Attachment of Salmonella spp. to pork meat

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Abstract
Five strains of Salmonella, one wildtype and four knock-out mutants (the prg, **f**hDC, yhjH and **f**iC genes) were investigated based on their probability to attach and subsequently detach from a surface of pork fillet. The attachment followed by detachment was measured and modelled for two different contact times using cells coming from either a planktonic or an immobilized state of growth. The results showed that the probability of detachment generally decreased when the contact time increased and that the highest difference between contact times was achieved when the cells were grown planktonic.

Introduction
Severe infections caused by Salmonella contaminated food products still pose a threat to human health. A critical step in transferring Salmonella from animals to the consumer is usually through the slaughter process where contamination is almost unavoidable. A better understanding of the behaviour of Salmonella in food production environments is needed for optimization of the production and thereby minimizing the contamination. Attachment is an important prerequisite for adhesion and persistence of Salmonella in the food production chain. The adhesion of bacteria to a surface is in**f**uenced by many factors such as surface composition, roughness, charge and hydrophobicity of both the surface and the cells (Tresse et al. 2007). It is also affected by cell surface structures such as **f**agella and fimbriae (Dickson and Koohmaraie 1989, Li and Mclandsborough 1999). The surface structure typically results in the formation of colonies due to the immobilization of the bacteria. Previous studies have shown that certain bacteria under stressed conditions grow differently when they are immobilized as colonies, compared to their behaviour during planktonic growth (Brocklehurst et al. 1995, 1997). Using the IFR Gel Cassette System (Brocklehurst et al. 1995), which allows the study of immobilized cells, it is possible to mimic growth on biological surfaces so that growth in food (as immobilized cells) and planktonic growth (cells in solution) can be compared under controlled conditions.
In this study, the Gel Cassette System was used together with a developed meat surface model in order to study whether Salmonella immobilized as colonies reacts differently according to the probability of detachment when transferred to a surface of pork meat compared to cells grown planktonic. From a wildtype Salmonella serotype Typhimurium, four knock-out strains (deletion of the prg and **f**hDC operon and the yhjH and **f**iC genes; all involved in the **f**agellum biosynthesis pathway) were constructed. The knock-out strains were investigated and compared to the wildtype strain with respect to their ability to detach from a pork fillet surface during a blotting series.

Material and Methods
Strains
Strains used in this study are listed in Table 1.The deletion of the prg operon and the yhjH gene were made in Salmonella serotype Typhimurium 4/74 as described by Datsenko and Wanner (2000).

Table 1. List of strains

Salmonella Typhimurium Strain	Origin
4/74	Supplied from Gitte Knudsen (Bowden et *al.* 2010)
4/74::Δ*prg*::kan^R	This study
4/74::Δ*yhjH*::kan^R	This study
4/74::Δ*fliC*::chor^R	Supplied by Maj-Britt Nielsen, University of Copenhagen
4/74::Δ*flhDC*::kan^R	Supplied by Maj-Britt Nielsen, University of Copenhagen

Preparation of inocula

Bacteria were grown in 8 ml of Luria-Bertani (LB) broth at 25°C for 24 h. An 100× dilution were made in 8 ml LB and the bacteria were grown again at 25°C for 24 h. Inocula for the cassettes were prepared by a 1000× dilution of the culture in maximum recovery diluent (MRD), which contained 1 g peptone (Fluka) and 8.5 g sodium chloride (Sigma) dissolved in 1 litre, pH 7.0. Two different media were used for the gel cassettes; LB media (for planktonic growth) and LB with 29.3% pluronic (for immobilized growth).

Cassettes

The Gel Cassette System for immobilized growth of bacteria was obtained as a kit 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 containing bacteria culture was transferred into the cassettes by a sterile pipette. The filled cassettes were incubated at 25 °C for either 16 h (LB) or 18.25 h (LB with pluronic).

Sterile meat pieces. Pork fillet was chosen as a model surface and obtained from a local retailer. The packages were sprayed with 70% ethanol, opened and the fillet was scalded with boiling water. Slices of meat were cut from the fillet to a desired thickness (approximately 1 cm) under sterile conditions. Pieces of meat were punched out with a meat stamp with a diameter of 30 mm.

Detachment

After incubation, 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 and blended in a Stomacher Lab Blender (Seward) for 1 min at high speed. 50 ml of the suspension were spun down at 6500 rpm for 7 min at 10°C and the resulting pellet was dissolved in 6 ml cooled MRD. An 250× dilution were made in MRD and 100 µl of this dilution were spread onto the surface of two meat pieces and incubated for 2 and 60 min at room temperature. After incubation, the meat piece was transferred to a beaker containing 100 ml MRD and was shaken for 1 min at 250 rpm. The meat piece was transferred to an XLD plate (Oxoid, Basingstoke, UK) 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 and so on for a total of 16 plates. After each move, the liquid remaining on the plate surface was spread out. The plates were incubated at 37°C for 24 h and colonies were counted. The detachment rate was calculated as described by Garrood et al. (2004). In short, the log10CFU/plate was plotted against the plate number and the detachment rate calculated from the slope of the resulting linear relationship.

Results

The effect of contact time and preceding growth conditions on the numbers of bacteria detaching from the surface of a pork fillet by a blotting series are shown in Table 2. From the data, the slopes (x) and coefficients (R2) of the straight lines representing the probability of detachment from the surface of a pork fillet were obtained by linear regression (Table 2).

All mutant strains, except the **Δf**iC strain, demonstrated a detachment probability that decreases when the contact time was increased. The largest differences between 2 and 60 min for all strains (except the wildtype strain, were no difference was seen) was observed when the cells have grown planktonic before being applied to the meat surface. There was a significant difference in the detachment rate for the different contact times for the two knock-out strains **Δ**prg and **Δf**hDC, with the **Δf**hDC having the highest difference, when they were grown planktonic and they also differed from the wildtype.

Table 2. Coefficients describing the detachment probability of different Salmonella Typhimurium strains to pork fillet

Strain	Contact time (min)	Planktonic		Immobilized	
		x[a]	R²[b]	x	R²
4/74	2	0.045 (0.028-0.062)[c]	0.737	0.062 (0.042-0.082)	0.810
	60	0.045 (0.033-0.058)	0.839	0.078 (0.068-0.089)	0.962
4/74::Δ*prg*	2	0.125 (0.104-0.146)	0.933	0.063 (0.052-0.073)	0.931
	60	0.088 (0.077-0.099)	0.963	0.065 (0.053-0.077)	0.922
4/74::Δ*yhjH*	2	0.063 (0.045-0.081)	0.825	0.090 (0.068-0.113)	0.914
	60	0.056 (0.041-0.070)	0.843	0.075 (0.048-0.102)	0.772
4/74::Δ*fliC*	2	0.075 (0.053-0.097)	0.883	0.063 (0.039-0.087)	0.752
	60	0.098 (0.063-0.132)	0.839	0.085 (0.066-0.104)	0.886
4/74::Δ*flhDC*	2	0.177 (0.151-0.203)[d]	0.989	0.058 (0.029-0.087)	0.671
	60	0.089 (0.063-0.115)	0.853	0.054 (0.030-0.078)	0.743

[a] *Probability of detachment of a bacterium from a meat surface during a single blotting event*
[b] *Coefficient of determination of the linear fit to the data, with 1 being perfect fit to the data*
[c] *An uncertainly estimate interval using a 95% confidence interval*
[d] *Data only based on the first 6 plates*

Discussion

In the present study, it has been demonstrated how different strains with deleted attachment genes detach from a pork surface when they have been immobilized in gel compared to planktonic growth before addition to the meat surface using different contact times. When applying cells that has been grown in a planktonic state, an increase in contact time decreased the probability of detachment, indicating an increasing difficulty in detaching the cells from the meat surface, which consists with previous reports. The detachment probability of Listeria monocytogenes from potato decreased over the first 2 min but then remained constant up to 60 min (Garrood et al. 2004). Another experiment with the probability of detachment of Campylobacter jejuni from stainless steel showed that the detachment decreased when the contact time increased (Nguyen et al. 2010).

However, in the experiments with cells being immobilized before contact with the meat, no significant differences were found between the detachment probabilities for the two contact times. Furthermore, the Δprg, Δ**f**iC and Δ**f**hDC strains had lower detachment rates than the one for the planktonic growth, in contrary to what was seen for the wildtype and ΔyhjH. There were no significant differences in the detachment probabilities for the Δprg, Δ**f**iC and Δ**f**hDC strains compared to the wildtype. This result could indicate that other attachment genes have been up-regulated during immobilization in compensation for loss of prg, **f**iC or **f**hDC. The up-regulation of other attachment genes might be a process that takes some time, especially for the Δprg and Δ**f**hDC strains, which can be noted when comparing to the data for the planktonic growth. The data for the wildtype and ΔyhjH strains suggests that the attachment genes might already be down-regulated when they are applied to the meat surface, and therefore are the following attachment a bit weaker due to a slower start on expression. This correlates with the findings of Wang et al. (2004) that found transcription of genes involved in the **f**agellum biosynthesis to drop after 4 h, when Salmonella was grown on 0.6% agar. In this study cells have been immobilized for 18.25 h before being applied to the meat.

The planktonic growth of the Δprg and Δ**f**hDC strains, both have detachment probabilities that are significant different from the wildtype strain. This finding correlates with previous experiments where it has been shown that the **f**hDC operon is the activator of the **f**agellum biosynthesis pathway which includes prg, **f**iC and yhjH (Frye et al. 2006). However on basis of this knowledge, a larger difference would have been expected for the Δ**f**hDC strain. The prg operon being dependent on the activation of **f**hDC, explains the lower detachment probability for the Δprg strain compared to the Δ**f**hDC strain. Studies have shown that the entire prg operon is required for the process of assembling the **f**agella (Kimbrough et al. 2000), which can explain the higher detachment probability seen.

Conclusion

The results indicate that an increase in contact time of the cells to the meat surface result in better attachment. This tendency seems to be of higher impact for planktonic cells compared to immobilized cells. Deletion of the prg and **f**hDC operons has the highest in**f**uence on the detachment probability compared to the wildtype when grown planktonic. To further investigate these findings, real-time PCR are being used to look at the gene expression of seven selected attachment genes in the knock-out strains. This is to see if there is a change in the gene expression in the knock-out strains compared to what is seen in the wildtype. The findings of changed detachment properties in the Δprg and Δ**f**hDC strains can used for further investigations in which factors that can reduce the gene expression. This knowledge can then be transferred to the industry and thereby be used for lowering the contamination of Salmonella in the slaughter process.

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