was observed for any of the four matrices. The corn gluten meal and soybean flour seemed to the most difficult to extract DNA from, consistent with Wielinga et *al*. (Wielinga et al., 2011). The poor extraction from some of the matrices can originate from different sources; insufficient lysis of the cells and inhibitors from the matrix itself that not only hinders the extraction procedure but also in the PCR reaction. Both the matrices and protocol were found to have a significant effect on the PCR amplification ($P < 0.05$).

A reason for insufficient extraction of DNA and thereby poor detection is improper disruption of the cells, i.e. the release of DNA from the cell. The two kits PowerSoil and PowerPlant Pro are based on the same buffer systems and technology but in this study bead beating was performed in different ways with the two kits. With PowerSoil, the vortex adapter recommended in the protocol was used, whereas for the PowerPlant Pro, a standard bead beater was used. The PowerPlant Pro outperformed PowerSoil, giving PCR product for

three of the four matrices (Table 1) indicating that the bead beating procedure used for the PowerSoil kit might not be optimal for these samples.

The better performance of the MasterPure kit compared with the PowerPlant Pro kit could be caused by the left-overs of food/feed particles in the supernatant after the initial centrifugation step. The PowerPlant Pro kit is a column-based extraction where the nucleic acids are captured in the column after the disruption of the cell walls. If some food or feed particles are left in the lysate, the column can be clogged and thereby hinders the proceeding steps of the DNA extraction. The MasterPure kit, on the other hand, uses lysis buffers which to some extent not only lyse the cells walls but also digests the food/feed particles left in the supernatant. The lysis of food/feed particles together with the proceeding protein precipitation makes the MasterPure more able to handle particle contaminations in the sample.

Other things that might interfere with the detection are inhibitors left in the extracted DNA. DNA extraction methods can be evaluated on their ability to remove the inhibitors that might interfere in the downstream applications, i.e. PCR (Hedman & Rådström, 2013; Wilson, 1997). The lack of PCR amplication for some of the samples could be a result of PCR inhibition by carry-over of different compounds in the extracted DNA. In DNA extracts failing to yield visible PCR amplicons dilution of the extracts prior PCR amplification have been shown to lower the effect of the inhibitors on the PCR reaction (Eckhart et al., 2000; Scipioni et al., 2008). However, dilution of the DNA extracts did not result in additional visible amplicons for any of the extraction methods (data not shown). Furthermore, the dilution of the MasterPure extracts resulted in a lack of visible amplicons for the low concentrations. With low levels of initial contamination, dilution will decrease the concentration of PCR inhibitors while simultaneously also dilute the template level below the detection level. The MasterPure kit uses isopropanol for precipitation of the DNA, which has been shown to remove more inhibitors compared to other extraction methods (Hänni et al., 1995).

3.2 Survival of *B. cereus* after lysis

Due to the good performance of the MasterPure extraction kit, this was chosen as the extraction method for the evaluation of the direct sequencing approach on food and feed samples. One of the critical steps in the direct sequencing method is the DNA extraction, particularly the disruption of the cells. In order to evaluate the survival of the cells after the lysis step, the number of CFUs was determined after the lysis step. *B. cereus* F2085/98 showed higher survival (around 0.2%) after the lysis compared with *B. cereus* NVH0597-99 (less than 0.1%). In

another study, the detection level of *B. cereus* F2085/98 was found to be 10-fold lower compared with *B. cereus* NVH0597-99 (Hansen et al., 2013). This indicates that the *B. cereus* F2085/98 strain might be more difficult to disrupt compared with *B. cereus* NVH0597-99 strain. Even though the two *B. cereus* isolates are of very close resemblance, they do have some differences in their heat activation of the spores (Fricker et al., 2011). The spores of *B. cereus* F2085/98 are more heat resistant compared with those of *B. cereus* NVH0597-99 (Fricker et al., 2011), so if spores has been produced, DNA extraction would be more difficult for *B. cereus* F2085/98 giving a higher detection level.

3.3 Direct sequencing

The direct sequencing approach of a universally conserved 16S rDNA gene region (Trosvik et al., 2007) was used for classification of the total bacterial flora in ten food and feed samples artificially inoculated with *B. cereus*.

Out of the 276 samples inoculated with *Bacillus* sequenced, spectra were obtained from 162 samples (data not shown), which was used for further analysis. This gives a success rate of 61%. Comparing with the study by Hansen et *al*. 2013, where spectra of 85% of the samples were obtained, the success rate is lower. This might originate from the fact that bottled water used in that study is a rather clean matrix containing a minimum of inhibitors. In contrast, the food and feed samples used in this study have a more complex structure including fragments and different inhibitors that can interfere with both the DNA extraction and also the PCR reactions (Rossen et al., 1992). No sequences were obtained for the WPC 35 UF matrix for any of the *B. cereus* strains. This matrix contains 35% protein (Wielinga et al., 2011), which is higher compared with the other matrices. High protein concentrations are known to inhibit the PCR reaction (Rossen et al., 1992), which could explain the lack of sequence products for this matrix.

The length of the sequences varied from 130 to 215 nucleotides. The shorter sequence products could originate from inhibition of the amplification, as discussed above. The length seems to be dependent on the *B. cereus* strains inoculated in the samples as samples inoculated with *B. cereus* NVH0597-99 overall produced longer sequences compared with samples inoculated with *B. cereus* F2085/98 (data not shown). The matrix also seemed to influence the length of the sequences, which implies that inhibitors might be interfering with the sequencing reaction. An ANOVA analysis revealed that both the matrix and strain had a significant effect ($P < 0.05$) on the length of the sequence products.

The obtained sequences were searched in the Ribosomal Database Project (RDP) database (Cole et al., 2009) to verify if the sequences could be identified as *B. cereus*. Out of the 162 sequences, only five sequences were identified to belong to the class of *Bacilli*. The five samples originate from both low and high inoculation levels together with different matrices meaning that no final conclusion could be drawn.

3.3.1 Principal component analysis

The direct sequencing method has previous been evaluated on bottled water and was found to be able to distinguish between the two strains of *B. cereus*, i.e. F2085/98 and NVH0597-99, and different types of bottled water based on the composition of the background flora (Hansen et al., 2013).

The mixed spectra of the 162 samples were first trimmed to around 130 nucleotides, then transformed into frequencies of pentamers and PCA was performed (Rudi et al., 2006). The two first principle components (PCs) explained together 7% of the total variance in the data set, with PC1 representing 4%. The explained variance is very low compared with other studies using the same method (Arboleya et al., 2012; Hansen et al., 2013; Wang et al., 2009). The PCA plots are visualized in Figure 1 and Figure 2, where the different matrices are split in separate plots. When looking at the PCA plots (Figure 1 and Figure 2), there is a slight tendency of the *B. cereus* NVH0597-99 inoculated samples to be located further to the left in the plot, whereas most of the *B. cereus* F2085/98 inoculated samples are located to the right, indicating that the PC1 separates the strains. However, the separation of the two *B. cereus* strains seems to be influenced by the matrix, as a better separation of the strains are seen for the matrices corn gluten meal (Figure 1A), whey powder Belgium (Figure 1B) and heat-treated corn grain (Figure 1D) compared with the other matrices (Figure 1C and Figure 2A-C).

The samples inoculated with *B. thuringiensis* are distributed over the entire plot; however the highest inoculation concentration, i.e. 10^6 CFU/100 mg, is located in the upper left corner as the only ones of the *B. thuringiensis* inoculated samples (Figure 1B). The samples with mixtures of *B. cereus* NVH0597-99 and *B. thuringiensis* are located in the lower left corner of the PCA plot (Figure 1B). The *B. thuringiensis* does not separate from the *B. cereus* strains, indicating that the method might not be able to distinguish between them which properly is because of their close resemblance (Helgason et al., 2000; Rasko et al., 2005). The

sample inoculated with *Salmonella,* group together with the samples with mixture of *B. cereus* NVH0597-99 and *B. thuringiensis*.

The low variance explained by the PCs could be due to the short length of the sequences. The shorter the sequences are the less genetic information is gained. A relationship between sequence length and phylogenetic error has been reported (Pollock et al., 2002). The results show that the method could not provide reliable conclusions on the ability to distinguish or detect *B. cereus* in the food and feed samples.

4 Conclusion

The direct sequencing method was evaluated for the use as a fast identification method for a potentially unknown pathogen in food and feed samples, using *B. cereus* as a model. Five commercial DNA extraction kits were evaluated for extraction of *B. cereus* from four food and feed samples. The results demonstrate that the performance of the kits varied to a large extent. The PCA could not clearly distinguish between the two *B. cereus* strains and based on the sequences it was only possible to classify five samples as belonging to the class of Bacilli. The results show that the method could not provide reliable conclusions on the ability to distinguish or detect *B. cereus* in the food and feed samples. For the method to be useful in detection of bacteria in food and feed samples, additional optimization and validation are therefore needed.

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Table 1 The performance of the different commercial DNA extraction kits. The performance was based on the ability to extract and amplify DNA in PCR from *B. cereus* F2085/98 inoculated in food and feed samples.

Extraction method[A]	Corn gluten meal		Wheat flour		Soybean flour		SMP low heat	
	10^4	10^6	10^4	10^6	10^4	10^6	10^4	10^6
PrepSEQ	--[B]	--	++	++	--	--	++	++
prepGEM	--	--	-	++	--	--	--	--
PowerPlant Pro	--	--	++	++	++	++	++	++
PowerSoil	--	--	--	--	--	--	--	--
MasterPure	++	++	++	++	++	++	++	++

[A] Extractions were performed in duplicates

[B] Purified DNA from each sample was PCR amplified. +: positive PCR amplification; −: negative PCR amplification.

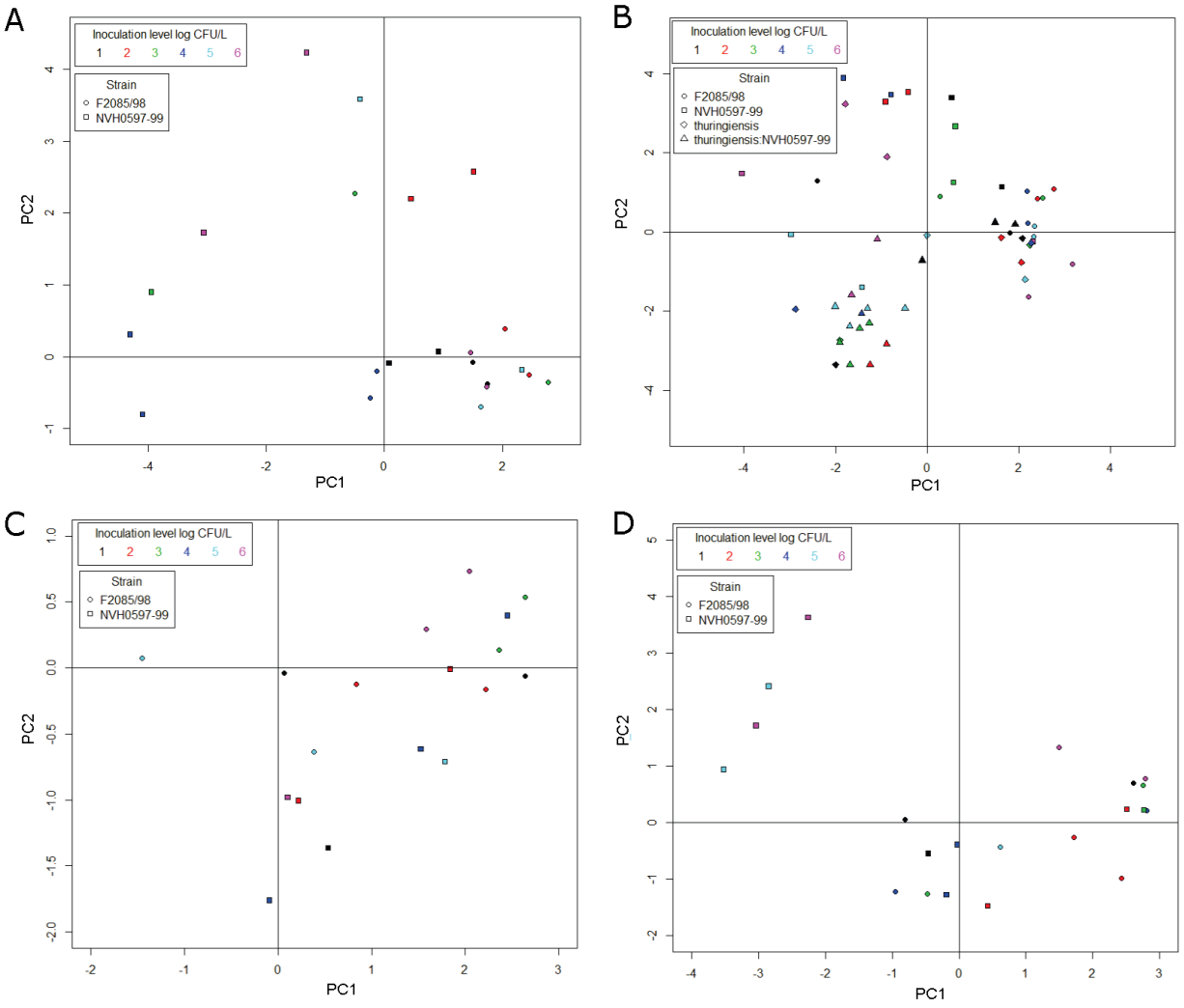

Figure 1 Principle component analysis (PCA) score plot. The principle component 1 (PC1) was plotted against PC2 for each matrix with A. Corn gluten meal, B. Whey powder (Belgium), C. Wheat flour and D. Soybean flour. The fill coloring of the samples represents the level of artificial inoculation and symbols the strain inoculated.

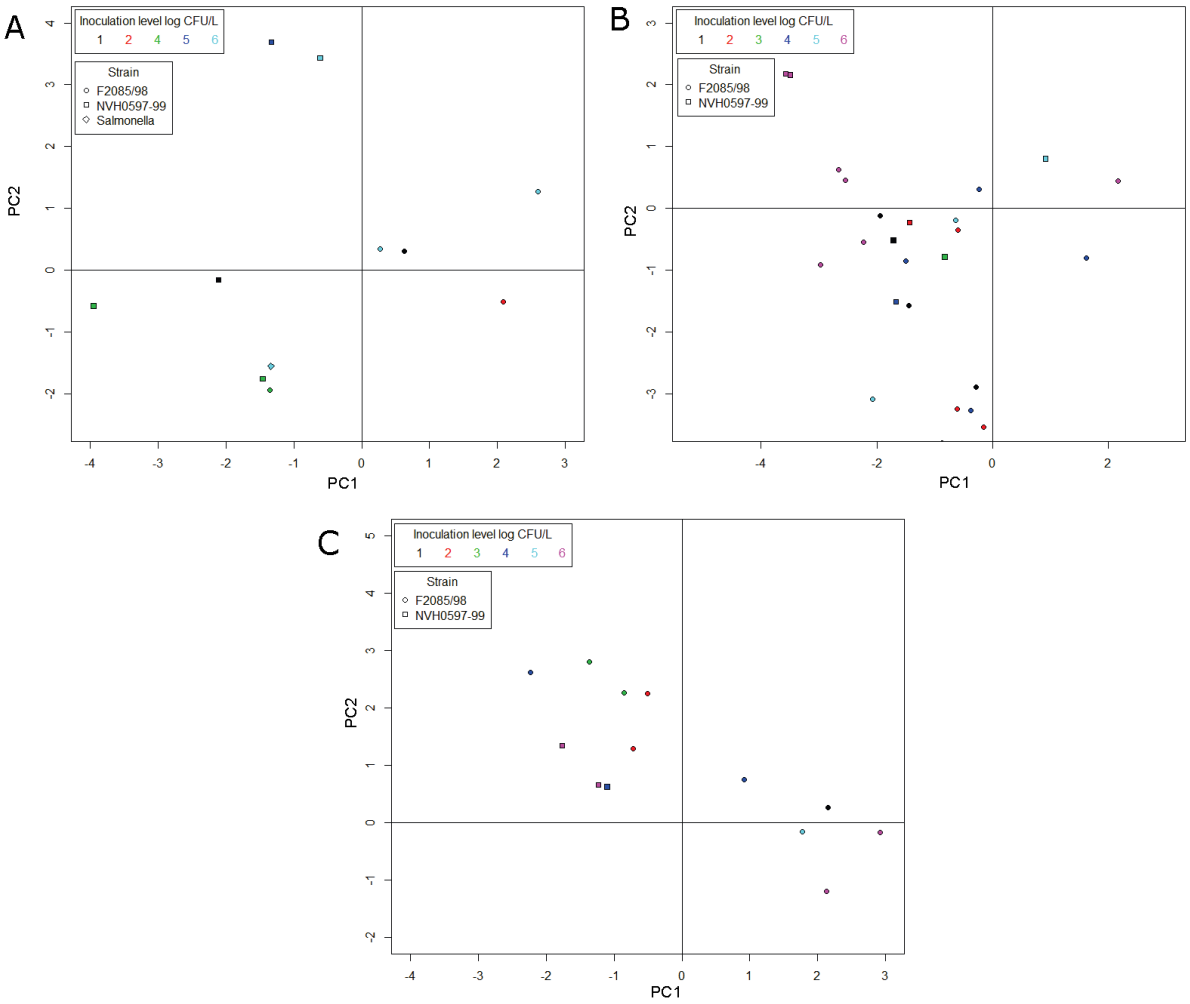

Figure 2 Principle component analysis (PCA) score plot. The principle component 1 (PC1) was plotted against PC2 for each matrix with A. Heat-treated corn grain, B. SMP high, medium and low heat, C. Whey powder (Ireland). The fill coloring of the samples represents the level of artificial inoculation and symbols the strain inoculated.

5 Supplementary data

Table S1 Overview of the samples used for this study.

Inoculation levels (CFU/100 mg)[A]	Strain	Matrix[B]
10, 10^2, 10^3, 10^4, 10^5, 10^6	*B. cereus* F2085/98	Corn gluten meal
10, 10^2, 10^3, 10^4, 10^5, 10^6	*B. cereus* F2085/98	Whey powder Belgium
10, 10^2, 10^3, 10^4, 10^5, 10^6	*B. cereus* F2085/98	Wheat flour
10, 10^2, 10^3, 10^4, 10^5, 10^6	*B. cereus* F2085/98	Soybean flour
10, 10^2, 10^3, 10^4, 10^5, 10^6	*B. cereus* F2085/98	Heat-treated corn grain
10, 10^2, 10^3, 10^4, 10^5, 10^6	*B. cereus* F2085/98	SMP high heat
10, 10^2, 10^3, 10^4, 10^5, 10^6	*B. cereus* F2085/98	SMP medium heat
10, 10^2, 10^3, 10^4, 10^5, 10^6	*B. cereus* F2085/98	SMP low heat
10, 10^2, 10^3, 10^4, 10^5, 10^6	*B. cereus* F2085/98	WPC 35 UF
10, 10^2, 10^3, 10^4, 10^5, 10^6	*B. cereus* F2085/98	Whey powder Ireland
10, 10^2, 10^3, 10^4, 10^5, 10^6	*B. cereus* NVH0597-99	Corn gluten meal
10, 10^2, 10^3, 10^4, 10^5, 10^6	*B. cereus* NVH0597-99	Whey powder Belgium
10, 10^2, 10^3, 10^4, 10^5, 10^6	*B. cereus* NVH0597-99	Wheat flour
10, 10^2, 10^3, 10^4, 10^5, 10^6	*B. cereus* NVH0597-99	Soybean flour
10, 10^2, 10^3, 10^4, 10^5, 10^6	*B. cereus* NVH0597-99	Heat-treated corn grain
10, 10^2, 10^3, 10^4, 10^5, 10^6	*B. cereus* NVH0597-99	SMP high heat
10, 10^2, 10^3, 10^4, 10^5, 10^6	*B. cereus* NVH0597-99	SMP medium heat
10, 10^2, 10^3, 10^4, 10^5, 10^6	*B. cereus* NVH0597-99	SMP low heat
10, 10^2, 10^3, 10^4, 10^5, 10^6	*B. cereus* NVH0597-99	WPC 35 UF

10, 10^2, 10^3, 10^4, 10^5, 10^6	*B. cereus* NVH0597-99	Whey powder Ireland
10, 10^2, 10^3, 10^4, 10^5, 10^6	*B. thuringiensis*:*B. cereus* NVH0597-99 (1:1)	Whey powder Belgium
10, 10^2, 10^3, 10^4, 10^5, 10^6	*B. thuringiensis*:*B. cereus* NVH0597-99 (1:3)	Whey powder Belgium
10, 10^2, 10^3, 10^4, 10^5, 10^6	*B. thuringiensis*	Whey powder Belgium
10, 10^2, 10^3, 10^4, 10^5, 10^6	*S.* Typhimurium	WPC 35 UF
10, 10^2, 10^3, 10^4, 10^5, 10^6	*S.* Typhimurium	Heat-treated corn grain

[A] All samples were run in duplicates

[B] The matrices are from Wielinga et *al*. 2011

Chapter 5

Discussion and perspective

5 Discussion and perspectives

The presence of microorganisms in foods is unavoidable and presents a significant threat to the public health worldwide. In order to accommodate the customer demand to food quality and food safety, improved methods for diagnostics of foodborne pathogens are essential. Moreover, methods that can detect and characterize foodborne pathogens present in food products or the food production chain are crucial for public health. Furthermore is rapid response to potential threats, either as a deliberately or accidently act, vital for the public safety. The focus of this thesis was to evaluate and optimize some of the current nucleic acid based methods for their potential application in detection and characterization of foodborne pathogens in order to improve food safety. The nucleic acid based diagnostic methods are gaining more and more attention due to their valuable advantages compared to the culture-dependent standard methods.

5.1 Characterization of *Salmonella* in pork processing

During the pork slaughtering process, the overall number of pig carcasses associated *Salmonella* decreased due to scalding, singeing and chilling. However, re-contamination of carcasses from insufficient cleaning of equipment accounts for approximately 30% of all *Salmonella* on pig carcasses (van Hoek et al., 2012).

The persistence of *Salmonella* in food production chains has been suggested to be a result of bacterial attachment and surface colonization (Swanenburg et al., 2001; Vestby et al., 2009). In addition, the physiological state of *Salmonella* has been shown to have an impact on the ability for *Salmonella* to attach to a pork meat surface (Paper I and Paper II), and subsequent the possibility for causing cross contamination in the slaughter-line. Cells that were grown immobilized prior application on the meat surface were found to be more easily removed (Paper I and Paper II). In the pork processing, *Salmonella* might appear in an immobilized state on the pork surfaces posing a risk for cross contamination. It was found that a *S.* Rissen isolate had the easiest to detach from the pork meat surface after immobilized growth (Paper II), which might explain a recurring contamination of *S.* Rissen on a carcass splitter in the pork slaughter-line found by van Hoek et *al.* (van Hoek et al., 2012). Additionally *S.* Rissen was the second most frequently isolated serotype from the carcasses at the end of the

slaughter-line (van Hoek et al., 2012). Lower attachment strength to a surface, whether it is meat or the production environment, gives rise to higher chance of cross contamination, whereas the stronger attachment to a surface makes decontamination steps more difficult. The results supports previous studies where loosely attach cells were more easily removed under decontamination (Tamblyn et al., 1997; Vestby et al., 2009). The results obtained in Paper I further showed that not only the growth state was important for the attachment potential but also the time of adhesion plays a role. An increase in contact time of the cells to a pork meat surface resulted in stronger attachment, which is consistent with related studies with *Campylobacter jejuni* (Nguyen et al., 2010). This implies that any decontamination strategies have to be applied within short time for decreasing the strength whereby the *Salmonella* might attach to the equipment, enabling better cleaning and thereby improving food safety.

Furthermore, was it found that the deletion of either of the two operons *flhDC* and *prg* in *S.* Typhimurium was accompanied by a decrease in attachment capability on a pork meat surface as planktonic cells (Paper I**)**. However, with immobilized cells the deletion of either operon lead to an increase in attachment strength compared with the wildtype. Conditions in the production environment that would alter the expression of these two genes could affect how decontamination steps should be performed. To further investigate how the growth state impacts the expression of attachment related genes, qRT-PCR analysis of the expression of six genes over a time course was conducted, comparing the expression in the knock-out strains relative to a wildtype strain (Paper I**)**. It showed that overall were most of the genes investigated down-regulated in the knock-out strains and continued to be for the three hours investigated. However, for full understanding of the impact of growth conditions on gene level, other genes should be investigated.

The use of qRT-PCR for gene expression studies as in (Paper I**)** has some drawbacks, since looking at multiple genes can be quite time-consuming and labor-intensive. In addition, the observed change in expression does not tell anything about the subsequent effect on the bacteria. With gene expressions studies, it is only possible to say what a particular condition does to the expression of the genes, but it cannot say anything about the functionally consequences it have on the cells. In addition, since not all bacteria have been investigated in depth, most gene expression patterns can only be compared with similar studies for other related microorganisms.

If specific genes can be connected to the source of isolation in the food production line, development of more optimized processing environments can be made, which eventually can decrease cross contamination, thereby increase food safety. DNA microarray based diagnostics allow for the comparison of a relatively large number of bacterial strains and to identify strain specific genes (Garaizar et al., 2006; Kostrzynska & Bachand, 2006). In Paper II, genotyping DNA microarray (Grønlund et al., 2011; Huehn et al., 2009) was applied for investigation of the use in tracing *Salmonella* in the slaughter-line based on the gene content of *Salmonella* isolates from a pork slaughter-line (van Hoek et al., 2012). Six genes were identified as being source specific, however when used for clustering, these failed in grouping by the different sources of isolation. The grouping was very much influenced by the serotype of the isolates. Also including attachment related genes, i.e. pathogenicity, fimbria and mobility genes, did not change the grouping of the isolates with regard to their serotype. One of the *S*. Derby isolates had stronger attachment ability on a pork meat surface compared with the other *S*. Derby isolates. The stronger attachment might be explained by the presence of a pathogenicity associated gene, *cdtB*, which plays a role in invasion and survival of *Salmonella* in host environments (Haghjoo & Galán, 2004; Skyberg et al., 2006). A *S*. Rissen isolate with low attachment ability was in contrast found to lack two fimbriae gene, *safC* and *lpfD*, involved in the adhesion to the intestinal epithelium (Weening et al., 2005). Even though the microarray utilized contains probes detecting genes associated with pathogenicity, antibiotic resistance, mobility, metabolism and serotyping (Huehn et al., 2009), it was not possible to identify specific genes that could distinguish the *Salmonella* isolates based on the different sources of isolation. The probes on the array cover a wide range of *Salmonella* genes; however, it still might not capture the true difference in the gene content of all the isolates investigated. As the detectable genes are restricted to the probes present on the array, it is not possible to identify new genes which mean that the information obtained can be limited. Thus, an alternative method for investigating the difference in gene content could be whole genome sequencing or microarray, which could be used for identification of novel genes specific for the strains, which then can be related to the isolation source.

In addition to knowing how the growth state might influence the persistence of *Salmonella* in the slaughter-line, understanding of how *Salmonella* response to stress conditions, such as pH and temperature, found in the production line is essential. By using whole genome gene expression microarray in Paper III it was possible to investigate the full tran-

scriptional response of *Salmonella* to temperature and acid variations. It was found that the heat shock was accompanied by an increase in resistance to heat and acid inactivation conditions. If *Salmonella* cells become more resistant after a treatment, e.g. temperature and acid, which commonly are used for decontamination in the slaughter-line, later decontamination steps might be more difficult and subsequently lead to a higher risk of contamination of the food products.

5.2 Direct sequencing approach for detection of *B. cereus*

For investigations of potential outbreaks, more generic methods are needed such as the metagenomics based direct sequencing approach evaluated in Paper IV and Paper V for identification of unknown pathogens. The method was first evaluated on bottled water due to the low complexity of the sample matrix and thereafter in food and feed model samples.

In Paper IV, a metagenomics based direct 16S rDNA target sequencing approach was evaluated as a diagnostic tool for screening of unknown bacteria, using *B. cereus* artificially inoculated in bottled water without prior cultivation as a model. The sequence data obtained was analyzed by PCA and MCR. The results revealed that the method was able to detect *B. cereus* at levels of 10^5-10^6 CFU/L. Consequently, the method was found to be a good candidate as a method for detection of *B. cereus*, and for screening of other bacterial contaminants, in water samples. This is further supported by the fact that the detection limit found for the method is in the same range as other published PCR methods for detection of *B. cereus* (Fricker et al., 2007; Oliwa-Stasiak et al., 2010). In addition, it has been shown that samples implicated in *B. cereus* outbreaks contain $>10^3$ CFU/ml or g food (Ehling-Schulz & Messelhäusser, 2012), which would be detectable using this method. From the PCA analysis, it was seen that the two strains of *B. cereus*, used in the study, could be separated at high inoculation concentrations despite their close sequence resemblance. The results in Paper IV also revealed that background flora in the bottled water used in the study varied between the different water suppliers. This is in accordance with previous reports (Casanovas-Massana & Blanch, 2011), showing that the difference in background flora could be connected to the difference in the water producing country. The different water types were found to affect the

detection level of *B. cereus,* which could originate from a difference in the chemical composition of the water.

The direct sequencing approach is based on total DNA extraction from the samples of interest and therefore is the initial DNA extraction a key factor that in the end can affect the results of the analysis, particularly the detection level. The bottled water analyzed in Paper IV is a rather clean matrix without many inhibitors. However, some inhibitors seemed to be present based on the results obtained, e.g. different detection levels of *B. cereus* were observed depending on the water supplier. This variation seemed to be linked to the chemical composition of the water samples, particularly the concentration of Mg^{2+} and Ca^{2+}. Excess of Mg^{2+} and Ca^{2+} are known to inhibit PCR amplification (Bickley et al., 1996; Rossen et al., 1992) and it was observed that the water samples with the highest concentration of these two compounds, had the highest detection level.

The capability of the method applied in Paper IV, was further evaluated on food and feed samples of various types in Paper V. As food and feed samples are more complex matrices compared with water, an optimization of the initial DNA extraction was applied in Paper V. With complex matrices, problems can arise in the extraction step such as cell lysis efficiency and quality of the extracted DNA, which both are of highest importance for the performance of the method. Different extractions methods have their own distinctive limitations. Many extraction kits are often only effective on one or a limit number of matrices, which was observed in Paper V. Of the five DNA extraction kits evaluated on food and feed samples in Paper V, only one of them was able to extract DNA of high enough quality for producing a PCR amplicon for all the tested matrices. Another factor that can affect the method's capability is unfavorable substances in the DNA extracts. Water, food and feed all contains substances that can inhibit the PCR amplification. Another critical step in the DNA extraction is the proper lysis of the cells. In Paper V, the survival of the *B. cereus* after the lysis step was investigated. Only a limited number of *B. cereus* cells survived the lysis step, however, a difference between the two *B. cereus* strains, F2085/98 and NVH0597-99, was observed. The survival of *B. cereus* F2085/98 seemed to be slightly higher compared with *B. cereus* NVH0597-99. The higher survival is consistent with the higher detection level of *B. cereus* F2085/98 seen in Paper IV, even though different lysis protocols were applied. Even though the DNA extraction protocol works on the vegetative cells of *B. cereus*, the lysis of the

spores produced by *B. cereus* are more difficult (Driks, 2003). This might pose a problem if the method has to be applied on spores and therefore this has to be evaluated further.

The application of the direct sequencing approach in Paper V seemed more difficult. The separation of the two *B. cereus* strains was not as clear as seen in Paper IV, which seemed to be affected by the different matrices. In addition was it only possible to detect *Bacillus* in a few of the artificially contaminated samples. The influence of the matrix and the low detection of *Bacillus* indicates the importance of a proper DNA extraction, as consistent with other studies (Dauphin et al., 2009; Gulledge et al., 2010; Wielinga et al., 2011).

The separation of the two *B. cereus* strains by the PCA visualization, particular seen in Paper IV, but also some indications in Paper V, indicates that the method has the possibility for distinguishing of *B. cereus* from other closely related species such a *B. anthracis* and *B. thuringiensis* which normally pose a problem (Helgason et al., 2000; Rasko et al., 2005). Even though 16S rDNA gene sequences can be used for species identification, there are some limitations in discriminating closely related species, due to high sequence similarity (Chakravorty et al., 2007; Fox et al., 1992). Other studies have shown that the 16S-23S rDNA intergenic spacer region could be an alternative to the 16S rDNA gene as it has higher sequence variation (García-Martínez et al., 1999; Xu & Côté, 2003). With the progress and lowering in cost of whole genome sequencing, this technique might take over the species identification (Didelot et al., 2012).

In outbreak situations, where the identity of the agent might not be known, methods such as PCR and microarray fail in rapid identification as a larger number of assays and runs are needed in order to determine the actual species, which can be laboratory demanding and time-consuming. Even though mixed-genome microarray has been developed for identification of multiple pathogens at the time (Wilson et al., 2002), it is still rather laboratory demanding with extraction of DNA, labeling and hybridization (Kostrzynska & Bachand, 2006). The technique used in Paper IV and Paper V might be useful as it does not require prior knowledge about the agent. However, considerations have to be taken in order to avoid bias caused by the DNA extraction. In addition, if an outbreak is caused by viruses or toxins, the method will not be able to detect it.

5.3 Concluding remarks

The work present in this thesis focuses on characterization and detection of *Salmonella* and *B. cereus* in relation to improve food safety by application of molecular diagnostic methods. The study contributes to the better understanding of the behavior of *Salmonella* in the pork processing and which factors that might influence the persistence and adaption. This information can be used for control of *Salmonella* by contributing to developments of more specific control measures and treatments within the food production-line and thereby improve food safety. In addition, a method for direct detection of *B. cereus* in different biological matrices was evaluated and found promising with the potential to be adapted for screening of any bacteria. This makes the method useful in outbreaks situations where the causing agent might be unknown.

The field of molecular diagnostics of foodborne pathogens is constantly moving forward in further optimization of existing technologies and the development of new methods. The future trends in detection methods points towards lab-on-chip systems which integrates the process from DNA extraction to analysis within one small instrument. The advantage of lab-on-chips is the possibility for portable devices for on-site detection, enabling faster detection, increasing the food safety and quality. Another technique that will be used more frequently is whole genome sequencing. The use of whole genome sequencing has already shown its potential in detection of foodborne pathogens, but it will keep moving rapidly forward with respect to the use in food safety as the price for sequencing are going down and the technology continues to be further developed.

Chapter 6

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6 References

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