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LPS structure influences protein secretion in *Salmonella enterica*

Running title: *Salmonella* LPS mutants and protein secretion

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Abstract

In this study we have compared protein secretion in the wild type of *S. Typhimurium* and the *rfaC* mutant. We found out that the *rfaC* mutant was defective in protein secretion. In addition, the *rfaC* mutant was defective in its invasion into an IPEC-J2 porcine epithelial cell line and also in motility in semisolid agar. Consistent with this, reduced flagella numbers were observed in the *rfaC* mutant. In the *rfaC* mutant, there were no defects in flagellin expression as detected by western blot and immune electron microscopy which demonstrated equal amounts of flagellin in the cytoplasm of both the *rfaC* mutant and the wild-type *S. Typhimurium*. However, in the wild-type strain only, the flagellin was assembled to spatially restricted areas on the inner side of cytoplasmic membrane. The oligosaccharide core of LPS is therefore required for the assembly of flagella and T3SS secretion machinery followed by protein secretion.

Keywords

Salmonella; LPS; *rfaC*; protein secretion; motility; SPI-1

Introduction

Salmonella enterica is a pathogen of many bird and mammalian species including humans. Its pathogenicity is dependent on type III secretion systems (T3SS) and nearly all the proteins found in nutrient rich media after *S. enterica* culture are those transported by T3SS (Komoriya et al., 1999). There are 3 different T3SS in *S. enterica* (Hautefort et al., 2008). The first one is used for the transport of flagellar proteins and the assembly of flagella on the surface of bacterial cells. The remaining two T3SS are tightly associated with *S. enterica* virulence. The T3SS encoded by *Salmonella* pathogenicity island 1 (SPI-1) is involved in *S. enterica* invasion into epithelial cells (Kaniga et al., 1995) and the last T3SS encoded by *Salmonella* pathogenicity island 2 (SPI-2) is involved in intracellular survival of *S. enterica* (Cirillo et al., 1998).

To protect humans or farm animals against *Salmonella* infections, different live or inactivated vaccines have been tested. Besides the vaccination against *Salmonella* infections, *S. enterica* is also frequently used as a vector for the expression and delivery of heterologous antigens (Panthel et al., 2005). However, while the attenuation and immunogenicity have been tested experimentally many times, the pattern of protein secretion has not been addressed thoroughly. It is known that mutants defective in the synthesis of full-sized LPS show impaired motility (Parker et al., 1992; Karasova et al., 2009) which may indicate a defect in the secretion of flagellar proteins. In this study we were therefore interested in the secretion of flagellar and SPI-1-encoded T3SS proteins by *rfaC* mutant of *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*). This mutation conveys defect in the synthesis of the oligosaccharide core of LPS and therefore produce an oligosaccharide core free LPS (Parker et al., 1992; Sirisena et al., 1992; Genevaux et al., 1999). We found out that secretion of flagellar and SPI-1-encoded proteins required the presence of an intact oligosaccharide core

of LPS and that this defect is caused most likely by the inability to set up the T3SS translocation machinery.

Material and Methods

Bacterial strains and growth condition

S. Typhimurium strain LT2, its *rfaC* mutant and the *rfaC* mutant complemented with a recombinant plasmid encoding *rfaC* has been used in this study. The deletion of *rfaC* in *S. Typhimurium* was constructed exactly as described previously for *S. Enteritidis* (Sebkova et al., 2008). For complementation, the *rfaC* gene was amplified by PCR using forward (CTCGGCAAAAGACCATGAAG) and reverse (TCCACAATAGGTTTGGGATG) primers and the resulting PCR product was cloned into pCR2.1 using the TOPO cloning system according to the instructions of the manufacturer (pCR2.1 TOPO Cloning kit, Invitrogen). After selection by PCR using primers flanking the plasmid-insert junction, the pRfaC plasmid was electroporated into *S. Typhimurium rfaC* mutant. All the strains were stored in LB broth with 10% glycerol at -70 °C and when in use, the strains were grown in LB broth or LB agar at 37 °C supplemented with antibiotics if appropriate. Motility was determined by inoculation of 1 µl of 18-hour-old culture into the centre of Petri plate with 0.3% LB agar and by measuring the diameter of bacterial growth 8 hours later.

SDS-PAGE and protein identification

Protein secretion was determined by SDS-PAGE. All the strains and mutants were grown for 18 hours in LB medium at 37 °C to reach OD_{600nm} 0.8. To characterise total cellular protein, 120 µl of bacterial culture was pelleted by centrifugation (7000g, 15 min, 4 °C), resuspended in SDS-PAGE loading buffer (20% SDS, 25% glycerol, 0.5% β-mercaptoethanol, 0.06 M Tris-HCl, pH 6.8, 0.15% bromphenol blue), heated at 95 °C for 10 minutes and loaded onto

12% polyacrylamide gel. Secreted proteins were precipitated from 20 ml filtered (Millex GP filter 0.22 μ m, Millipore) bacterial culture supernatant with trichloroacetic acid (13% final concentration). After 1 hour incubation on ice, the precipitated proteins were centrifuged (7000g, 10 min, 4 °C) and washed twice with ice-cold acetone. Finally the protein pellet was air dried and dissolved in 200 μ l of 10% SDS. Total protein was determined by BCA assay and protein samples were diluted to 100 μ g/ml concentration. In the case of proteins secreted by the *rfaC* mutant we were unable to get proteins at a concentration of 100 μ g/ml and all the proteins available were therefore loaded onto polyacrylamide gel. SDS-PAGE was performed using Mini Protean II system (Bio-Rad) and gels were stained with Coomassie brilliant blue. Individual protein bands were cut out from the polyacrylamide gel and identified by MALDI-TOF/TOF exactly as described previously (Straskova et al., 2009).

Invasion tissue culture cells

The IPEC-J2 porcine epithelial cell line was grown on a 24-well microplate at 37 °C under 5% CO₂ atmosphere. When the cultures reached semiconfluent growth, *S. Typhimurium* and the *rfaC* mutant from the 6 hour old LB culture were added at a multiplicity of infection equal to 5. The numbers of adhered and invaded *S. Typhimurium* cells were determined by a standard gentamicin protection assay – counts of total adhered and invaded bacteria were determined 2 hours post *Salmonella* inoculation after lysing the cells with Triton X-100 (1% v/v) and the counts were expressed as percentage of the adhered bacteria out of inoculum. To determine the counts of invaded bacteria, gentamicin (final concentration 100 μ g/ml) was added to kill extracellular bacteria for 2 hours and after that, the tissue culture cells were lysed with 1% Triton X-100 to release intracellular bacteria. The released bacteria were serially diluted and plated on LB agar plates for overnight culture at 37 °C.

Western blot analysis

SDS-PAGE-separated proteins were electroblotted onto a HybondTM-P PVDF membrane according to the instructions of the manufacturer (Amersham, GE Healthcare). After blocking with 0.1 % Tween and 0.5% casein hydrolysate in PBS, the membrane was incubated with primary monovalent H:i antibody (Bio-Rad) diluted 1:100 in PBS for 1 hour. After washing with 0.1 % Tween in PBS, the membrane was incubated for another 1 hour with a secondary anti-rabbit antibody conjugated with horse-radish peroxidase. 3,3'-Diamino-benzidine-tetrahydrochlorid (Serva) was finally used for the colorimetric signal development.

Microarray analysis

Microarray chips were prepared by spotting 5' amino linker modified 70mer oligonucleotides covering both the *S. Typhimurium* and *S. Typhi* genomes (The Salmonella Genus AROS V1.0, Operon, Germany). The oligonucleotides were resuspended in Micro Spotting Solution Plus buffer (Telechem International, Inc., Sunnyvale, USA) and spotted onto Nexterion Slide E Epoxysilane Coated Substrate microarray glasses (Nexterion, Jena, Germany). For RNA preparation, both strains were grown statically in 10 ml of LB broth for 16 hours at 37 °C, bacterial cultures were pelleted and total RNA was purified using RNeasy Mini kit (Qiagen). At least 5 µg of RNA was reverse transcribed with Cy3 or Cy5-labeled dCTP and hybridised to the microarray slides, which were subsequently washed and scanned exactly as described previously (Sebkova et al., 2008). Expression profiles were obtained from 3 independent experiments, each performed in dye-swap design. Data were normalised using statistical software R (Wit and McClure, 2004). Only the spots flagged as of good quality were included into the analysis and were further examined with three approaches: i) t-test with subsequent q-value package for p-value multiple adjustment (Dabney and Storey, 2002), ii) *limma* linear modeling package (Smyth, 2004) and iii) *smida* package of Significance Analysis of

Microarrays (Tusher et al., 2001). Full raw microarray data have been deposited in the NCBI GEO database under accession number GSE19878.

Negative staining of bacterial cells from liquid culture

Bacterial strains from 18-hour-old LB cultures grown at 37 °C were centrifuged at 2500g and washed with 0.1 M HEPES pH 7.4. A single drop of culture was placed on formvar-coated copper grids and the bacteria were allowed to attach for 5 min. In the next step, the bacteria were stained with 1% ammonium molybdate and observed with a Philips EM 208 transmission electron microscope under an acceleration of 90 kV. At least 150 different cells were observed and the numbers of cells with zero, 1-2, and 3 or more flagella was determined.

Detection of flagellin in ultrathin sections

One ml of 18-hour-old culture was centrifuged at 2500g, washed with HEPES and the pellet was resuspended in 100 µl of 4% gelatine warmed to 45 °C. After gelatine solidification, each sample was cut into 1 to 3 mm³ blocks which were fixed with a mixture of 3% glutaraldehyde and 2% paraformaldehyde (1:1), and prestained with 0.25% ruthenium red for one hour. Samples were then dehydrated with acetone and embedded in Lowicryl K4M (Sigma). Embedded samples were UV photopolymerized for 2 days at room temperature and 100 nm ultrathin sections were prepared using a LKB ultramicrotome. The ultrathin sections were transferred to formvar-coated copper grids and incubated for 10 min in blocking solution (0.5M NaCl containing 0.1% BSA, 0.05% Tween and 5% fetal bovine serum, pH 7.5) for 10 min. The grids were first allowed to react with 10x diluted rabbit monovalent H:i antiserum (BioRad) for 20 min followed by rinsing 3 times with distilled water. In the next step, the grids were incubated for 20 min with a drop of anti-rabbit antibody conjugated with 10 nm

colloidal gold (Sigma) diluted 1:10 in blocking solution. Finally, the labeled ultrathin sections were stained with uranyl acetate and lead citrate and observed with a transmission electron microscope.

Results

Protein secretion in the rfaC mutant of S. Typhimurium

Major secreted proteins identified by MALDI-TOF/TOF in the wild type strain included SipA, FlgK, FliC, FliD, SipC, InvJ and FlgL, similar to Komoriya et al. (1999). On the other hand, none of the flagellar or SPI-1-encoded proteins were detected in culture supernatants of the *rfaC* mutant. Complementation with the pRfaC plasmid restored the ability of the mutant to secrete proteins, although the profile of the complemented mutant was slightly different from the secretion profile of the wild type *S. Typhimurium* (Fig. 1). Similar to observations of Sheng et al. (2008), complementation affected the size of secreted flagellin but we did not study this in more detail.

Motility and flagella expression

Since defects in secretion of flagellar proteins should correlate with defects in motility, motility in 0.3% agar was tested in next experiment. The *S. Typhimurium rfaC* mutant exhibited a defect in motility as the diameter of growth 8 hours post inoculation was around 20 mm while the diameter of the growth halo of the wild type *S. Typhimurium* reached values around 65 mm (Fig. 2). Complementation with pRfaC plasmid only partially restored motility of the *rfaC* mutant in 0.3% agar (Fig. 2) which could be caused by the overexpression of the RfaC from the multicopy plasmid pCR2.1.

In the next step we therefore studied the presence of flagella on the surface of the *rfaC* mutant directly by electron microscopy. Majority of the wild type *S. Typhimurium* expressed flagella on the bacterial surface whilst only around 8 % cells of the *rfaC* mutant expressed flagella and the number of non-flagellated cells in the *rfaC* mutant reached 92 %. Complementation with pRfaC plasmid fully restored flagella expression on the surface of the cell (Fig. 3). In addition, a distinct electron dense capsule was observed in the *rfaC* mutant, consistent with previous reports on the overexpression of capsule in rough mutants (Parker et al., 1992).

Cell invasion

Since defects in secretion of SPI-1-encoded proteins should correlate with defects in cell invasion, invasion of IPEC-J2 cell line was tested in the next experiment. The *rfaC* mutant tended to be more adherent to the IPEC-J2 cell line, consistent with our previous report (Matiasovic et al., 2011). The *rfaC* mutant of *S. Typhimurium* was also disabled in its invasion of the same cell line and this defect could be complemented with the pRfaC plasmid (Fig. 4).

Gene expression in S. Typhimurium rfaC mutant

Next we were interested whether the decrease in protein secretion could be caused by a decrease in gene transcription since reports of altered transcription in rough mutants have been published (Bauer and Welch, 1997; Nagy et al., 2006). To test this, we compared gene expression in the wild type *S. Typhimurium* and the *rfaC* mutant by microarray analysis. Although fluorescent signals of 3963 spots (out of 6144 spots present on chips) were classified by the ScanArray Express software as of good quality, none of them was differentially expressed in the wild type *S. Typhimurium* and the *rfaC* mutant after their 18-hour growth in LB broth.

Western blot analysis

The absence of protein secretion with a pattern of maintained transcription suggested that the secreted proteins may accumulate in the bacterial cytoplasm. To address this, we separated secreted and whole cellular proteins of *S. Typhimurium* by SDS-PAGE, and performed western blot analysis using anti-flagella antibodies. Western blot confirmed that significantly more FliC protein was secreted by the wild type strain than by the *rfaC* mutant, as expected based on a visual inspection of Coomassie blue stained polyacrylamide gels. However, when the FliC protein was detected by western blot in total cellular protein, less FliC was observed in the wild type *S. Typhimurium* than in the *rfaC* mutant (Fig. 5).

Immune electron microscopy of *S. Typhimurium rfaC* mutant

Our results suggested that the defect in the *rfaC* mutant was at the level of protein translocation across the inner and outer membranes. To test this, we finally analysed the spatial distribution of flagellin inside *S. Typhimurium* cells using ultrathin sections and immune electron microscopy. Specific gold-labeled antibodies confirmed that both the wild type *S. Typhimurium* and the *rfaC* mutant expressed flagella antigens since these could be detected in both these strains. However, while the flagellin in the *rfaC* mutant was randomly distributed along the inner surface of the cytoplasmic membrane, in the wild type *S. Typhimurium* the flagellin was clustered to spatially discerned loci (Fig. 6). This indicated that incomplete LPS of the *rfaC* mutant did not allow the flagellar machinery to set up properly and transport flagellin out of the cell.

Discussion

In this study we have shown that mutation in *rfaC* gene cause defects in protein secretion, motility or cell invasion in *S. Typhimurium*. Similar to many previous reports (Parker et al., 1992; Genevoux et al., 1999; Nagy et al., 2006; Sheng et al., 2008), but different from Toguchi et al. (2000) and Inoue et al. (2007), the *rfaC* mutant was defective in motility. The different results in the two studies are likely to be associated with a different strain background and particular experimental procedures used. Mutant in *rfaC* produce an oligosaccharide core free LPS (Parker et al., 1992; Sirisena et al., 1992; Genevoux et al., 1999) suggesting that the oligosaccharide LPS core is important for the assembly of flagella and protein secretion including cell invasion. Using microarray analysis, we excluded the hypothesis of significantly altered RNA transcription in the *rfaC* mutant, although this was surprising considering the many defects associated with *rfaC* mutations (Nagy et al., 2006; Karasova et al., 2009) and the fact that Nagy et al. (2006) determined the expression of *rfaH* and *rfaG* mutants in *S. Typhimurium* by microarray analysis and observed a decrease of SPI-1 and flagellar gene expression. We therefore checked the quality of spots containing the probes complementary to SPI-1 gene mRNA individually and even this additional verification step did not indicate any difference in the expression of these genes in the wild type *S. Typhimurium* and the *rfaC* mutant. We concluded that there is no or very small decrease in the expression of SPI-1 genes in the *rfaC* mutant, and that the differences in the results of microarray analysis can be explained by different culture conditions and time of growth prior to RNA purification when shaken cultures were used by Nagy et al. whereas we used a static overnight culture instead.

Using western blot analysis and immune electron microscopy we next excluded the hypothesis of significantly altered translation or protein stability in the *rfaC* mutant because western blot analysis clearly indicated that the amount of the FliC protein was either the same

or even slightly higher in the cytoplasm of the *rfaC* mutant than in the cytoplasm of the wild type *S. Typhimurium*. This was also confirmed by immune electron microscopy using ultrathin sections and we therefore excluded defects at the level of protein expression as a major reason for the aberrant behavior of the *rfaC* mutant in protein secretion. The only difference could be seen in the distribution of flagellin inside the cells of the wild type strain and the *rfaC* mutant. In the wild type *S. Typhimurium*, the flagellin was localised to several spatially restricted areas while no such clustering was evident in the *rfaC* mutant suggesting that the absence of flagellar protein secretion could be caused by the inability of flagellar transport apparatus to assemble correctly.

The presence of FliC in the absence of protein secretion is quite puzzling since based on the current knowledge of hierarchal control of gene expression in flagella assembly, the FliC should not be expressed before the antisigma factor FlgM is secreted out of a cell through a polymerised flagellar hook (Karlinsey et al., 2000b). The size of the LPS molecule is around 10 nm, of which 2.4 nm is the size of lipid A anchoring the LPS molecule into the outer membrane (Kastowsky et al., 1992). The flagellar hook extends approx. 50 nm from the bacterial surface (Moriya et al., 2006) and the SPI1 injectisome is of approx. the same size as the flagellar hook (Kubori et al., 2000). This means that if the injectisome or hook polymerisation was initiated, the process would continue above the LPS molecule and therefore independent of its structure. It is therefore likely that incomplete LPS did not allow initiation of injectisome or flagellar hook polymerisation immediately at the outer membrane of *S. Typhimurium* where it can be influenced by LPS structure. Furthermore, if the hook-basal body is not formed, FlgK protein cannot be secreted and may remain associated with the FlgN chaperone (Bennett et al., 2001). Interestingly, if the FlgN is associated with FlgK, it is not available for positive regulation of FlgM expression (Karlinsey et al., 2000a), which may

result in an expression of FliC. FliC can therefore be expressed and accumulate in the cytoplasm even in the tightly controlled flagella expression system, as has been documented for the double *flgG-L* and *flgN* mutant (Karinsey et al., 2000a).

Conclusion

In this study we have found that mutant in *rfaC* gene exhibited defects in protein secretion via T3SS. The oligosaccharide core of LPS was required for the assembly of flagella and the SPI-1 encoded secretion apparatus.

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Figure legends

Figure 1. Protein secretion in *S. Typhimurium* LT2, its *rfaC* mutant and the *rfaC* mutant complemented with pRfaC. Sizes of molecular weight marker (lane M) and proteins identified by MALDI-TOF/TOF are indicated.

Figure 2. Motility of *S. Typhimurium* LT2, its isogenic *rfaC* mutant and the mutant complemented with recombinant pRfaC plasmid in 0.3% LB agar. Since more strains were inoculated in a single plate for comparison, the assay in this figure was interrupted 3 hours post inoculation when the *rfaC* mutant still appeared as completely non-motile. However, after 8 hours of motility assay a small amount of growth similar to that described by Genevaux et al. (1999) was observed.

Figure 3. Presence of flagella on the surface of *S. Typhimurium*, *rfaC* mutant and the *rfaC* mutant complemented by recombinant pRfaC plasmid determined by electron microscopy (Panel A). Panel B, numbers of cells without any flagella (white columns), cells expressing 1 or 2 flagella (grey columns), and cells expressing 3 or more flagella (black columns).

Figure 4. Adhesion and invasion of the *rfaC* mutant of *S. Typhimurium* into an IPEC-J2 porcine epithelial cell line. Panel A, adhesion of *S. Typhimurium* *rfaC* mutant and the mutant complemented by the pRfaC plasmid. Panel B, invasion of *S. Typhimurium* *rfaC* mutant and the mutant complemented by the pRfaC plasmid. * - significantly different by t-test ($P < 0.05$) from appropriate wild type strain control.

Figure 5. SDS-PAGE (Panel A) and western blot analysis using anti flagellin H:i antibody (Panel B) of secreted and total cellular proteins in wild type *S. Typhimurium* (STM LT2 wt), *rfaC* mutant and *rfaC* mutant complemented with recombinant pRfaC plasmid. Lane M, molecular weight standard.

Figure 6. Immune electron microscopy in ultrathin section of the wild type *S. Typhimurium* (panel A) and its isogenic *rfaC* mutant (Panel B) using anti-flagella antibody. Unlike the *rfaC* mutant, in the wild type strain, spatially restricted clusters of flagellin indicated by arrows were observed.

STM LT2
rfaC
pRfaC

STM LT2
rfaC

STM LT2 wt

A

B

1 μm