

Article

Roles for *wbtC*, *wbtI*, and *kdtA* Genes in Lipopolysaccharide Biosynthesis, Protein Glycosylation, Virulence, and Immunogenicity in *Francisella tularensis* Strain SCHU S4

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Abstract: Using a strategy of gene deletion mutagenesis, we have examined the roles of genes putatively involved in lipopolysaccharide biosynthesis in the virulent facultative intracellular bacterial pathogen, *Francisella tularensis* subspecies *tularensis*, strain SCHU S4 in LPS biosynthesis, protein glycosylation, virulence and immunogenicity. One mutant, $\Delta wbtI$, did not elaborate a long chain *O*-polysaccharide (OPS), was completely avirulent for mice, and failed to induce a protective immune response against challenge with wild type bacteria. Another mutant, $\Delta wbtC$, produced a long chain OPS with altered chemical and electrophoretic characteristics. This mutant showed markedly reduced glycosylation of several known glycoproteins. Additionally this mutant was highly attenuated, and elicited a protective immune response against systemic, but not respiratory challenge with wild type SCHU S4. A third mutant, $\Delta kdtA$, produced an unconjugated long chain OPS, lacking a detectable core structure, and which was not obviously expressed at the surface. It was avirulent and elicited partial protection against systemic challenge only.

Keywords: *Francisella tularensis*; lipopolysaccharide; glycosylation; virulence

1. Introduction

The gram negative coccobacillus, *Francisella tularensis*, is a facultative intracellular bacterial pathogen of multiple mammalian species including humans [1]. Two subspecies, subsp. *holarctica*, and the more virulent subsp. *tularensis*, can cause severe infection, tularemia, in humans. In particular, disseminated infection, typhoidal tularemia, following inhalation of subsp. *tularensis*, had a mortality rate of 30%–60% in the pre-antibiotic era [1]. Consequently, subsp. *tularensis* was developed as a biological warfare agent [2]. During the past several years, multiple virulence factors of *F. tularensis* have been revealed [3,4]. Loss of virulence genes results in a spectrum of attenuations and abilities of the mutant strains to elicit a protective immune response against challenge with wild type strains. Our interest has been to develop attenuated strains of subsp. *tularensis* strain, SCHU S4, which can be used as live vaccines. To this end, we have produced approximately 100 deletion mutants over the past several years including mutants missing genes involved in lipopolysaccharide (LPS) biosynthesis. The latter are the subject of the present study.

F. tularensis elaborates LPS with a polymeric *O*-polysaccharide (OPS). The *O*-antigen repeat unit (–4–)- α -D-GalpNAcAN-(–1-4)- α -D-GalpNAcAN-(1-3)- β -D-QuipNAc-(1-2)- β -D-Quip4NFo-(1-)_n, is unique to subsp. *holarctica* and subsp. *tularensis*, and is distinct from the *O*-antigen of the related, but clinically irrelevant *novicida* subspecies [5,6]. Recently, it was shown that polymerization of the *O*-antigen occurs via a Wzy-dependent mechanism [7]. OPS is a critical virulence factor for the pathogen, possibly by conferring resistance to killing by serum or macrophages, or by allowing stealthy infection [8]. A putative *F. tularensis* OPS biosynthesis gene locus has been proposed consisting of genes *wbtA*-to-*wbtN* encoding protein homologs of sugar epimerases, transferases, dehydrogenases, and flippases known to be involved in LPS biosynthesis in other gram negative bacteria [9]. Mutants with defective *wbtA*, *wbtI*, *wbtM* genes and so called “grey variant” mutants of the empirically attenuated *holarctica* strain, LVS, lack polymeric OPS and are further reduced in virulence for mice [10–14]. In contrast, a *wbtC* transposon mutant of LVS expressed high molecular weight LPS with altered electrophoretic characteristics compared to wild type [13], but its relative virulence was not investigated. In subsp. *tularensis*, a Δ *wbtDEF* mutant failed to elaborate OPS and was highly attenuated [8]. In addition to OPS, LPS biosynthesis also requires conjugation of 3-deoxy-D-manno-octulosonic acid (KDO) to Lipid A to allow for subsequent attachment of the core region [15]. Mannose is the major sugar present in the *F. tularensis* LPS core [16].

The aim of the present study was to characterize the broad impact of three selected mutants in LPS biosynthetic genes upon bacterial virulence, immunogenicity as well as biochemical characteristics such as protein glycosylation and LPS biosynthesis. This study was carried out entirely in the highly virulent subsp. *tularensis* strain SCHU S4. The results show that genes from the LPS biosynthetic pathway, in addition to contributing to LPS biosynthesis have impact upon bacterial protein glycosylation, virulence and their potential for use as live vaccines.

2. Results

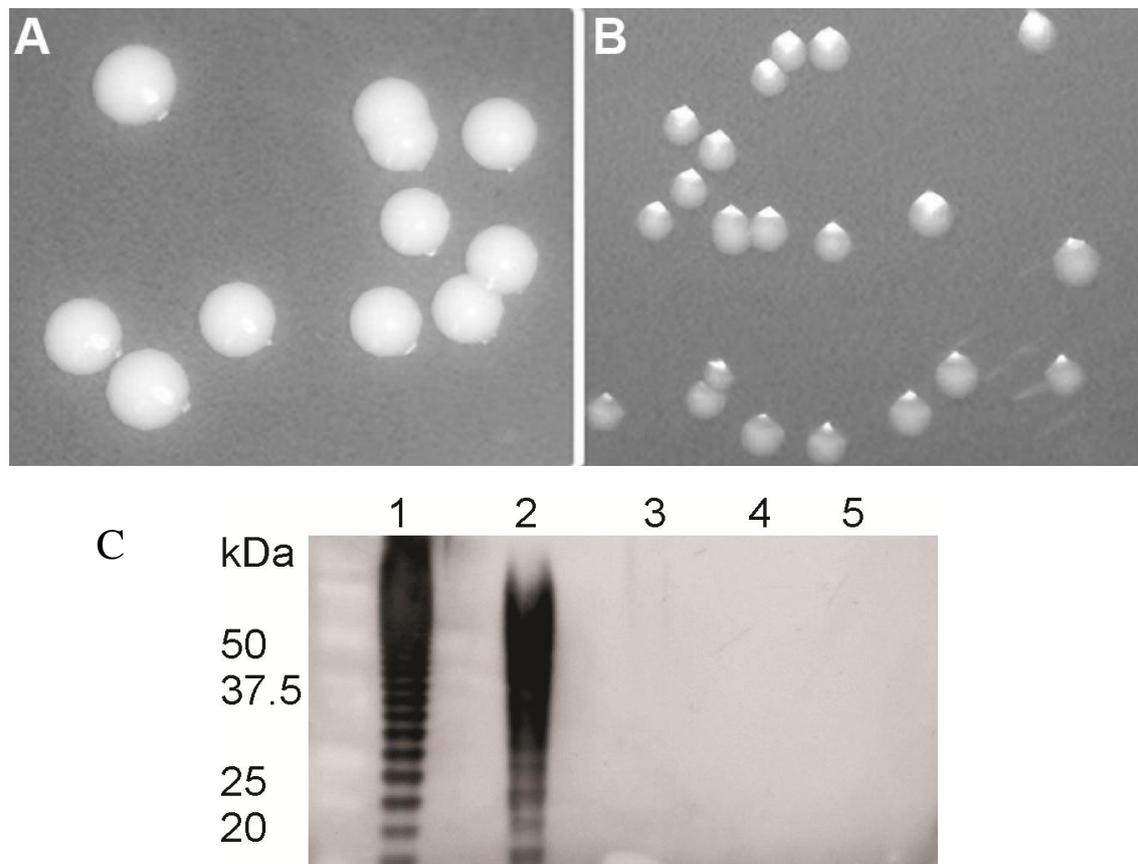
Deletion mutants in the current study included Δ *wbtI*, Δ *wbtC* and Δ *kdtA*. The gene product, WbtI, is annotated as a sugar transamine/perosamine synthetase, WbtC as a UDP-sugar epimerase and KdtA as

a KDO transferase. Immunological, biochemical and animal virulence studies were carried out to determine the broad impact of these gene deletions.

2.1. LPS Analysis

The mutants $\Delta wbtI$ and $\Delta wbtC$ had the same colony morphology as wild type bacteria on CHAH medium, whereas $\Delta kdtA$ colonies were flat and dry, and lacked the characteristic green halo that encompasses normal colonies on CHAH (Figure 1a and 1b). Additionally, the $\Delta kdtA$ colonies were much smaller, in keeping with our previous findings that this mutant grows more slowly in liquid culture [3]. Furthermore, it was recently shown that $\Delta kdtA$ was much more sensitive than wild type bacteria to killing by normal serum (Sjostedt, manuscript submitted). Moreover, specific rabbit antiserum raised against whole bacteria only agglutinated SCHU S4 and $\Delta wbtC$ (data not shown). Western blots were performed on purified LPS of SCHU S4, mutant strains, and *F. novicida* (Figure 1c). Only wild type and $\Delta wbtC$ LPS were reactive, with subtly different staining patterns. Note that, in our hands when purified using hot phenol method, unconjugated *O*-antigen is not observed.

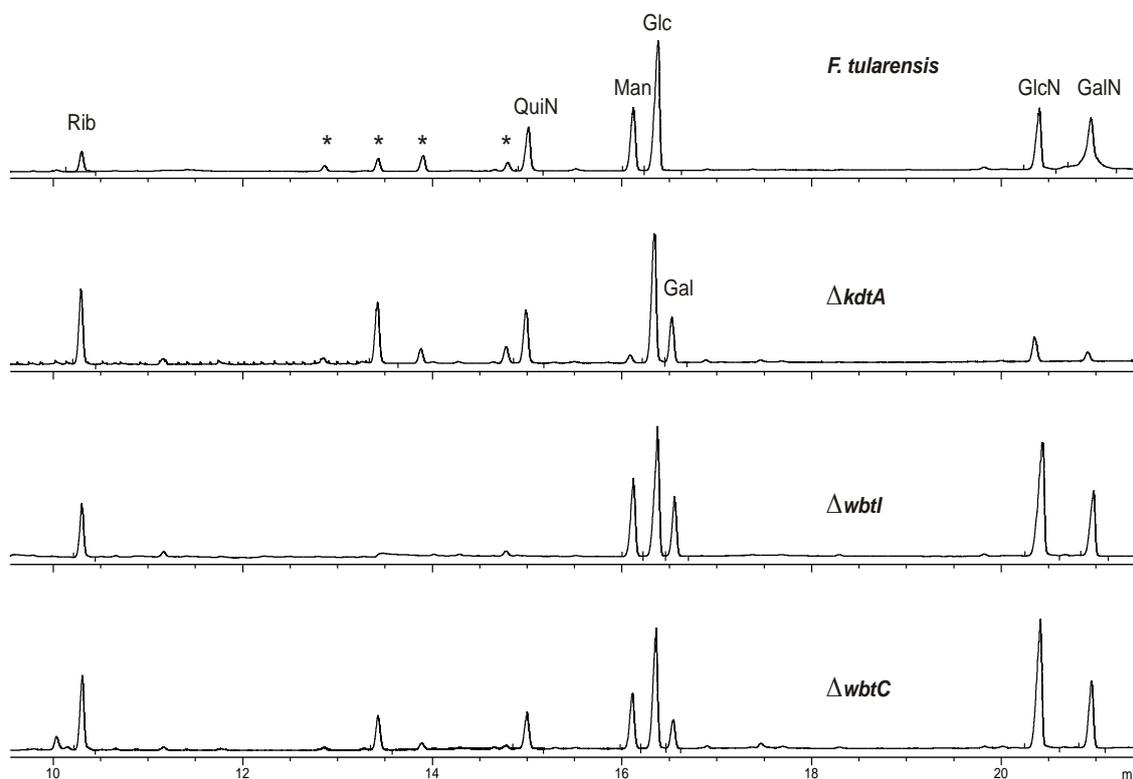
Figure 1. Colony morphology of wild type, (a) and $\Delta kdtA$ (b) *F. tularensis* SCHU S4 on CHAH medium. (c) Western blots of purified lipopolysaccharide (LPS) from SCHU S4 (lane 1), $\Delta wbtC$ (lane 2) $\Delta wbtI$ (lane 3) $\Delta kdtA$ (lane 4), and *F. novicida* (lane 5).



Next, chemical structural analyses were performed on the purified LPS. Gas chromatography (GC) analysis of sugar alditol acetates showed that LPS from wild type SCHU S4 and $\Delta wbtC$ contained all of the expected sugars. However, Qui4NFm (4,6-dideoxy-4-formamido-D-glucose), a major

component of normal OPS, was absent from $\Delta wbtI$, and mannose, a major component of the core, was essentially absent from $\Delta kdtA$, (Figure 2). This was confirmed by sugar analysis of whole bacteria (Figure S1). ^1H NMR analysis of OPS (Figure 3) showed wild type and $\Delta kdtA$ contained identical OPS structures, but $\Delta wbtC$ spectra contained some irregularities. No evidence of OPS was found in $\Delta wbtI$. In keeping with these data, mass spectrometry analyses of sugar alditol acetates from whole bacteria digested with proteinase K, DNAase, and RNAase showed again that mannose, an LPS core sugar, was essentially absent from the $\Delta kdtA$ (Figure S1). This demonstrated that this observation was not an artifact of the LPS isolation method employed. In addition, mass spectrometry analyses showed that each strain had a common lipid A structure, and SDS-PAGE showed that there was no *O*-antigen linked to it in either $\Delta kdtA$ or $\Delta wbtI$ (Figures S2 and S3).

Figure 2. Gas chromatography traces of alditol acetates, obtained after hydrolysis of LPS. $\Delta wbtI$ contained no QuiN, $\Delta kdtA$ contained a very small amount of mannose, but normal amounts of the QuiN. The $\Delta wbtC$ spectrum was similar to normal *F. tularensis*. Ribose probably originated from RNA contamination, glucose from contaminating glucans is always present in *F. tularensis* LPS preparations. All mutants contained galactose of unknown origin. Asterisks denote components with sugar-like mass spectra, but could not be identified.



Previous work by our group [6] has shown that the OPS repeat unit of *F. tularensis* has the composition QuiNAc (2-acetamido-2,6-dideoxy-D-glucose), Qui4NFm (4,6-dideoxy-4-formamido-D-glucose) and two moles of GalNAcAN (2-acetamido-2-deoxy-D-galacturonamide) or GalNAcAN-QuiNAc-GalNAcAN-Qui4NFm. An MS survey scan of OPS from SCHU S4 showed a prominent ion at m/z 793. MS/MS fragmentation of the monoisotopic ion at m/z 793 showed major fragment ions at

m/z 620, 404 and 217 (Figure 4a). These corresponded to sequential losses of monosaccharides Qui4NFm (173 Da, m/z 793–620), GalNAcAN (216 Da, m/z 620–404), QuiNAc (187 Da, m/z 404–217), with the oxonium ion peak of GalNAcAN observed at m/z 217. Several minor fragment ions corresponded to disaccharide components (for example m/z 433, 216–216) or losses of water (m/z 775).

Figure 3. ^1H NMR spectra of *O*-antigens from $\Delta wbtC$, $\Delta kdtA$ and wild-type SCHU S4 (40–45 °C, 500 MHz).

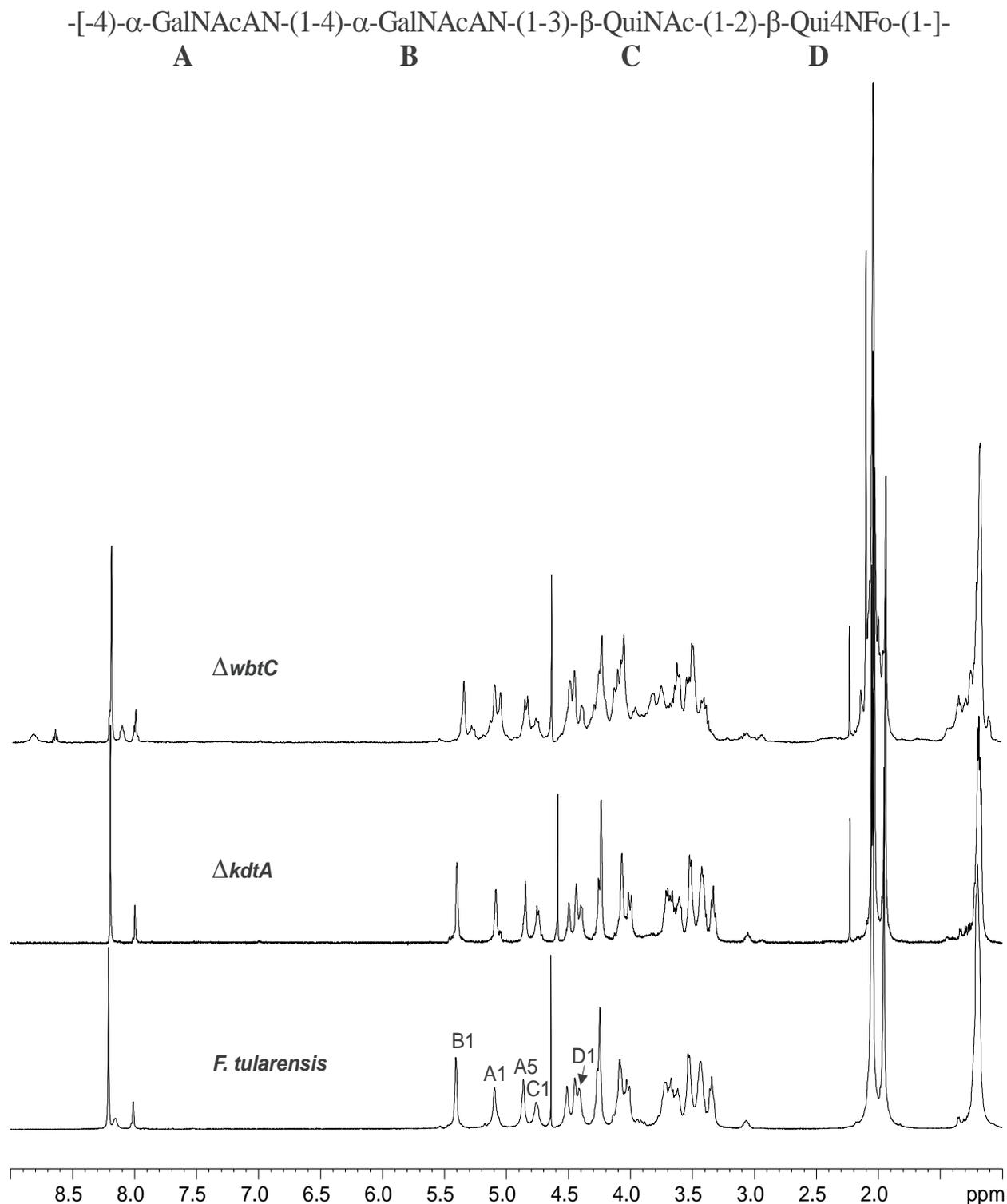
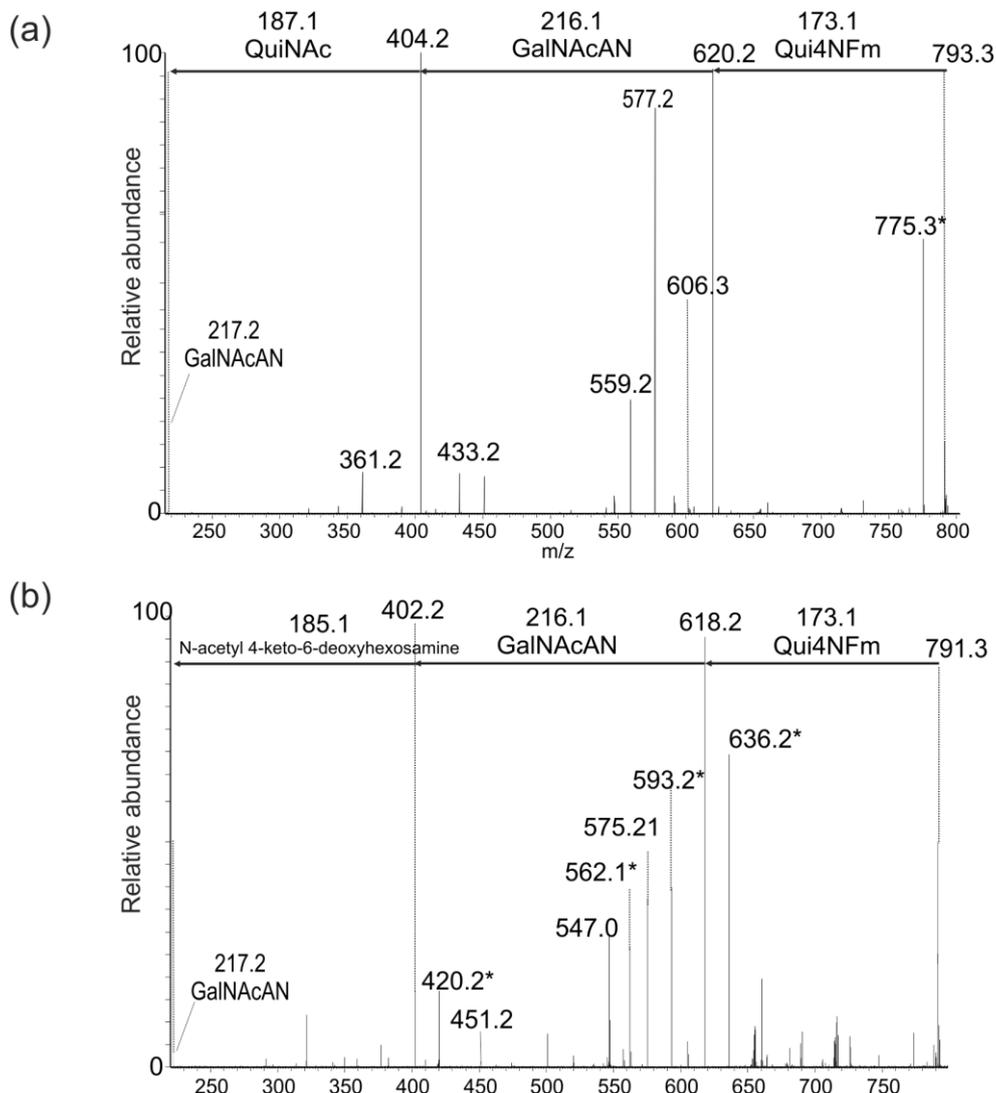


Figure 4. Positive ion ESI MSMS spectra of OPS. MS/MS spectra of *O*-polysaccharide (OPS) from (a) *Francisella tularensis* and (b) SCHU S4 $\Delta wbtC$.

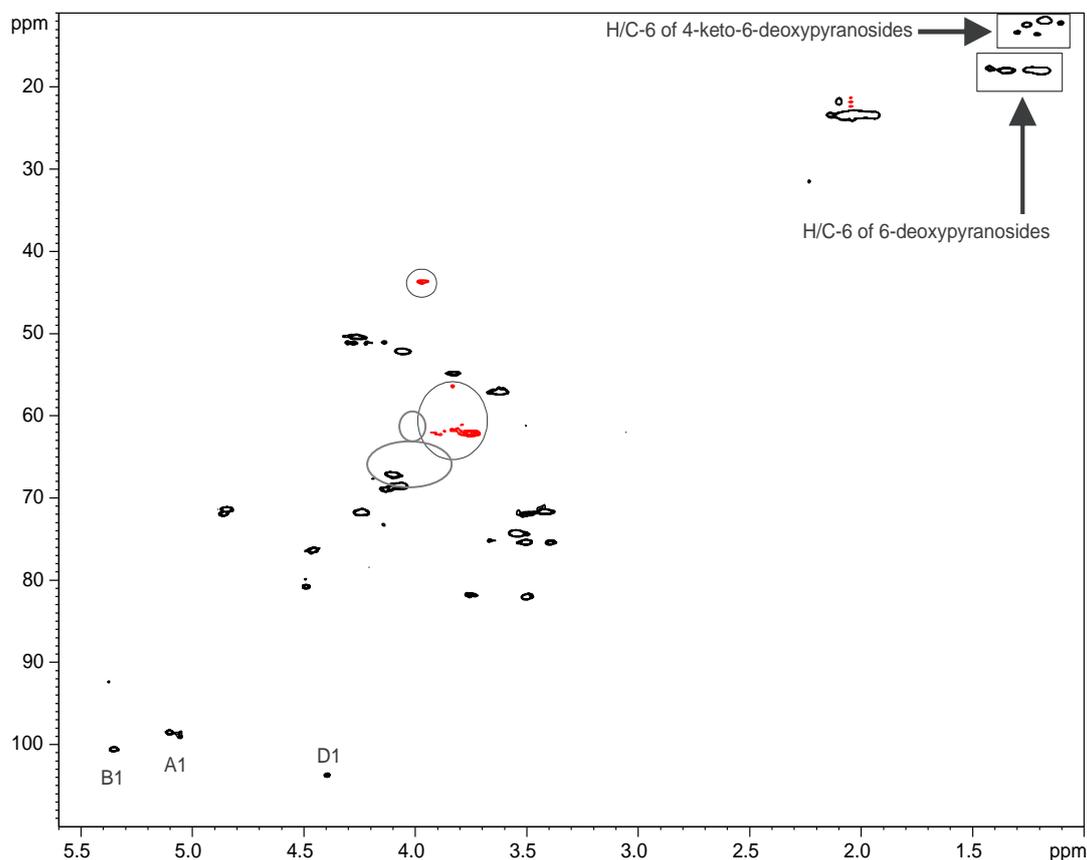


The mass spectrum of SCHU S4 $\Delta kdtA$ OPS gave an identical fragmentation pattern to that of wild type OPS (data not shown). By contrast, the mass spectrum of the $\Delta wbtC$ OPS had a peak at m/z 791 and a peak of much lower intensity at m/z 793. MS/MS of the ion at m/z 793 showed an identical fragmentation to that of wild type OPS. In contrast, the MS/MS spectrum of the ion at m/z 791 showed a different fragmentation pattern compared to that of the wild type OPS (Figure 4b). Of particular note, many of the fragment ions were shifted by -2 amu, compared to the wild type mass spectrum (notably m/z 402, 618) (Figure 4b). Sequential losses of Qui4Fm and GalNAcAN were observed, giving rise to fragment ions at m/z 618 and 402. Differing from wild type OPS, a loss of 185 Da was observed (m/z 402–217) instead of 187 (QuiNAc). Other OPS fragment ions in the $\Delta wbtC$ mass spectrum were also 2 amu less than corresponding fragments in the wild type OPS spectrum (notably m/z 402, 618, 791). Interestingly, these major fragment ions in the $\Delta wbtC$ spectrum were also accompanied by fragment ions +18 amu greater, likely corresponding to a hydrated form of the keto-group of *N*-acetyl 4-keto-6-deoxyhexosamine (m/z 420, 636, 809). Taken together, these data indicate that OPS of SCHU S4 $\Delta wbtC$ is comprised of Qui4Fm-GalNAcAN-unknown sugar- GalNAcAN. The unknown sugar of

mass 185 Da differs from QuiNAc by the loss of the mass of two hydrogen ions, therefore likely corresponds to the addition of *N*-acetyl 4-keto-6-deoxyhexosamine (185 Da) instead of *N*-acetyl 6-deoxyhexosamine (QuiNAc, 187 Da), *N*-acetyl 4-keto-6-deoxyhexosamine is the precursor of QuiNAc.

Next, $\Delta wbtC$ OPS was analyzed using 2D NMR methods (Figure 5). The data supported the mass spectrometry analyses, confirming the presence of *N*-acetyl 4-keto-6-deoxyhexosamine in this mutant. The HSQC spectrum contained a signal at 1.17/11.8 ppm (H/C), characteristic for *N*-acetyl 4-keto-6-deoxyhexosamine. HMBC spectrum showed that C-4 of this monosaccharide gives a signal at 95.0 ppm, typical for a hydrated keto group. H/C-5 was found at 3.54/74.6 ppm. TOCSY spectrum showed only one correlation from methyl group signal at 1.17 ppm, which agreed with the absence of a proton at C-4. These data were in close agreement with those presented by others [17] describing *N*-acetyl 4-keto-6-deoxyhexosamine.

Figure 5. HSQC spectrum of $\Delta wbtC$ OPS. Circled signals (CH_2 groups) belong to impurities.

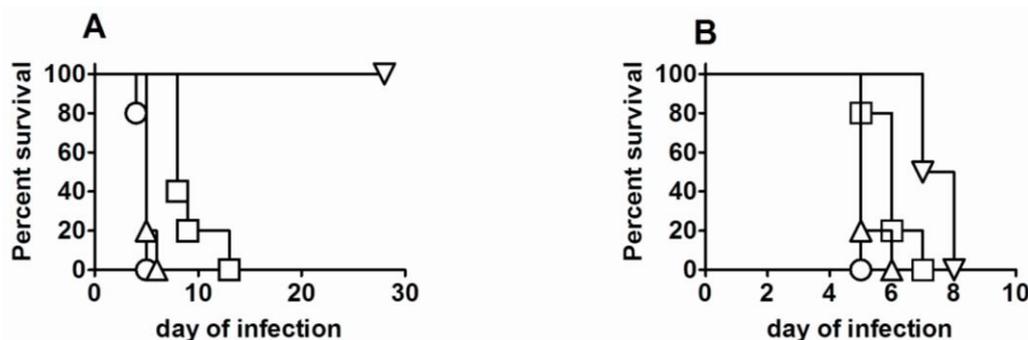


2.2. Virulence and Immunogenicity of Mutants

The lethal dose of wild type SCHU S4 for mice by either the ID or aerosol route is <10 CFU, which kills them within 7 days [14,18]. In a previous preliminary study, we showed that the ID LD₅₀ of $\Delta wbtI$ or $\Delta kdtA$ for BALB/c mice was $>10^3$ CFU, and mice that recovered from this challenge were not protected against a subsequent ID or aerosol challenge with SCHU S4 [3]. We extend these findings in the present study wherein BALB/c mice were challenged ID with up to 10^7 CFU of one or other mutant strain. All mice survived challenge with $\Delta wbtI$ or $\Delta kdtA$, but 3/5 mice died after challenge with

10^7 CFU of $\Delta wbtC$. Mice immunized with $\Delta wbtI$ or $\Delta kdtA$ displayed no overt inflammation at the puncture site, and remained healthy throughout. In contrast, mice immunized with 10^7 CFU of $\Delta wbtC$ developed large areas of necrosis at the site of inoculation, and the mice that died became severely sick beforehand. Mice immunized with 10^3 or 10^5 CFU of $\Delta wbtC$ all survived. Some of the mice that survived ID inocula were subsequently challenged ID with 1000 CFU (>100 LD₅₀) of wild type SCHU S4. All naïve mice and all the mice immunized with 10^7 CFU of $\Delta wbtI$ died by day 5 of challenge. The median time to death of mice immunized with 10^7 CFU of $\Delta kdtA$ was 8 days and this was significantly longer than the controls ($P = 0.003$ by Log-rank test). In contrast, 100% of mice immunized with 10^3 of $\Delta wbtC$ survived this challenge (Figure 6). Mice that survived immunization with 10^7 CFU of one or other mutant were challenged with a low dose aerosol (~ 20 CFU) of SCHU S4. Naïve mice all died by day 5, all mice immunized with $\Delta wbtI$ died between days 5–6, all mice immunized with $\Delta kdtA$ died between days 5–7, and the 2 mice that survived ID immunization with 10^7 CFU of $\Delta wbtC$ died on days 7 and 8. The latter two groups survived significantly longer than naïve mice ($P = 0.014$).

Figure 6. Survival of mice immunized with mutant strains to challenge with wild type SCHU S4. Naïve mice (circles), mice immunized with $\Delta kdtA$ (squares), $\Delta wbtI$ (triangle), or $\Delta wbtC$ (inverted triangle) were challenged six weeks later with (A), 1000 CFU SCHU S4 ID or (B), a low dose aerosol of ~ 20 CFU SCHU S4, and their survival was monitored.



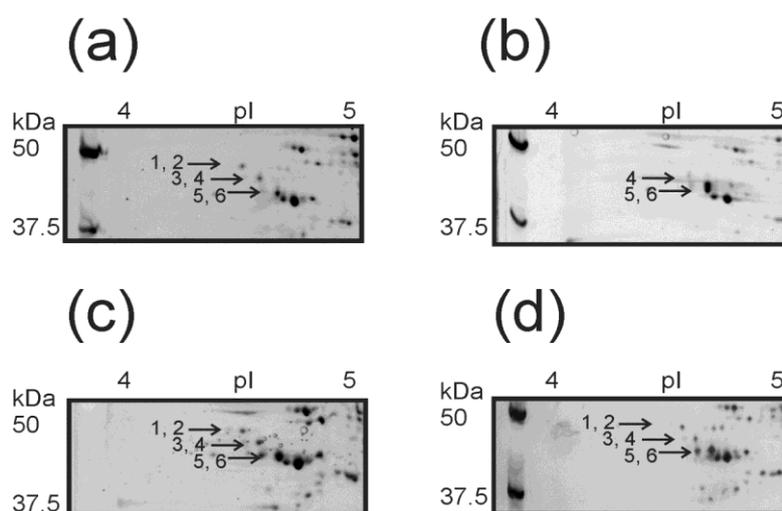
2.3. Involvement of *WbtC* in Protein Glycosylation

Because $\Delta wbtC$ appeared to have essentially normal LPS, its degree of attenuation was unexpected. Therefore, we examined the proteome of SCHU S4 $\Delta wbtC$ by 2D-PAGE for evidence of other defects. 2D-PAGE has the advantage of readily showing protein isoforms differing by mass or isoelectric point and is amenable to staining with glycoreactive stains, making rapid differences in protein glycosylation readily discernible.

We found differences in protein levels and glycosylation in previously reported glycoproteins, when comparing the proteome of $\Delta wbtC$ and the other three strains. For example, we have previously characterized in detail the carbohydrate modification of a disulfide isomerase, DsbA [19] and shown that the protein migrates on 2D-PAGE to six distinct protein spots (Figure 7a). Glyco-staining showed that spots annotated 1–4 in Figure 7a, but not spots 5 and 6 were glycosylated (data not shown). We speculate that isoforms 1–4 likely correspond to varying degrees of protein glycosylation. The mutants $\Delta wbtI$ and $\Delta kdtA$ showed a similar distribution of isoforms of DsbA as SCHU S4 (Figure 7c and 7d).

Interestingly, the $\Delta wbtC$ mutant did not elaborate the same isoforms of DsbA as the other three strains; protein spots annotated as 1–3 were not observed in the $\Delta wbtC$ mutant (Figure 7b). However, three spots (denoted 4–6 in Figure 7) were visible in the $\Delta wbtC$ mutant, and migrated to almost identical MW/pI as those observed in SCHU S4. In particular, spot 6 migrated to a molecular mass of 38.5 kDa/pI 4.8, close to the values predicted from the translated gene sequence of DsbA. The identity of this protein was confirmed as DsbA, by tandem mass spectrometry of the in-gel tryptic digest and the absence of detectable glycopeptides strongly suggests that this corresponds to the unglycosylated form of the protein. Two protein spots of low abundance, were also observed in the $\Delta wbtC$ mutant, migrating to MW/pI values of 41.5 kDa/pI 4.75 (spot 5) and 43.0 kDa/ pI 4.75 (spot 4). Tandem mass spectrometry of the tryptic digests of each spot from the $\Delta wbtC$ mutant confirmed the spots to be DsbA, and MS/MS spectra showed very low levels of a peptide, modified via *O*-linkage with the previously observed 1156 Da glycan moiety [19]. Measurement of the comparative protein spot intensity between SCHU S4 and the $\Delta wbtC$ mutant showed spots 5 and 6 to be present at similar levels in SCHU S4 and the $\Delta wbtC$ mutant. The only glycosylated form of the protein observed in the $\Delta wbtC$ mutant, spot 4, was present at approximately 3 fold lower levels compared with SCHU S4. Further analysis revealed only a few differences in the proteome of SCHU S4 $\Delta wbtC$ by 2D-PAGE when compared to wild type. This included differential glycosylation of another glycoprotein reported to harbor the same modification, the pilin subunit PilA [20]. The protein migrates to several protein spots on 2D-PAGE, with molecular masses/isoelectric points of 17.6 kDa/ pI 3.5, 17.6 kDa/ pI 3.9, 15.8 kDa/pI 3.6, 15.8 kDa/pI 3.95. Typically, we have observed all glycoforms to react with glycostain, however very weakly with protein staining reagents such as silver. In all but the $\Delta wbtC$ mutant we observed the same profile of PilA glycoreactivity compared with wild type (data not shown). By comparison the $\Delta wbtC$ mutant showed comparable area of observable reactivity with glycostain and no detectable protein stain. This strongly suggested that the glycosylation of PilA was negatively impacted by the deletion of the *wbtC* gene. Due to the weak reactivity of the protein isoforms of PilA, the comparative protein intensities were not measured. Other reported glycoproteins [21] were not detected in our hands using 2D-PAGE and glycostain.

Figure 7. 2DE gels of *F. tularensis* strains (a), wild type SCHU S4, (b), $\Delta wbtC$, (c), $\Delta wbtI$, (d), $\Delta kdtA$. Arrows mark isoforms 1-to-6 of glycoprotein DsbA.



3. Discussion

The ability to elaborate a long chain OPS is key to the virulence of *F. tularensis* subsp *tularensis* [8] and is confirmed herein. The OPS gene locus was annotated (*wbtA*-to-*wbtN*) and putative gene functions were assigned based upon homology to genes from other bacteria known to be involved in LPS biosynthesis [9]. In particular, many of the genes within the *F. tularensis* OPS locus were found to be homologous to the *O*-antigen genes of *Pseudomonas aeruginosa* serotype O6, which expresses a similar *O*-antigen repeat structure [22].

With particular relevance to the current study, the *F. tularensis wbtC* gene product has high homology to bacterial UDP-glucose 4-epimerase enzymes, especially *wbpV* of *Pseudomonas aeruginosa*. *WbpV* has been shown to have a role in UDP-QuiNac biosynthesis [23]. Similar to many epimerase/dehydratase enzymes, *wbtC* has putative NAD-binding domains which are thought to be important for dehydratase activities [24]. The *wbpV* null mutant in *P. aeruginosa* was deficient in B-band LPS production [23]. This does not appear to be the case in SCHU S4, where deletion of the *wbtC* gene still results in an *O*-antigen repeat structure visualised by 1D-PAGE, albeit with a somewhat different banding pattern to that observed in wild type LPS. A shift in LPS electrophoretic mobility was previously observed in a *wbtC* mutant of *F. tularensis* LVS [13]. A more recent study showed that a monoclonal antibody specific for wild type OPS failed to recognize the *O*-antigen expressed by the LVS *wbtC* mutant [25] implying that the OPS from the latter had altered structure.

The sugar detected as a component of the SCHU S4 $\Delta wbtC$ OPS in the current study, N-acetyl 4-keto-6-deoxyhexosamine, is a biosynthetic intermediate of the QuiNac pathway, and its presence suggests a mutation in the enzyme reducing UDP-4-keto-product into UDP-QuiNac. By MS, two peaks were detected in *wbtC* OPS, the minor peak (m/z 793) corresponds to the presence of QuiNac with the dominance of its precursor (major peak m/z 791), N-acetyl 4-keto-6-deoxyhexosamine. The former could result from the presence of another 4-reductase enzyme, involved in the biosynthesis of another deoxy sugar. Studies in other bacteria, for example *Rhizobium etli* showed that mutation of the sugar epimerase/reductase resulted in replacement of QuiNac in LPS with its 4-keto derivative [26]. The latter study also detected low levels of QuiNac in the OPS.

Several recent reports have described the modification of several *Francisella* proteins with glycan moieties in different subsp [19–21,27], with reports of putative roles of genes within the *O*-antigen biosynthetic locus [27] and other putative biosynthetic gene clusters [19]. Interestingly, $\Delta wbtC$ also showed an absence or marked decrease in abundance of the glycosylated isoforms of the DsbA homologue, which others have shown to be a putative lipoprotein [28]. The same group showed that deletion of the *dsbA* gene from SCHU S4 resulted in complete attenuation. Several recent publications show that glycoproteins of type A and B strains are modified with a 1156 Da glycan moiety [25–27]. These prior works have shown that a PglA homologue [25] and LPS biosynthetic genes *wbtDEF* [27] are putatively involved in protein glycosylation in *Francisella*. Here we show a role for *wbtC* in protein glycosylation. The utilization of common enzymes in LPS biosynthesis and protein glycosylation is not unique to *F. tularensis*. For instance, others have shown that *P. aeruginosa* [29] and *Aeromonas caviae* [30] use the same transferase in LPS biosynthesis and protein glycosylation to incorporate a common sugar. Additionally, *Helicobacter pylori* was recently shown to use a homolog of a protein glycosylation transferase in its OPS biosynthesis [31]. It was also recently shown [32] that

Campylobacter jejuni uses the same enzyme to modify its LPS and flagella rod protein. However, MS indicates that OPS and *F. tularensis* glycan do not contain any common sugars by monosaccharide mass (not shown). Thus, the basis for the involvement of *wbtC* in both processes remains unknown; one possibility is that sugar precursors are drawn from the OPS biosynthetic pathway into the glycosylation pathway for DsbA. In this regard, it is interesting to note, that *F. novicida* does not possess the *wbtC* gene [8], and its DsbA protein homologue appears to be glycosylated with different sugars than those found in the *F. tularensis* protein [19]. Interestingly, glycosylation of DsbA in the *wbtC* mutant was not completely abolished, with observable low levels of modification with the same glycan chain. This suggests that a second enzyme with homology to WbtC is participating in protein glycosylation. Previous work identified a second glycan biosynthetic gene cluster, encompassing the FTT0789-FTT0800 [26]. One of the genes, within the second polysaccharide gene cluster, FTT0791, is a putative UDP-Hex epimerase with some homology to WbtC. When the corresponding gene was disrupted, the mutant strain showed no detectable presence of modified form of the protein [26]. Some bacteria have been reported to harbor several copies of these epimerase genes; it is likely that this enzyme may also have some activity in protein glycosylation. Indeed, there is much that remains to be deciphered regarding the mechanisms of protein glycosylation in Francisella, with reports of PglA homologue responsible for glycosylation in several strains [20,27].

The *wbtI* gene product has high homology to sugar transamine/perosamine synthetases and was proposed to be involved in the biosynthesis of the fourth sugar in the *F. tularensis* O-antigen repeat, Qui4Fm [9], specifically, in amination of the sugar moiety. In this study, deletion of the gene resulted in lack of detectable O-antigen reactivity by 1D-PAGE. Another study generated a mutant of LVS with a single amino acid substitution in the *wbtI* gene (WbtIG191V). This resulted in disruption of *wbtI* enzymatic activity, and loss of OPS, including inability to detect the O-antigen specific sugar QuiNAc [11]. The *kdtA* gene has high homology to 3-deoxy-D-manno-octulosonic-acid transferase enzymes and harbours two conserved glycosyltransferase domains. In *E. coli*, KdtA is the enzyme responsible for the attachment of the two 3-deoxy-D-manno-octulosonic acid residues to lipid A. The core sugars and OPS are then added to KDO2-lipid A, to yield mature LPS [33]. Our analyses showed that OPS is still synthesized by the SCHU S4 $\Delta kdtA$ mutant, but mature LPS is not formed. Recently, others have shown that *F. tularensis* possesses a microcapsule consisting of polymerized O-antigen [25]. However, the serum agglutination results reported herein, suggest that the O-antigen found in the $\Delta kdtA$ mutant is not expressed at the surface.

Others have previously shown that Francisella mutants that fail to elaborate O-antigen are much more susceptible than wild type bacteria to being killed by normal human serum [34–36], in particular the, OPS must be conjugated to Lipid A-core in order to protect the pathogen from serum-mediated killing. Herein we observed that mice that recovered from infection with mutant $\Delta wbtC$ were fully protected against a subsequent intradermal challenge with wild type SCHU S4, whereas mice infected with $\Delta kdtA$ were partially protected and those infected with $\Delta wbtI$ were completely unprotected from such challenge. Previously, our group has shown that dual vaccination with an O-antigen deletion mutant of LVS and an O-antigen conjugated to tetanus toxoid, but not with either material alone could protect mice from systemic challenge with SCHU S4 [18]. We hypothesize that the *kdtA* mutant has partially replicated this effect because it produces unconjugated O-antigen, unlike *wbtI* which fails to elaborate it at all. None of the SCHU S4 mutants elicited any significant protection against

aerosol challenge when administered via the skin, the sole route of administration recommended for vaccinating humans with LVS.

4. Experimental Section

4.1. Bacterial Strains

Wild type SCHU S4 was originally isolated from a human patient [37]. For the present study it was obtained from a stock (FSC237) maintained at Umea University, Sweden. Mutants $\Delta wbtI$ and $\Delta kdtA$ have been described previously [3]. An in-frame deletion of *wbtC* was generated by allelic exchange using the previously described method [3]. For the present study, stock cultures of all strains were prepared by growing them as confluent lawns on cystine heart agar supplemented with 1% (w/v) hemoglobin (CHAH). Bacteria were harvested after 48 h incubation at 37 °C into freezing medium consisting of modified Mueller Hinton broth containing 10% w/v sucrose [38]. Stocks were aliquotted in volumes of 1 mL and stored at -80 °C at a concentration of 10^9 – 10^{11} CFU/mL.

4.2. Isolation and Analysis of LPS

LPS and free polysaccharides were isolated from plate-grown bacteria by standard methods that we have previously used for *F. tularensis* and *F. novicida* [5,16]. Briefly, cells were extracted with 45% phenol for 30 min at 80 °C, dialyzed, nucleic acids precipitated by addition of AcOH up to 10% and centrifugation at 5000 g. Solutions were exhaustively dialyzed against water and freeze dried. For gas chromatography, the resulting LPS or polysaccharide was hydrolyzed with 3M trifluoroacetic acid (120 °C, 3h) and sugars converted to alditol acetates by standard methods. For NMR analysis, LPS or polysaccharides were hydrolyzed with AcOH and *O*-chain isolated by gel chromatography as previously described [5]. ^1H and ^{13}C NMR spectra were recorded using a Varian Inova 500 MHz spectrometer for samples in D_2O solutions at 25–45 °C with acetone internal reference (2.23 ppm for ^1H and 31.5 ppm for ^{13}C) using standard pulse sequences double quantum filtered correlation spectroscopy (DQCOSY), total correlation spectroscopy (TOCSY) (mixing time 120 ms), nuclear overhauser effect spectroscopy (NOESY) (mixing time 400 ms), heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond correlation (HMBC) (80 ms long range transfer delay).

Mass spectrometry analyses were carried out using an LTQ Orbitrap XL mass spectrometer (Thermo, Waltham, MA, USA). *O*-antigen solutions were diluted two-fold with a solution of 25% methanol, 1% formic acid and infused into the mass spectrometer at 1 $\mu\text{L}/\text{min}$. MS/MS spectra were recorded on putative glycan related fragment ions under the following conditions: RF lens 1 120 V, CE 28. Resolution was typically 80,000 (50% valley definition). Collision-induced dissociation MS/MS analysis was performed on the glycan ions to confirm their identity.

4.3. De-O-Acetylation of Cell Samples

Bacterial strains were harvested from growth on CHAH and killed with 2% phenol for a minimum of two hours. The cell pellets were then harvested by centrifugation for 20 mins at 13,000 g at 7 °C. Cell pellets were water washed and spun again before removing supernatant and lyophilizing. Lyophilised samples were treated with 0.35 mg/mL proteinase K for 2 hours at 37 °C and 65 °C for

10 minutes. Samples were lyophilized again and treated with 10 uL of 0.1 mg/mL RNAase and 0.2 mg/mL of DNAase in 20 mM ammonium acetate. After 4 hours at 37 °C, samples were lyophilized once more and a portion removed for 1D-PAGE and mass spectrometry studies. To the remainder of the sample, 200 uL of hydrazine was added under nitrogen. Samples were incubated at 37 °C for 1.5 hours before quenching on dry ice with acetone. Samples were pelleted using a benchtop centrifuge, supernatant discarded and the pellet resuspended in acetone. Mass spectrometry studies were carried out using an ABSciex 4700 MALDI TOF/TOF mass spectrometer.

4.4. 1D-PAGE and Western Blotting Analysis of LPS

Lyophilised LPS was solubilised in Milli-Q and mixed with Laemmli buffer prior to separation by SDS-PAGE (12% acrylamide). Gels were transferred to PVDF membranes, as described in our recent work [39] and LPS was visualised by immunoblotting with a 1:2000 dilution of polyclonal antiserum obtained from mice immunised with BSA-conjugated *O*-antigen [6]. The secondary antibody, HRP conjugated goat anti-mouse IgG, (Perkin-Elmer Life and Analytical Sciences, Woodbridge, Ontario, Canada) was used at a dilution of 1:5000. Reactivity was visualised using the ECL kit (GE Healthcare) as per the manufacturer's instructions. Material extracted from whole killed cells was separated using 1D-PAGE as described and visualized using the Pro-Emerald Q LPS Glycostain kit (Invitrogen), as per the manufacturer's instructions. Stained gel images were captured using a FluorS Scanner (Biorad, Hercules, CA, USA) and PDQuest software (Biorad, Hercules, CA, USA).

4.5. 2D-PAGE Analysis

Francisella strains were plated for single colony growth on CHAH. At 72 h of incubation, 200 colonies of one or other strain were resuspended in 12 times the estimated pellet volume of lysis solution (7 M urea, 2 M thiourea, 1% (w/v) DTT, 4% (w/v) CHAPS and 0.5% (w/v) ASB-14 [40]). Protein concentrations of the extracts were assayed using the RC-DC protein assay (Biorad, Hercules, CA, USA) or using a modified Bradford Assay (Biorad, Hercules, CA, USA). Whole cell lysates were separated using immobilised pH gradient strips (IPG), either linear pH 4–7, 17 cm (Biorad, Hercules, CA, USA). 100 µg of each protein solution was diluted with the lysis solution, with 0.5% v/v pH 3–10 Biolytes (Biorad, Hercules, CA, USA) and 0.003% Orange G (Biorad, Hercules, CA, USA). Proteins were loaded onto the IPG strips by in-gel rehydration overnight. Isoelectric focusing was conducted as described previously [40]. The second dimension was carried out with 12% polyacrylamide gels (190 × 190 × 1.5 mm) using the Protein Iixi System (Biorad, Hercules, CA, USA) at 24 mA per gel for 5 hours. Gels were first stained with Emerald Q Glycostain (Invitrogen, Burlington, ON, Canada) to visualise glycoproteins and subsequently with Sypro Ruby for protein visualisation. Stained gel images were captured using a FluorS Scanner (Biorad, Hercules, CA, USA) and PDQuest software (Biorad, Hercules, CA, USA).

4.6. Protein Identification

Spots on 2DE gels were excised, digested with trypsin, and the resulting peptides analysed by nano-electrospray tandem mass spectrometry (nLC-MS/MS) as recently described [41]. The peaklist

files of MS2 spectra of the excised protein spots were searched against the NCBI *F. tularensis* database (2007.09.05) with 12283 entries using the MASCOT™ search engine (version 2.2.0) (Matrix Science, London, UK) for protein identification. The mass tolerance for precursor ions was ± 0.8 Da and the mass tolerance for fragment ions was ± 0.15 Da with trypsin. A cut-off ions score of 30 was used to indicate identity and in addition, all spectral matches were verified manually. Unmatched MS2 spectra were examined manually to determine the sequence of peptide y and b type ions.

4.7. Biological Studies

Female BALB/c mice were purchased from Charles River Laboratories (St. Constant, Quebec) and entered experiments at 6–8 weeks of age. Mice were maintained and used in accordance with the recommendations of the Canadian Council on Animal Care Guide to the Care and Use of Experimental Animals. Intradermal (ID) inocula were injected into a fold of skin in the mid-belly in a volume of 0.05 mL saline. Aerosol challenges were performed using an InTox Products nose-only exposure chamber as previously described [42]. Survival curves were constructed using GraphPad Prism 5 software (Graphpad Software, La Jolla, CA) and compared for statistical significance by Log-rank test using GraphPad Prism 5 software. To test the susceptibility of the mutants to serum, they were suspended in Chamberlains Defined Medium [43] in the presence or absence of 10% unheated normal human serum. Colony counts were performed at 0 and 4h. For the bacterial agglutination assay, 20 μ L of culture suspended in saline was admixed with 5 μ L of Francisella-specific rabbit antiserum (Difco, BD Biosciences, Mississauga, ON, Canada).

5. Conclusions

In the present study, we show that deletion of select genes from the *Francisella tularensis* subsp *tularensis* LPS biosynthetic locus results in not only changes in LPS structure, but more subtle effects upon protein glycosylation and bacterial virulence. In particular, deletion of the *wbtI* gene from SCHU S4, results in an avirulent mutant that does not express OPS, and fails to elicit a protective immune response. In contrast, deleting gene *wbtC*, led to a more subtle effect on OPS and also interfered with glycosylation of several known glycoproteins. This mutant retained slight virulence and elicited full protection against systemic, but not aerosol challenge with wild type SCHU S4. We show too that a mutant missing a homolog of a gene, *kdtA*, that encodes KDO transferase, lacked an obvious core region and produced free polymeric OPS that did not appear to be elaborated at the surface of the pathogen. It was avirulent, but elicited partial protective immunity against systemic challenge with wild type bacteria. This work contributes to a broader understanding of the complexity of the biology of this bacterium, and underlines the work still required for the design of an effective live vaccine.

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Conflict of Interest

The authors declare no conflict of interest.

References

1. Sjostedt, A. Tularemia: History, epidemiology, pathogen physiology, and clinical manifestations. *Ann. N. Y. Acad. Sci.* **2007**, *1105*, 1–29.
2. Dennis, D.T.; Inglesby, T.V.; Henderson, D.A.; Bartlett, J.G.; Ascher, M.S.; Eitzen, E.; Fine, A.D.; Friedlander, A.M.; Hauer, J.; Layton, M., *et al.* Tularemia as a biological weapon: Medical and public health management. *JAMA* **2001**, *285*, 2763–2773.
3. Kadzhaev, K.; Zingmark, C.; Golovliov, I.; Bolanowski, M.; Shen, H.; Conlan, W.; Sjostedt, A. Identification of genes contributing to the virulence of francisella tularensis schu s4 in a mouse intradermal infection model. *PLoS One* **2009**, *4*, e5463.
4. Meibom, K.L.; Charbit, A. The unraveling panoply of francisella tularensis virulence attributes. *Curr. Opin. Microbiol.* **2010**, *13*, 11–17.
5. Vinogradov, E.; Conlan, W.J.; Gunn, J.S.; Perry, M.B. Characterization of the lipopolysaccharide o-antigen of francisella novicida (u112). *Carbohydr. Res.* **2004**, *339*, 649–654.
6. Conlan, J.W.; Shen, H.; Webb, A.; Perry, M.B. Mice vaccinated with the o-antigen of francisella tularensis lvs lipopolysaccharide conjugated to bovine serum albumin develop varying degrees of protective immunity against systemic or aerosol challenge with virulent type a and type b strains of the pathogen. *Vaccine* **2002**, *20*, 3465–3471.
7. Kim, T.H.; Sebastian, S.; Pinkham, J.T.; Ross, R.A.; Blalock, L.T.; Kasper, D.L. Characterization of the o-antigen polymerase (wzy) of francisella tularensis. *J. Biol. Chem.* **2010**, *285*, 27839–27849.
8. Thomas, R.M.; Titball, R.W.; Oyston, P.C.; Griffin, K.; Waters, E.; Hitchen, P.G.; Michell, S.L.; Grice, I.D.; Wilson, J.C.; Prior, J.L. The immunologically distinct o antigens from francisella tularensis subspecies tularensis and francisella novicida are both virulence determinants and protective antigens. *Infect. Immun.* **2007**, *75*, 371–378.
9. Prior, J.L.; Prior, R.G.; Hitchen, P.G.; Diaper, H.; Griffin, K.F.; Morris, H.R.; Dell, A.; Titball, R.W. Characterization of the o antigen gene cluster and structural analysis of the o antigen of francisella tularensis subsp. Tularensis. *J. Med. Microbiol.* **2003**, *52*, 845–851.
10. Hartley, G.; Taylor, R.; Prior, J.; Newstead, S.; Hitchen, P.G.; Morris, H.R.; Dell, A.; Titball, R.W. Grey variants of the live vaccine strain of francisella tularensis lack lipopolysaccharide o-antigen, show reduced ability to survive in macrophages and do not induce protective immunity in mice. *Vaccine* **2006**, *24*, 989–996.
11. Li, J.; Ryder, C.; Mandal, M.; Ahmed, F.; Azadi, P.; Snyder, D.S.; Pechous, R.D.; Zahrt, T.; Inzana, T.J. Attenuation and protective efficacy of an o-antigen-deficient mutant of francisella tularensis lvs. *Microbiology* **2007**, *153*, 3141–3153.

12. Raynaud, C.; Meibom, K.L.; Lety, M.A.; Dubail, I.; Candela, T.; Frapy, E.; Charbit, A. Role of the wbt locus of francisella tularensis in lipopolysaccharide o-antigen biogenesis and pathogenicity. *Infect. Immun.* **2007**, *75*, 536–541.
13. Maier, T.M.; Casey, M.S.; Becker, R.H.; Dorsey, C.W.; Glass, E.M.; Maltsev, N.; Zahrt, T.C.; Frank, D.W. Identification of francisella tularensis himar1-based transposon mutants defective for replication in macrophages. *Infect. Immun.* **2007**, *75*, 5376–5389.
14. Sebastian, S.; Dillon, S.T.; Lynch, J.G.; Blalock, L.T.; Balon, E.; Lee, K.T.; Comstock, L.E.; Conlan, J.W.; Rubin, E.J.; Tzianabos, A.O., *et al.* A defined o-antigen polysaccharide mutant of francisella tularensis live vaccine strain has attenuated virulence while retaining its protective capacity. *Infect. Immun.* **2007**, *75*, 2591–2602.
15. Raetz, C.R.; Reynolds, C.M.; Trent, M.S.; Bishop, R.E. Lipid a modification systems in gram-negative bacteria. *Annu. Rev. Biochem.* **2007**, *76*, 295–329.
16. Vinogradov, E.; Perry, M.B.; Conlan, J.W. Structural analysis of francisella tularensis lipopolysaccharide. *Eur. J. Biochem.* **2002**, *269*, 6112–6118.
17. Schoenhofen, I.C.; McNally, D.J.; Vinogradov, E.; Whitfield, D.; Young, N.M.; Dick, S.; Wakarchuk, W.W.; Brisson, J.R.; Logan, S.M. Functional characterization of dehydratase/aminotransferase pairs from helicobacter and campylobacter: Enzymes distinguishing the pseudaminic acid and bacillosamine biosynthetic pathways. *J. Biol. Chem.* **2006**, *281*, 723–732.
18. Sebastian, S.; Pinkham, J.T.; Lynch, J.G.; Ross, R.A.; Reinap, B.; Blalock, L.T.; Conlan, J.W.; Kasper, D.L. Cellular and humoral immunity are synergistic in protection against types a and b francisella tularensis. *Vaccine* **2009**, *27*, 597–605.
19. Thomas, R.M.; Twine, S.M.; Fulton, K.M.; Tessier, L.; Kilmury, S.L.; Ding, W.; Harmer, N.; Michell, S.L.; Oyston, P.C.; Titball, R.W., *et al.* Glycosylation of dsba in francisella tularensis subsp. Tularensis. *J. Bacteriol.* **2011**, *193*, 5498–5509.
20. Egge-Jacobsen, W.; Salomonsson, E.N.; Aas, F.E.; Forslund, A.L.; Winther-Larsen, H.C.; Maier, J.; Macellaro, A.; Kuoppa, K.; Oyston, P.C.; Titball, R.W., *et al.* O-linked glycosylation of the pila pilin protein of francisella tularensis: Identification of the endogenous protein-targeting oligosaccharyltransferase and characterization of the native oligosaccharide. *J. Bacteriol.* **2011**, *193*, 5487–5497.
21. Balonova, L.; Hernychova, L.; Mann, B.F.; Link, M.; Bilkova, Z.; Novotny, M.V.; Stulik, J. Multimethodological approach to identification of glycoproteins from the proteome of francisella tularensis, an intracellular microorganism. *J. Proteome Res.* **2010**, *9*, 1995–2005.
22. Knirel, Y.A.; Skvortsov, I.M.; Shashkov, A.S.; Dmitriev, B.A.; Kochetkov, N.K.; Stanislavsky, E.S.; Mashilova, G.M. Somatic antigens of pseudomonas aeruginosa. The structure of the o-specific polysaccharide chains of p. Aeruginosa o11 (lanyi) lipopolysaccharides. *Eur. J. Biochem.* **1985**, *150*, 551–557.
23. Belanger, M.; Burrows, L.L.; Lam, J.S. Functional analysis of genes responsible for the synthesis of the b-band o antigen of pseudomonas aeruginosa serotype o6 lipopolysaccharide. *Microbiology* **1999**, *145* (Pt 12), 3505–3521.
24. Wyk, P.; Reeves, P. Identification and sequence of the gene for abequose synthase, which confers antigenic specificity on group b salmonellae: Homology with galactose epimerase. *J. Bacteriol.* **1989**, *171*, 5687–5693.

25. Apicella, M.A.; Post, D.M.; Fowler, A.C.; Jones, B.D.; Rasmussen, J.A.; Hunt, J.R.; Imagawa, S.; Choudhury, B.; Inzana, T.J.; Maier, T.M., *et al.* Identification, characterization and immunogenicity of an o-antigen capsular polysaccharide of francisella tularensis. *PLoS One* **2010**, *5*, e11060.
26. Forsberg, L.S.; Noel, K.D.; Box, J.; Carlson, R.W. Genetic locus and structural characterization of the biochemical defect in the o-antigenic polysaccharide of the symbiotically deficient rhizobium etli mutant, ce166. Replacement of n-acetylquinovosamine with its hexosyl-4-ulose precursor. *J. Biol. Chem.* **2003**, *278*, 51347–51359.
27. Balonova, L.; Mann, B.F.; Cervený, L.; Alley, W.R.; Chovancova, E.; Forslund, A.L.; Salomonsson, E.N.; Forsberg, A.; Damborsky, J.; Novotny, M.V., *et al.* Characterization of protein glycosylation in francisella tularensis subsp. Holarctica; identification of a novel glycosylated lipoprotein required for virulence. *Mol. Cell Proteomics* **2012**, *11*, M111.015016.
28. Qin, A.; Scott, D.W.; Thompson, J.A.; Mann, B.J. Identification of an essential francisella tularensis subsp. Tularensis virulence factor. *Infect. Immun.* **2009**, *77*, 152–161.
29. Castric, P.; Cassels, F.J.; Carlson, R.W. Structural characterization of the pseudomonas aeruginosa 1244 pilin glycan. *J. Biol. Chem.* **2001**, *276*, 26479–26485.
30. Tabei, S.M.; Hitchen, P.G.; Day-Williams, M.J.; Merino, S.; Vart, R.; Pang, P.C.; Horsburgh, G.J.; Viches, S.; Wilhelms, M.; Tomas, J.M., *et al.* An aeromonas caviae genomic island is required for both o-antigen lipopolysaccharide biosynthesis and flagellin glycosylation. *J. Bacteriol.* **2009**, *191*, 2851–2863.
31. Hug, I.; Couturier, M.R.; Rooker, M.M.; Taylor, D.E.; Stein, M.; Feldman, M.F. Helicobacter pylori lipopolysaccharide is synthesized via a novel pathway with an evolutionary connection to protein n-glycosylation. *PLoS Pathog.* **2010**, *6*, e1000819.
32. Cullen, T.W.; Trent, M.S. A link between the assembly of flagella and lipooligosaccharide of the gram-negative bacterium campylobacter jejuni. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 5160–5165.
33. Clementz, T. The gene coding for 3-deoxy-manno-octulosonic acid transferase and the rfaq gene are transcribed from divergently arranged promoters in escherichia coli. *J. Bacteriol.* **1992**, *174*, 7750–7756.
34. Sorokin, V.M.; Pavlovich, N.V.; Prozorova, L.A. Francisella tularensis resistance to bactericidal action of normal human serum. *FEMS Immunol. Med. Microbiol.* **1996**, *13*, 249–252.
35. Clay, C.D.; Soni, S.; Gunn, J.S.; Schlesinger, L.S. Evasion of complement-mediated lysis and complement c3 deposition are regulated by francisella tularensis lipopolysaccharide o antigen. *J. Immunol.* **2008**, *181*, 5568–5578.
36. Sandstrom, G.; Lofgren, S.; Tarnvik, A. A capsule-deficient mutant of francisella tularensis lvs exhibits enhanced sensitivity to killing by serum but diminished sensitivity to killing by polymorphonuclear leukocytes. *Infect. Immun.* **1988**, *56*, 1194–1202.
37. Eigelsbach, H.T.; Braun, W.; Herring, R.D. Studies on the variation of bacterium tularensis. *J. Bacteriol.* **1951**, *61*, 557–569.
38. Baker, C.N.; Hollis, D.G.; Thornsberry, C. Antimicrobial susceptibility testing of francisella tularensis with a modified mueller-hinton broth. *J. Clin. Microbiol.* **1985**, *22*, 212–215.

39. Twine, S.M.; Petit, M.D.; Fulton, K.M.; House, R.V.; Conlan, J.W. Immunoproteomics analysis of the murine antibody response to vaccination with an improved francisella tularensis live vaccine strain (lvs). *PLoS One* **2010**, *5*, e10000.
40. Twine, S.M.; Mykytczuk, N.C.; Petit, M.; Tremblay, T.L.; Lanthier, P.; Conlan, J.W.; Kelly, J.F. Francisella tularensis proteome: Low levels of asb-14 facilitate the visualization of membrane proteins in total protein extracts. *J. Proteome Res.* **2005**, *4*, 1848–1854.
41. Twine, S.M.; Reid, C.W.; Aubry, A.; McMullin, D.R.; Fulton, K.M.; Austin, J.; Logan, S.M. Motility and flagellar glycosylation in clostridium difficile. *J Bacteriol.* **2009**, *191*, 7050–7062.
42. Conlan, J.W.; Chen, W.; Shen, H.; Webb, A.; KuoLee, R. Experimental tularemia in mice challenged by aerosol or intradermally with virulent strains of francisella tularensis: Bacteriologic and histopathologic studies. *Microb. Pathog.* **2003**, *34*, 239–248.
43. Chamberlain, R.E. Evaluation of live tularemia vaccine prepared in a chemically defined medium. *Appl. Microbiol.* **1965**, *13*, 232–235.

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