Deletion of the *Bacillus anthracis* *capB* homologue in *Francisella tularensis* subspecies *tularensis* generates an attenuated strain that protects mice against virulent tularaemia

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As there is currently no licensed vaccine against *Francisella tularensis*, the causative agent of tularaemia, the bacterium is an agent of concern as a potential bioweapon. Although *F. tularensis* has a low infectious dose and high associated mortality, it possesses few classical virulence factors. An analysis of the *F. tularensis* subspecies *tularensis* genome sequence has revealed the presence of a region containing genes with low sequence homology to part of the *capBCADE* operon of *Bacillus anthracis*. We have generated an isogenic *capB* mutant of *F. tularensis* subspecies *tularensis* SchuS4 and shown it to be attenuated. Furthermore, using BALB/c mice, we have demonstrated that this *capB* strain affords protection against significant homologous challenge with the wild-type strain. These data have important implications for the development of a defined and efficacious tularaemia vaccine.

INTRODUCTION

Due to its high infectivity and ability to cause severe disease and morbidity, *Francisella tularensis*, the causative agent of tularaemia, has been designated a category A biological agent (Saslaw *et al.*, 1961b; Khan *et al.*, 2000; Oyston *et al.*, 2004). *F. tularensis* subspecies *tularensis*, previously known as type A (Olsufiev *et al.*, 1959), is recognized as the most virulent biotype and has an infectious dose in humans of as low as 10 c.f.u. via the airborne route (McCrumb, 1961). *F. tularensis* subspecies *holarctica*, or type B, can also cause disease, but is less virulent than *F. tularensis* subspecies *tularensis* strains (Chen *et al.*, 2003). At present, there is no licensed vaccine for the prevention of tularaemia although it has been demonstrated that an undefined live attenuated strain of *F. tularensis* subspecies *holarctica* confers reasonable protection against injected challenges with *F.*

tularensis subspecies *tularensis* (Saslaw *et al.*, 1961a, b). The fact that an attenuated mutant of *F. tularensis* can induce protective immunity suggests that the development of live vaccines based on defined mutations in virulence factors is a feasible approach to vaccine development. Few virulence determinants have been proposed and relatively little is known about virulence mechanisms of this bacterium (Larsson *et al.*, 2005), although intracellular survival seems to be key to pathogenesis (Oyston, 2008). To date, the deletion of only seven loci, *purMCD*, *dsbA* and *dsbB*, *wbtDEF*, FTT0918, FTT1103 and *clpB* has been shown to attenuate this highly virulent strain in a mouse model of infection (Pechous *et al.*, 2008; Qin *et al.*, 2008, 2009; Straskova *et al.*, 2009; Thomas *et al.*, 2007; Twine *et al.*, 2005). *F. tularensis* subspecies *tularensis* lacks a type III secretion system, plasmids, toxins and other classical virulence factors thus prompting an empirical need to identify the virulence determinants of this pathogen.

An analysis of genome sequence data from attenuated and virulent strains of *F. tularensis* has revealed the presence of a conserved *capBCA* region with low levels of sequence homology to part of the *capBCADE* operon of *Bacillus anthracis* (Larsson *et al.*, 2005; Makino *et al.*, 1989; Ravel *et al.*, 2009). In *B. anthracis*, the *capBCADE* operon encodes the synthetic machinery for the assembly of capsular poly-D-glutamic acid (PGA), with deletion of this operon in *B. anthracis* resulting in an attenuated phenotype (Drysdale

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Abbreviations: IFN- γ , gamma interferon; IL-2, interleukin 2; MLD, median lethal dose; PGA, poly-D-glutamic acid; TNF- α , tumour necrosis factor alpha.

A figure of serum sensitivity data is available as supplementary data with the online version of this paper.

et al., 2005). While the deletion of *cap* genes in low virulence strains of *F. tularensis* has demonstrated a role *in vivo* (Su *et al.*, 2007; Weiss *et al.*, 2007), this has, however, not yet been investigated in the highly virulent *F. tularensis* subspecies *tularensis*. Our studies and those of others have shown that deletion of *pilA* and of *hfq*, in different species and in strains of *F. tularensis*, results in a more marked attenuation in the less virulent *holarctica* subspecies than in the more virulent *tularensis* subspecies (Meibom *et al.*, 2009). Thus, we wished to study the phenotype of a CapB-deficient mutant of *F. tularensis* subspecies *tularensis* with respect to virulence. In a study by Su and colleagues, the *cap* locus containing *capB*, *capC* and a putative transporter FTL_1412 (FTT0807) was all deleted (Su *et al.*, 2007). As the function of FTL_1412 (FTT0807) has not yet been determined it cannot be ruled out that this mutation could have played a role in the observed attenuation of the mutant strain. In this study, we have demonstrated that a homologue of the *B. anthracis cap* biosynthetic locus plays a role in the virulence in the highly virulent SchuS4 strain. In addition, this *capB* mutant was able to induce a protective immune response, suggesting that the *cap* locus may be a potential target for mutation in the development of a live attenuated tularaemia vaccine. As such, this mutation has already been combined into an avirulent mutant of *F. tularensis* subspecies *tularensis* strain SchuS4 (Conlan *et al.*, 2010). This work conclusively demonstrates the role of this gene in the virulence of *F. tularensis* subspecies *tularensis* strain SchuS4.

METHODS

Bacterial strains, plasmids and media. The bacterial strains and plasmids used in this study are listed in Table 1. Strains of *F. tularensis* were cultured on blood cysteine glucose agar (BCGA) supplemented with 10 ml (10% w/v) histidine l^{-1} , on modified Thayer Martin (TM) agar (BBL GC agar base) (Becton Dickinson) supplemented with 1% (w/v) haemoglobin and 1% (v/v) BBL IsoVitalEx (Becton Dickinson) and 50 µg polymyxin B sulphate ml^{-1} , or in liquid culture in Chamberlain's defined media (Chamberlain, 1965) or modified cysteine partial hydrolysate (MCPH) broth. *Escherichia coli* were cultured on Luria-Bertani plates or broth. Chloramphenicol was added as required to growth media at 20 µg ml^{-1} for both *E. coli* and *F. tularensis*.

General DNA manipulation. DNA extraction and manipulation procedures were performed as described by Sambrook *et al.*, 1989). Unless otherwise stated, enzymes for the manipulation of DNA and reagents for the detection of the DIG-labelled probe for Southern blotting were obtained from Roche Diagnostics. Chemicals were obtained from Sigma, and culture media were obtained from Oxoid. Oligonucleotides were supplied by MWG BioTech and are listed in Table 2.

Construction of *F. tularensis* subspecies *tularensis* SchuS4 $\Delta capB::Cam$. The suicide plasmid for inactivation of *capB* was constructed as follows. Regions of DNA flanking the *capB* gene of *F. tularensis* were PCR amplified from *F. tularensis* subspecies *tularensis* SchuS4 genomic DNA using the primer pairs P1/P2 and P3/P4. The PCR products were digested with *BstEII* and ligated together. This ligation mixture was used as template in a PCR using the primer pair

P1/P4. The 2085 bp product from this reaction was cloned into the plasmid pGEM-T Easy to give the plasmid pSMP32. The chloramphenicol resistance cassette (*Cam*) was PCR amplified from the plasmid pKK202 (Norqvist *et al.*, 1996) with the primer pair *CamF*/*CamR* and cloned into the *BstEII* restriction of pSMP32 to generate pSMP39. This mutated allele of *capB* was excised from pSMP39 by *MluI* digestion and cloned into the *MluI* site of pSMP22 to give pSMP42. The suicide plasmid pSMP42 for deletion of *capB* was electroporated into the *E. coli* mobilizing strain S17 λ *pir* (Simon *et al.*, 1983). The plasmid was then introduced from the mobilizing strain to *F. tularensis* subspecies *tularensis* SchuS4 by conjugal transfer, as described by Golovliov *et al.* (2003). Transconjugants were selected on chloramphenicol and merodiploids arising from chromosomal integration of the suicide plasmid were resolved by plating on TM agar containing 5% (w/v) sucrose. Colonies were confirmed as mutants by Southern blotting. Briefly, genomic DNA of wild-type and SchuS4 $\Delta capB::Cam$ mutants was digested with *MluI* and *NcoI*, separated by agarose gel electrophoresis and transferred to a nylon membrane. The *MluI* insert of pSMP32 was labelled with DIG-11-dUTP during PCR amplification with the primer pair P1/P4 and used as a probe to hybridize to the membrane. DNA fragments to which the probe hybridized were detected by chemiluminescence [CSPD – disodium 3-(4-methoxy)spiro[1,2-dioxetane-3,2'-(5'-chloro)-tricyclo(3.3.1.1^{3,7})decan]-4-yl)phenylphosphate – substrate, 30 min exposure, X-ray film].

The use of chloramphenicol as a marker was approved for this specific study. All work undertaken in the UK is reviewed locally by a genetic manipulation safety committee on a project-by-project basis and is also approved nationally by our health and safety authorities.

Real-time PCR. Bacteria were cultured in MCPH broth at 37 °C with shaking at 180 r.p.m. until cells were growing exponentially. RNA extraction was performed using an RNeasy midi preparation kit (Qiagen). Contaminating DNA was removed using Turbo DNA-free (Ambion) and mRNA was reverse transcribed using an Omniscript reverse transcription kit (Qiagen). Oligonucleotide primers and probes were designed and supplied by Applied Biosystems. The probe used for the analysis of gene expression was located within the target gene. These sequences are shown in Table 2. Each reaction contained 1.25 µl TaqMan gene expression assay, 12.5 µl TaqMan PCR master mix, 6.25 µl nuclease-free water (Applied Biosystems) and 5 µl cDNA (500 ng). TaqMan analysis was performed in a MicroAmp optical 96-well plate using the ABI Prism 7000 sequence detection system (Applied Biosystems). Assays were performed in triplicate on cDNA samples, using *dnaA* as an endogenous control (Livak & Schmittgen, 2001) with a standard cycle protocol.

Intramacrophage survival assay. J774A murine macrophages were infected at an m.o.i. of 10 and incubated at 37 °C for 30 min. Extracellular bacteria were killed with 10 µg gentamicin ml^{-1} for 30 min. Cultures were maintained in L-15 tissue culture medium (Sigma-Aldrich) containing 2 µg gentamicin ml^{-1} at 37 °C, until harvesting at various times post-infection. Macrophages were disrupted by the addition of 1 ml dH₂O and aspiration 30 times. Bacteria were enumerated by plating them on to BCGA and incubating for 3 days at 37 °C.

In vivo studies. Female BALB/c mice (6–8 weeks old; Charles River Laboratories) were injected subcutaneously with 100 µl PBS containing 10^4 c.f.u. *F. tularensis* subspecies *tularensis* SchuS4, *F. tularensis* subspecies *tularensis* SchuS4 $\Delta capB::Cam$ or *F. tularensis* subspecies *holarctica* LVS. Several investigators have determined that the median lethal dose (MLD) of *F. tularensis* subspecies *tularensis* is <10 c.f.u. in the BALB/c mouse, irrespective of route of administration (Saslaw *et al.*, 1961a, b). For protection studies, immunized and naive mice were challenged on day 56 post-vaccination with 10^3 c.f.u. *F. tularensis*

Table 1. Bacterial strains and plasmids used in this study

Bacteria or plasmid	Characteristic	Reference or source
Strains		
<i>E. coli</i> S17 λ pir	<i>thi thr leu tonA lacY supE recA::RP4-2-Tc::Mu, Kn::Tn7</i>	Simon <i>et al.</i> (1983)
<i>F. tularensis</i> subsp. <i>tularensis</i> SchuS4	Virulent strain	Human ulcer, 1941, Ohio, FSC237
<i>F. tularensis</i> subsp. <i>holarctica</i> LVS	Avirulent strain – basis of attenuation not known	Original NDBR lot 4 vaccine ampoule produced during the 1960s
<i>F. tularensis</i> subsp. <i>tularensis</i> SchuS4 Δ capB::Cam	capB inactivation mutant	This study
Plasmids		
pGEM-T Easy	Commercial vector	Promega
pSMP32	pGEM-T Easy containing <i>capB</i> flanking regions	This study
pSMP22	Mobilizable suicide vector	Thomas <i>et al.</i> (2007)
pSMP42	Plasmid for deletion of <i>capB</i> , contains insert from pSMP39 cloned into pSMP22	This study
pSMP39	pSMP32 containing chloramphenicol gene	This study
pKK202	Plasmid to amplify chloramphenicol gene	Norqvist <i>et al.</i> (1996)

subspecies *tularensis* SchuS4 via the subcutaneous route. For colonization studies, groups of four mice were killed at 3, 7, 14 and 31 days following inoculation with the bacteria. Spleens were removed and the number of bacteria per spleen determined by serial dilution in PBS followed by microbiological culture on BCGA agar plates for 96 h at 37 °C. All procedures were carried out in accordance with UK Home Office guidelines. Animals were closely observed over a 35 day period for the development of clinical signs. Humane end points were strictly observed, so that if an animal displayed irreversible signs of tularaemia, it was promptly culled, thus avoiding undue distress.

Inactivation of bacteria. Overnight cultures of *F. tularensis* subspecies *holarctica* LVS grown on BCGA at 37 °C were inactivated by irradiation with 30 kGy per vial (Isotron). Before and after irradiation, 10% of the vial contents were removed and plated on BCGA to assess viability. The concentration of protein in each vial was ascertained using a bicinchoninic acid protein assay (Pierce). Irradiated bacteria were stored at –20 °C prior to use in

immunization studies. *F. tularensis* subspecies *tularensis* SchuS4 was inactivated by heating at 65 °C for 1 h.

Humoral and cellular responses following immunization with the SchuS4 Δ capB::Cam mutant. Bacteria were grown for 48 h at 37 °C on modified TM agar. Female BALB/c mice (6–8 weeks old) were injected subcutaneously with 100 μ l PBS containing 10⁴ c.f.u. *F. tularensis* subspecies *tularensis* SchuS4 Δ capB::Cam, *F. tularensis* subspecies *holarctica* LVS or PBS alone. Blood was sampled from immunized and naive mice from the tail vein on day 34. Serum was analysed for anti-*F. tularensis* subspecies *holarctica* LVS antibodies using standard ELISA methodology. Briefly, individual serum samples were dispensed into microtitre plates pre-coated with either irradiated LVS or heat-killed *F. tularensis* subspecies *tularensis* SchuS4 (5 μ g bacterial protein ml^{–1} in PBS). Binding of serum antibody was detected with peroxidase-labelled secondary antibody to mouse IgG1 and IgG2a (Harlan-SeraLab). To facilitate a comparison of one subclass titre with another, standard solutions of each subclass antibody (Harlan-SeraLab) in the range of 0.2–50.0 ng ml^{–1} were

Table 2. Oligonucleotides used in this study

Restriction sites are indicated by underlined sequences.

Primer	Sequence
P1	5'-CTG <u>ACGCGT</u> -AGGCAGTGTGGTTATGGGTAG-3'
P2	5'-GAC <u>GCGTAACC</u> -CAAATACGACGACAATTAAC-3'
P3	5'-CTGGGTAACC-TCCAGCAAACCTCTTATATTTC-3'
P4	5'-TAGACGCGT-ACCCAATCAACCCAGTACAAG-3'
CamF	5'-GCTGGTTACC-TAAGAGGTTCCAACCTTTCAC-3'
CamR	5'-CTAGGTTACC-TTTAAGGGCACCAATAACTG-3'
capC F ABI	5'-GGACTATCTACAGGTGGTATGGTTGT-3'
capC R ABI	5'-CGTCTACCGTAAATAATCATAAATTTAGACATAAATCTGA-3'
capC probe	5'-CAGGAGCCCCCATCTC-3'
dnaA F ABI	5'-CTCAAAAAAGCTCATGATTTAGGTCAGAA-3'
dnaA R ABI	5'-AGCACCTTCTAGTTCTCTGACATTAGT-3'
dnaA probe	5'-CAGCAGCTTTTATTGC-3'

assayed. The standard curves generated enabled determination of the mean concentration of each IgG subclass in serum derived from the various treatment groups. At 40 days following immunization, groups of four immunized or naive mice were sacrificed and their spleens removed. Single cell suspensions of spleen cells were prepared in culture media (RPMI 1640) (Sigma) supplemented with 10 % heat inactivated fetal bovine serum (Sigma), 1 % penicillin/streptomycin/glutamine (Sigma) and 50 μ M 2-mercaptoethanol (Sigma). Cells were stimulated overnight in triplicate with either heat-killed *F. tularensis* subspecies *tularensis* SchuS4 (5 μ g protein ml⁻¹) in supplemented RPMI 1640 or supplemented RPMI 1640 alone. Interleukin 2 (IL-2), tumour necrosis factor alpha (TNF- α) and gamma interferon (IFN- γ) secretion from the cells was determined using cytokine bead array technology (BD Biosciences).

Statistics. Statistical differences in antibody response were determined using ANOVA, Kruskal–Wallis and Student–Newman–Keuls tests. Survival data were analysed using Kaplan–Meier plots and a Mantel–Haenszel log-rank test.

RESULTS

F. tularensis subspecies *tularensis* contains homologues of the *B. anthracis* *capB* and *capC* genes

During annotation of the genome of *F. tularensis* subspecies *tularensis* SchuS4 (Larsson *et al.*, 2005), we identified two adjacent genes, FTT0805 and FTT0806, that displayed 36 and 33 % sequence identity at the amino acid level, with the CapB and CapC proteins, respectively, of *B. anthracis* and *Staphylococcus epidermidis* (Fig. 1). In *B. anthracis*, CapB and CapC have been shown to be responsible for the synthesis of PGA, while CapA and CapE are involved in transportation (Candela & Fouet, 2006). The synteny between the *F. tularensis* *cap* locus, and the *cap* locus of *B. anthracis* and *S. epidermidis*, is not completely preserved with the notable absence in *F. tularensis* of homologues to *capADE*, the genes required for the secretion and anchoring of PGA (Fig. 1a). It has been proposed that the gene downstream of *capC* (FTT0807) be renamed *capF* (or *pgsF* if PGA is not anchored to the cell wall) as it may play a role in transport similar to the roles of *capA* and *capE* (Candela *et al.*, 2009). In a report of the production of PGA by the Gram-negative bacterium *Fusobacterium nucleatum*, PGA appeared to be secreted to the culture medium (Candela *et al.*, 2009) suggesting that the gene in *Fusobacterium nucleatum* does not encode a functional homologue of CapD of *B. anthracis*; the latter being involved in anchoring PGA to the cell wall (Candela & Fouet, 2005). Indeed, the gene identified in *F. tularensis* as a homologue of the *capD* of *B. anthracis* (Su *et al.*, 2007), which has less sequence homology than the *Fusobacterium nucleatum* homologue, has recently been shown to be *ggt* encoding a gamma glutamyltransferase involved in the acquisition of cysteine from glutathione in the LVS strain (Alkhuder *et al.*, 2009), and more recently we have demonstrated this in the SchuS4 strain (H. LeButt, P. M. Ireland, R. M. Thomas & P. C. F. Oyston, personal communication), supporting its

original annotation as *ggt* rather than *capD* (Larsson *et al.*, 2005).

Construction of a *capB*-deficient strain of *F. tularensis* subspecies *tularensis* SchuS4

To investigate the role of *cap* genes in *F. tularensis* subspecies *tularensis*, the *capB* gene (FTT0805) was chosen as a target for inactivation on the basis of its sequence homology to the CapB protein encoded by the *cap* virulence locus of *B. anthracis* strain 'Ames' and *S. epidermidis* 1457 (Kocianova *et al.*, 2005; Makino *et al.*, 1989) (Fig. 1b). Insertion of a Cam cassette resulted in a 1073 bp deletion of the *capB* gene, with 6 and 35 amino acids remaining at the N- and C-terminus, respectively. Allelic replacement mutants were confirmed by Southern blotting as demonstrated by the two predicted hybridizing fragments of 12.5 and 3.2 kb contained in the SchuS4 Δ *capB*::Cam mutant (Fig. 2). Our local advisory committees on dangerous pathogens and genetic modification prohibit the use of two antibiotic resistance markers in *F. tularensis* subspecies *tularensis* SchuS4; thus, preventing us from generating a complemented mutant strain. Therefore, to investigate whether insertion of the Cam cassette resulted in a disruption of downstream gene expression, expression of *capC*, the gene immediately downstream of *capB*, was examined by reverse transcription RT-PCR. RNA was isolated from exponentially growing cultures of *F. tularensis* subspecies *tularensis* SchuS4 strain and SchuS4 Δ *capB*::Cam, and reverse transcribed. Expression of *capC* was confirmed using the method described by Livak & Schmittgen (2001).

F. tularensis subspecies *tularensis* SchuS4 Δ *capB*::Cam mutant is attenuated and induces a protective immune response

Inactivation of *capB* did not impair the growth rate of *F. tularensis* subspecies *tularensis* SchuS4 Δ *capB*::Cam in MCPH broth, and replication within J774 murine macrophage cells was not significantly affected (Fig. 3). In addition, we did not observe a difference in serum sensitivity in SchuS4 Δ *capB*::Cam, compared to wild-type (Supplementary Fig. S1 available with the online journal). In order to determine whether *capB* is required for virulence in a murine model of infection, mice were infected subcutaneously with *F. tularensis* subspecies *tularensis* SchuS4 or SchuS4 Δ *capB*::Cam. Mice infected with 120 c.f.u. of strain SchuS4 succumbed to infection by day 4 post-infection. In contrast, 100 % of mice infected with 1.6×10^4 c.f.u. of SchuS4 Δ *capB*::Cam survived, and 80 % of mice infected with 1.6×10^5 or 1.6×10^6 c.f.u. of SchuS4 Δ *capB*::Cam survived (Fig. 4a). Therefore, the MLD was assumed to be $>1.6 \times 10^6$. This significant ($P=0.0026$) level of survival following deletion of a single gene demonstrates that *capB* is important for the virulence of *F. tularensis* subspecies *tularensis*. Our data indicate that the level of protection afforded by the SchuS4 Δ *capB*::Cam mutant is comparable to that of LVS as mice immunized

Fig. 1. (a) Schematic representation of the *cap* locus from *B. anthracis* 'Ames Ancestor', *F. tularensis* subspecies *tularensis* SchuS4 and *S. epidermidis* 1457. (b) Global alignment of the amino acid sequences of CapB from *B. anthracis*, *F. tularensis* and *S. epidermidis* performed by Clone manager using a BLOSUM 62 scoring matrix.

with 10^4 c.f.u. of *SchuS4* $\Delta capB::Cam$ were solidly protected when challenged subcutaneously with 10^3 c.f.u. wild-type *F. tularensis* subspecies *tularensis* *SchuS4* 56 days later ($P=0.0001$) (Fig. 4b).

When the kinetics of bacterial clearance were examined, none of the mice infected with strain SchuS4 survived

longer than 5 days post-inoculation, which precluded determination of splenic bacterial burdens on days 7, 14 and 31 post-inoculation (Fig. 5). However, at day 3 post-inoculation there was a highly significant ($P<0.001$) difference in the numbers of bacteria in the spleens of mice infected with strains SchuS4 and SchuS4 $\Delta capB::Cam$; as mice infected with the mutant had

Fig. 2. Southern blot of genomic DNA from wild-type and SchuS4 Δ capB::Cam mutant strains of *F. tularensis* subspecies *tularensis*. Genomic DNA strain SchuS4 (lane 1) and of two SchuS4 Δ capB::Cam mutants (lanes 2 and 3) were digested with *Mlu*I and *Nco*I, separated by agarose gel electrophoresis and transferred to a nylon membrane. DNA fragments to which the probe hybridized were detected by chemiluminescence.

substantially lower numbers of bacteria in their spleens. No viable bacteria were detected in the spleens of animals infected with SchuS4 Δ capB::Cam at 31 days post-inoculation; thus, indicating that the animals had effectively cleared the mutant.

Characterizing the protective immune response induced by *F. tularensis* subspecies *tularensis* SchuS4 Δ capB::Cam

In our studies, mice immunized with the SchuS4 Δ capB::Cam mutant responded with specific antibody and cell-mediated responses, similar to that observed for LVS, as assessed by immunoassay. ELISA confirmed that mice dosed with LVS or SchuS4 Δ capB::Cam responded with robust serum IgG2a antibody responses (Fig. 6). Significantly increased quantities of IL-2 and IFN- γ were secreted when spleen cells from animals immunized with SchuS4 Δ capB::Cam or LVS were restimulated *in vitro* with inactivated *F. tularensis* subspecies *tularensis* SchuS4 ($P < 0.05$) (Fig. 7a, b). No significant IL-2 or IFN- γ secretion was detected when spleen cells from animals immunized with SchuS4 Δ capB::Cam or LVS were co-cultured with media alone, thereby confirming a *Francisella*-specific cytokine recall response. TNF- α secretion during *ex vivo* stimulation with inactivated *F. tularensis* subspecies *tularensis* SchuS4 was also statistically increased ($P < 0.05$) when cells were derived from mice immunized with SchuS4 Δ capB::Cam or LVS (Fig. 7c). However, TNF- α was also secreted by spleen cells derived from PBS-treated mice

Fig. 3. (a) Growth of SchuS4 ($\blacktriangle$) and SchuS4 Δ capB::Cam ($\square$) in MCPH broth. Cultures were incubated at 37 °C with shaking at 180 r.p.m. and the optical density (at 590 nm) measured at various time points. Data are presented as means and SEM of triplicate cultures. (b) Replication of SchuS4 (black bars) and SchuS4 Δ capB::Cam (white bars) in J774 murine macrophage cells. Cells were infected at a multiplicity of 10, and intracellular bacteria harvested and enumerated at 0, 24 and 48 h post-infection. Data are presented as the mean and SEM of triplicate experiments.

during *ex vivo* stimulation. This probably reflects TNF- α production by macrophages and dendritic cells following activation by Toll-like receptor agonists present in the heat-inactivated *F. tularensis* subspecies *tularensis* SchuS4.

DISCUSSION

In the last 40 years, there have been few reports of capsule production by *F. tularensis* (Cherwonogrodzky *et al.*, 1994; Hood, 1977; Sandström *et al.*, 1988). Furthermore, the reports of capsule production by *Francisella* are contrasting, making it difficult to ascertain its nature. One of the first, and most detailed, reports on capsule of the *F. tularensis* stated that it was observed as an electron-transparent layer, and that analysis of this material revealed a carbohydrate content of 21% and a fatty acid content of 51% (Hood, 1977). In contrast, subsequent studies described a *Francisella* capsule as an electron-dense layer; although no compositional studies were conducted on this material (Sandström *et al.*, 1988). During the course of this study, we attempted several methods to visualize capsules

Fig. 4. (a) Survival following infection of BALB/c mice with 120 c.f.u. *F. tularensis* subspecies *tularensis* SchuS4 (○), 1.6×10^4 c.f.u. *F. tularensis* subspecies *tularensis* SchuS4 Δ capB::Cam (◇), 1.6×10^5 c.f.u. *F. tularensis* subspecies *tularensis* SchuS4 Δ capB::Cam (△) or 1.6×10^6 c.f.u. *F. tularensis* subspecies *tularensis* SchuS4 Δ capB::Cam (□). Groups of five mice were infected by the subcutaneous route and monitored for 14 days. (b) Survival following infection of BALB/c mice with 10^3 c.f.u. *F. tularensis* subspecies *tularensis* SchuS4 by the subcutaneous route. At 56 days prior to the challenge, mice were injected subcutaneously with either 10^4 c.f.u. *F. tularensis* subspecies *tularensis* SchuS4 Δ capB::Cam (■) ($n=5$), 10^4 c.f.u. *F. tularensis* subspecies *holarctica* LVS (▲) ($n=5$) or PBS (●) ($n=6$).

on *F. tularensis* grown under a range of conditions, but no such structure was observed (data not shown). Therefore, it is still not possible to say that the *cap* operon of *F. tularensis* is involved in capsule production, as is the case for the *cap* operon of *B. anthracis*, which is involved in the biosynthesis of a PGA capsule. As mentioned, it is possible that *F. tularensis* produces PGA through the function of the products of the *capB* and *capC* genes, although it might not be presented as a capsular structure. Although it has been proposed that FTT0807 may function as the cognate transporter in place of a CapA homologue (Candela *et al.*, 2009; Su *et al.*, 2007), preliminary analysis of SchuS4 and SchuS4 Δ capB::Cam extracts by NMR failed to identify PGA in culture supernatant precipitates and cell extracts (data not shown); therefore, it remains to be definitively confirmed that the *cap* locus is involved in PGA production. As a result, we focussed on evaluating the effect of mutation of the *capB* gene on virulence.

Fig. 5. Clearance kinetics following infection of BALB/c mice with 10^4 c.f.u. strain SchuS4 (black bar) or 10^4 c.f.u. SchuS4 Δ capB::Cam (white bars) by the subcutaneous route. Data are means ($\pm$ SD) from four individual spleens per treatment at each time point. No mice infected with 10^4 c.f.u. *F. tularensis* subspecies *tularensis* SchuS4 survived beyond day 5.

The detection of homologues of the *cap* locus of *B. anthracis* in *F. tularensis* subspecies *tularensis* suggested that they might also play a role in the virulence in this organism. Studies in low virulence strains of *F. tularensis* have demonstrated the *cap* locus is essential for virulence. In this study, the finding that a *capB* mutant of *F. tularensis* subspecies *tularensis* exhibits an MLD $>1.6 \times 10^6$ relative to <10 for the parent strain confirms that this previously Gram-positive bacteria specific virulence factor is also a virulence factor in the highly virulent Gram-negative *F. tularensis* subspecies *tularensis*.

The generation of defined attenuated mutants of *F. tularensis* subspecies *tularensis* is a rational approach in

Fig. 6. Levels of specific anti-*F. tularensis* subspecies *tularensis* SchuS4 IgG1 (black bars) and IgG2a (white bars) antibodies in the serum of BALB/c mice infected subcutaneously 34 days previously with 10^4 c.f.u. SchuS4 Δ capB::Cam or 10^4 c.f.u. LVS. Data are means ($\pm$ SEM) from five individual mice. Analysis of serum from PBS-treated mice gave mean absorbance (at 450 nm) measurements of 0.075 and 0.070 for IgG1 and IgG2a, respectively.

Fig. 7. (a) IL-2 secretion from spleen cells co-cultured *in vitro* with heat-killed *F. tularensis* subspecies *tularensis* SchuS4 (grey bars) or media alone (black bars). (b) IFN- γ secretion from spleen cells co-cultured *in vitro* with heat-killed *F. tularensis* subspecies *tularensis* (grey bars) or media alone (black bars). (c) TNF- α secretion from spleen cells co-cultured *in vitro* with heat-killed *F. tularensis* subspecies *tularensis* SchuS4 (grey bars) or media alone (black bars). Spleen cells were isolated from BALB/c mice that had been injected subcutaneously with PBS or infected 40 days earlier with 10^4 c.f.u. SchuS4 $\Delta capB::Cam$ or 10^4 c.f.u. LVS by the subcutaneous route. Data are means ($\pm$ sd) from four individual mice per treatment group.

tularaemia vaccine development (reviewed by Oyston, 2009); moreover, it is interesting to note that strains of *B. anthracis* that are deficient in the *cap* locus have been used in humans as vaccines against anthrax (Merabishvili *et al.*, 2006). In our studies, mice infected with SchuS4 $\Delta capB::Cam$ and subsequently challenged with the fully virulent strain SchuS4 were protected. The mutant did

not result in as high levels of colonization as the wild-type, but persisted at detectable levels for at least 2 weeks. Complete clearance was observed by a month post-immunization. Mice infected with the SchuS4 $\Delta capB::Cam$ mutant responded with specific antibody and cell-mediated responses as adjudged by immunoassay. As was the case with LVS, SchuS4 $\Delta capB::Cam$ immunized mice developed an IgG2a-dominated *F. tularensis* specific antibody response. Previous work with inactivated LVS has indicated a link between protection and the development of IgG2a antibodies with specificities for certain proteins within the *F. tularensis* proteome (Eyles *et al.*, 2007). Similarly, mice immunized with LVS or SchuS4 $\Delta capB::Cam$ had significant numbers of *F. tularensis* specific IFN- γ and IL-2 secreting T cells in their spleens, as compared with non-immunized animals. This is potentially important as, in common with many other intracellular pathogens, cell-mediated responses are known to play an important role in immunity to virulent forms of tularaemia, as demonstrated by passive transfer and cell depletion studies in rats and mice (Anthony *et al.*, 1989; Conlan *et al.*, 1994; Fortier *et al.*, 1991; Tärnvik, 1989). Similarly, it is known that individuals recovering from tularaemia often demonstrate pronounced delayed-type hypersensitivity reactions (Burke, 1977).

We have demonstrated that *F. tularensis* subspecies *tularensis* possesses *capB*, a homologue of the *B. anthracis* virulence gene, a finding that, to the best of our knowledge, has not previously been reported in any other pathogenic Gram-negative genus. In addition, the *capB* mutant can provide protection against substantial wild-type challenge that is equivalent to that of LVS. This work supports other reports on the feasibility of producing an effective genetically defined live *F. tularensis* vaccine from subspecies *tularensis* strain of *F. tularensis*, and provides a novel target that could be used in the generation of a multiple loci mutant for such a vaccine (Conlan *et al.*, 2010).

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