RelA regulates virulence and intracellular survival of *Francisella novicida*

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Analysis of the genome of *Francisella tularensis* has revealed few regulatory systems, and how the organism adapts to conditions in different niches is poorly understood. The stringent response is a global stress response mediated by (p)ppGpp. The enzyme RelA has been shown to be involved in generation of this signal molecule in a range of bacterial species. We investigated the effect of inactivation of the *relA* gene in *Francisella* by generating a mutant in *Francisella novicida*. Under amino acid starvation conditions, the *relA* mutant was defective for (p)ppGpp production. Characterization showed the mutant to grow similarly to the wild-type, except that it entered stationary phase later than wild-type cultures, resulting in higher cell yields. The *relA* mutant showed increased biofilm formation, which may be linked to the delay in entering stationary phase, which in turn would result in higher cell numbers present in the biofilm and reduced resistance to *in vitro* stress. The mutant was attenuated in the J774A macrophage cell line and was shown to be attenuated in the mouse model of tularaemia, but was able to induce a protective immune response. Therefore, (p)ppGpp appears to be an important intracellular signal, integral to the pathogenesis of *F. novicida*.

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INTRODUCTION

Francisella tularensis is an intracellular pathogen that causes the disease tularaemia. The organism can infect a wide range of hosts, including humans. Natural infection can occur following ingestion of contaminated food or water, bites by infected arthropod vectors, direct contact with infected animals or by inhalation (Ellis *et al.*, 2002). The ecological niche of *F. tularensis* has not been identified, and little is known about the mechanisms utilized by the organism to persist in nature. Many outbreaks of *F. tularensis* have been associated with streams, ponds, lakes and rivers, and the bacterium is capable of surviving for more than a year in water and mud (Parker *et al.*, 1951). Closely related *Francisella*-like organisms have been isolated from fish (Nylund *et al.*, 2006; Ostland *et al.*, 2006), which suggests that the natural niche of *Francisella* may be aquatic, although this remains to be confirmed.

Little is known regarding the molecular mechanisms of *Francisella* pathogenesis; however, as an intracellular pathogen, the ability to invade and multiply within macrophages is essential (reviewed by Oyston, 2008). Gene regulation in *Francisella* is poorly defined, and the elucidation of the genome sequence of *F. tularensis* has revealed few genes predicted to encode proteins with

regulatory functions (Larsson *et al.*, 2005). A 30 kb *Francisella* pathogenicity island (FPI) has been identified which plays a role in intracellular multiplication (Nano *et al.*, 2004). Disruption of many of the genes in the FPI, such as *iglA*, *iglC*, *pdpA* and *pdpD*, results in attenuation *in vitro* or *in vivo* (Baron & Nano, 1998; Golovliov *et al.*, 2003; Gray *et al.*, 2002; Lauriano *et al.*, 2004; Lindgren *et al.*, 2004; Nano *et al.*, 2004). Genes of the FPI have been shown to be regulated by MglA, encoded by *mglA* (Lauriano *et al.*, 2004), and SspA, encoded by *sspA* (Charity *et al.*, 2007). MglA and MglB are essential for intracellular replication and virulence in mice (Baron & Nano, 1998; Lauriano *et al.*, 2004), and share homology with the *Escherichia coli* SspA and SspB proteins, which are regulators of stationary phase transcription and are involved in stress survival (Williams *et al.*, 1994). Apart from MglA and SspA, few regulators have been identified in *Francisella*. Considering the wide range of cell types, including amoebae, that the organism can parasitize, and that it can survive extracellularly and within arthropod vectors, the organism must exploit its relatively small (1.8 Mbp) genome highly efficiently to respond to changing environments and stress. In this respect it is similar to another intracellular pathogen, *Neisseria gonorrhoeae*, which also possesses few regulators to mediate adaptation to environmental change (Parkhill *et al.*, 2000; Tettelin *et al.*, 2000).

Many bacterial species resist environmental stress in part by induction of the stringent response (Jain *et al.*, 2006),

Abbreviations: CV, crystal violet; FPI, *Francisella* pathogenicity island; MLD, median lethal dose; SPI, *Salmonella* pathogenicity island.

mediated by production of hyperphosphorylated guanosine diphosphate and triphosphate analogues, collectively termed (p)ppGpp. During nutrient starvation, uncharged tRNA molecules bind to the ribosome, which results in ribosome stalling. This activates ribosome-associated RelA and catalyses the production of pppGpp, which is subsequently converted to ppGpp. The ppGpp molecule binds to RNA polymerase to affect expression of a range of physiological systems, including σ^s , an alternative sigma factor. The net effect is a reduction in transcription, but a large number of σ^{70} -dependent genes are induced, as are proteins involved in proteolysis and amino acid biosynthesis. In some bacteria, inactivation of both *relA* and *spoT* is required to completely abolish (p)ppGpp synthesis; SpoT is a bifunctional enzyme capable of both synthesis and degradation of the signal molecule, so preventing uncontrollable accumulation of (p)ppGpp (Jain *et al.*, 2006). The *relA* and *spoT* genes are restricted to the betaproteobacteria and gammaproteobacteria (Mittenhuber, 2001), and it has been proposed that the RelA-dependent response is linked to amino acid starvation and that the SpoT-dependent response is linked to fatty acid metabolism in these bacteria (Battesti & Bouveret, 2009). In some pathogens regulation of (p)ppGpp synthesis is dependent on SpoT, and virulence in *Legionella pneumophila* is critically dependent on the degradation of (p)ppGpp by SpoT (Dalebroux *et al.*, 2009). Both *relA* and *spoT* genes have been annotated in the *Francisella novicida* U112 (Rohmer *et al.*, 2006) and *F. tularensis* SCHU S4 (Larsson *et al.*, 2005) genome sequences; however, the functions of these enzymes in *Francisella* have not yet been defined.

The stringent response, induced by nutrient limitation, has been demonstrated in a range of bacteria, including *Escherichia coli* (Magnusson *et al.*, 2005), *N. gonorrhoeae* (Fisher *et al.*, 2005), *Streptococcus suis* (Li *et al.*, 2009) and *Borrelia burgdorferi* (Bugrysheva *et al.*, 2003b), and it appears that most bacteria require the stringent response for stationary phase survival (Mouery *et al.*, 2006; Bugrysheva *et al.*, 2003a). In addition, RelA has been shown to be involved in regulation of other diverse phenotypes, including biofilm formation by *Streptococcus mutans* (Lemos *et al.*, 2004), osmotolerance of *Listeria monocytogenes* (Okada *et al.*, 2002), stress response, vancomycin tolerance and virulence of *Enterococcus faecalis* (Abranches *et al.*, 2009), and anaerobiosis in *Mycobacterium tuberculosis* (Sureka *et al.*, 2007).

The stringent response has been shown to play an important role in the life cycle and virulence of intracellular pathogens. For example, in *Legionella pneumophila*, (p)ppGpp triggers a switch between the avirulent replicative phase and the virulent motile phase (Hammer & Swanson, 1999), while in *M. tuberculosis*, (p)ppGpp accumulation is important for the transition into latency (Ojha *et al.*, 2000; Primm *et al.*, 2000). In addition, the expression of specific virulence genes has been shown to be modified by RelA: in *Vibrio cholerae*, inactivation of RelA results in decreased expression of cholera toxin and the toxin-coregulated pilus (Haralalka *et al.*, 2003), and a

Salmonella enterica *spoT/relA* mutant has reduced expression of HilA, the central regulator of the *Salmonella* pathogenicity island (SPI) 1 and SPI 2 virulence genes (Pizarro-Cerda & Tedin, 2004).

In light of the paucity of information on regulation of gene expression in *F. tularensis*, and with the growing evidence of the role of (p)ppGpp as a global regulator of phenotypes associated with both *in vivo* and environmental survival, this study was undertaken to determine the effect of inactivation of RelA on *F. novicida*.

METHODS

Bacterial strains and culture conditions. *F. novicida* strain U112 was obtained from the American Type Culture Collection. The strain was grown on blood cysteine glucose agar (BCGA) supplemented with 4% cysteine, 4% histidine, 5% glucose and 10% fresh filtered horse blood, or on Thayer–Martin agar (Milne *et al.*, 2007). Where required, media were supplemented with 100 μg polymyxin B ml^{-1} , 10 μg chloramphenicol ml^{-1} and 10% (w/v) sucrose. Growth of strain U112 in liquid culture was supported by Chamberlain's defined medium (CDM) (Chamberlain, 1965) with aeration, or modified Chamberlain's defined medium lacking serine (CDM⁻S) or with reduced PO₄ at 2 mM (CDM₂) or at 0.4 mM (CDM_{0.4}). *E. coli* strains JM109 (Promega) and S17- λ pir (Simon *et al.*, 1983) were grown in Luria–Bertani medium at 37 °C, supplemented as required with 30 μg chloramphenicol ml^{-1} .

DNA manipulation. Generally, the manipulation of DNA was carried out using standard protocols (Sambrook *et al.*, 1989). Genomic DNA was isolated using the Puregene DNA Isolation kit (Gentra Systems). Plasmid DNA was isolated using the Qiagen mini and maxi plasmid isolation kits. Primers and plasmids used in this study are described in Table 1. Southern blotting was performed under high-stringency conditions (Sambrook *et al.*, 1989).

Generation of the Δ relA mutant. Oligonucleotide primers (Table 1) were designed from the *F. tularensis* SCHU S4 genome sequence, such that an 1877 bp internal region would be deleted. DNA flanking the *relA* gene was amplified by PCR following 30 cycles of amplification (94 °C, 30 s; 55 °C, 30 s; 72 °C, 1 min) (Perkin Elmer 9600 GeneAmp PCR system). Each product was ligated into pGEM-T Easy according to the manufacturer's instructions (Promega) and transformed into *E. coli* JM109. The inserts were isolated following digestion with *EcoRI* and *BamHI* and linked in a three-way ligation with pUC18 which had been digested with *EcoRI*. The insert was subsequently subcloned into pUCB, a derivative of pUC18 wherein the *BamHI* restriction site had been abolished by treatment with Klenow fragment. The *BamHI* restriction site in primers relR1 and relF2 allowed the insertion of the chloramphenicol resistance cassette from pGEMCMBamH1 between the two flanks. The whole insert was isolated by digestion with *MluI* and ligated into similarly digested pPV2 J to generate pPVrel. The plasmid was transformed into *E. coli* S17- λ pir by electroporation, and mobilized into *F. novicida* strain U112 by conjugation. Conjugation was performed on BCGA at 25 °C overnight. The bacteria were then removed and plated on Thayer–Martin agar supplemented with polymyxin B (to select against the *E. coli*) and chloramphenicol. Sucrose selection was subsequently performed to identify recombinants in which the plasmid was excised. The mutant was confirmed by PCR and Southern blotting and designated *F. novicida* U112 Δ relA::cam.

The mutant was complemented by insertion of the entire coding sequence of the *F. tularensis* *relA* gene. The gene was amplified by

Table 1. Oligonucleotide primers and plasmids used in this study

Underlined sequences represent restriction sites introduced into oligonucleotide sequences to facilitate cloning.

Primer	Sequence (5'–3')	Restriction site included
relF1	<u>ACGCGTCTAAGATAGCCGTTAGTAGC</u>	<i>MluI</i>
relR1	<u>GGATCCGGGATGATAATGAAGAGGTC</u>	<i>BamHI</i>
relF2	<u>GGATCCCTGACCATCACTATCTAGAAG</u>	<i>BamHI</i>
relR2	<u>ACGCGTCCCAGCAATTAGGAACCTAC</u>	<i>MluI</i>
crelF	<u>GGTACCATGCAAGTTATTGACTCTA</u>	<i>KpnI</i>
crelR	<u>GGTACCATTATTAGCTGACCTCTTC</u>	<i>KpnI</i>
Plasmid	Description	Source or reference
pUC18	Amp ^R	
pUCB	pUC18 with inactivated <i>BamHI</i> restriction site	This study
pDM4	Cam ^R	Milton <i>et al.</i> (1996)
pGEMCAMPamHI	pGEM-T Easy (Promega) containing the Cam ^R cassette from pDM4 amplified by PCR to be flanked by <i>BamHI</i> restriction sites; Amp ^R	Quarry <i>et al.</i> (2007)
pRel12	pUC18 containing the <i>ΔrelA</i> insert	This study
pRelCam	pRel12 with chloramphenicol resistance cassette inserted at the <i>BamHI</i> restriction site between the two flanks of the deletion insert	This study
pPV2 J	Derivative of pPV2 with inactivated Cam ^R ; oriT; <i>bla</i> ; <i>sacB</i>	Quarry <i>et al.</i> (2007)
pPVrel	Donor plasmid for conjugation; <i>sacB</i> ; pPV2 J containing mutated <i>ΔrelA::cam</i> fragment from pRelCam	This study
pSMP6OriT	Stable plasmid for complementation studies in <i>Francisella</i> ; Tet ^R	Quarry <i>et al.</i> (2007)
pSMP6OriT.relA	Complementation plasmid expressing RelA driven by the <i>Francisella</i> GroES promoter	This study

PCR using primers crelF and crelR, and the product ligated into pSMP6OriT, conferring tetracycline resistance, which had been digested with *KpnI*, to give pSMP6OriT.relA. The plasmid was mobilized into the U112 *ΔrelA::cam* mutant by conjugation to give U112 *ΔrelA::cam* preLA. Transformants were selected for on Thayer–Martin agar supplemented with polymyxin B and tetracycline and confirmed by PCR.

(p)ppGpp detection assay. The method of Cashel (1994) was adapted to determine levels of (p)ppGpp produced by *Francisella*. Cells were passaged three times in 20 ml CDM[−]S, with aeration at 37 °C for 24 h each passage. Bacteria were subcultured into 20 ml reduced PO₄ CDM₂[−]S and incubated for 18 h with aeration at 37 °C. Cells were harvested by centrifugation at 2500 *g* for 10 min and washed twice with minimal PO₄ CDM_{0.4}[−]S. Cells were adjusted to OD₆₀₀ 0.1 and incubated with aeration at 37 °C to OD₆₀₀ ~0.2, at which point 1 μl [³²P]H₃PO₄ (Perkin-Elmer) was added to each culture to give a final concentration of 10 μCi ml^{−1} (0.37 MBq ml^{−1}). After further incubation for 2 h, 2.5 mg serine hydroxamate ml^{−1} was added to cultures to induce serine-starvation conditions. After 30 min incubation, nucleotides were extracted by adding an equal volume of ice-cold 13 M formic acid, and samples were subjected to three freeze–thaw cycles. Nucleotides were separated on polyethylenimine TLC cellulose plates (Sigma-Aldrich) and visualized by exposure on a phosphorimaging plate. Controls consisting of radiolabelled (p)ppGpp isolated from *E. coli* K-12 and GTP (Perkin Elmer) were run alongside.

Microtitre biofilm assay. Bacteria were prepared for biofilm experiments by harvesting cells from BCGA plates and resuspending them in CDM to OD₅₄₀ 0.2, which equated to a suspension of ~1 × 10⁹ c.f.u. ml^{−1}, and then diluted 1:10. The microtitre biofilm assay was performed as described by Stepanovic *et al.* (2000) with

minor modifications. Eight wells on a 96-well microtitre plate (Costar 3595) were each filled with 200 μl bacterial suspension. The outer wells were filled with distilled water to reduce evaporation from the test samples. Plates were incubated at 37 °C for 6 days. To measure biofilm formation, each well was washed three times with PBS, to remove all non-adherent cells. Remaining attached cells were fixed by the addition of 200 μl 99% methanol. Methanol was removed and plates were left to air dry. Biofilms were stained by the addition of 200 μl of 2% Hucker's crystal violet (CV) for 5 min. Excess stain was removed by pipette, followed by submersion of the plate in 300 ml fresh tap water, three times. Plates were air-dried, after which the dye bound to the adherent cells was resuspended by the addition of 200 μl 33% (v/v) glacial acetic acid. The A₅₇₀ of each well was measured using a microtitre plate reader (MultiSkan EX, Thermo), following 10 s shaking.

Intramacrophage survival assay. J774A murine macrophages were infected at an m.o.i. of 5 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, 5% CO₂ until harvesting at various times post-infection. Macrophages were disrupted by the addition of 1 ml distilled H₂O and aspiration 30 times. Bacteria were enumerated by plating onto BCGA and incubating for 3 days at 37 °C.

Determination of attenuation in mice. Groups of six female BALB/c mice (Charles River Laboratories) aged 6–8 weeks were challenged subcutaneously with ~10⁴, 10⁵ or 10⁶ c.f.u. of *F. novicida* U112 *ΔrelA::cam*. The bacteria for challenge were harvested after growth for 18 h at 37 °C on BCGA agar. Mice were challenged on day 56 post-vaccination with ~10² c.f.u. *F. novicida* U112 via the intraperitoneal route. Actual doses were determined by retrospective viable

counts. All procedures were carried out in accordance with UK Home Office guidelines. Animals were observed for signs to indicate humane end points, and these were used to direct culling.

Statistics. Statistical differences in cell densities were determined using two-way ANOVA, repeated measures ANOVA with Bonferroni's multiple comparisons test, or *t* test. Survival data were analysed using a Mantel–Cox log-rank test. All statistical analyses were performed using GraphPad Prism v5.01.

RESULTS

An *F. novicida* *relA* mutant is unable to produce (p)ppGpp under conditions of amino acid limitation

To investigate the role of RelA in *Francisella*, FTN_1518, annotated to encode a (p)ppGpp synthetase in *F. novicida* strain U112, was targeted for inactivation and a defined $\Delta relA::cam$ mutant was created. The mutant had an internal 1877 bp fragment of the *relA* gene deleted and replaced with a chloramphenicol resistance cassette. It was not expected that this would have polar effects, based on the orientation and separation of adjacent genes. However, to confirm that phenotypes were specifically due to inactivation of *relA*, the mutation was complemented by a constitutively expressed plasmid-encoded copy of the *relA* gene.

It has been reported that RelA-dependent synthesis of (p)ppGpp occurs during amino acid starvation in conjunction with growth arrest; therefore, serine starvation was induced by adding serine hydroxamate to cultures. This study determined that 2.5 mg serine hydroxamate ml^{-1} completely inhibited growth of *F. novicida* U112 (data not shown); therefore, these culture conditions were used to induce serine starvation. To confirm the function of RelA in *F. novicida*, the ability of the wild-type strain U112 $\Delta relA::cam$ and the complemented mutant strain U112 $\Delta relA::cam$ pRelA to produce (p)ppGpp was assessed. Bacteria were cultured in serine-free CDM supplemented with ^{32}P , and serine hydroxamate was added to exponentially growing cultures to induce starvation. Extraction and separation of nucleotides from these cultures by TLC demonstrated (p)ppGpp production by the wild-type strain during starvation conditions. Conversely, (p)ppGpp was undetectable in U112 $\Delta relA::cam$ under the same conditions, and the production of (p)ppGpp was restored when the mutation was complemented in U112 $\Delta relA::cam$ pRelA (Fig. 1).

F. novicida U112 $\Delta relA::cam$ has growth kinetics similar to those of the wild-type *in vitro*, but has delayed entry into stationary phase

In other bacteria, disruption of the *relA* gene can result in reduced growth rates and stationary phase growth defects (Lemos *et al.*, 2004; Gaynor *et al.*, 2005; Fisher *et al.*, 2005; Milne *et al.*, 2007; Mouery *et al.*, 2006); therefore, the effect

Fig. 1. (p)ppGpp accumulation in *F. novicida* U112 (lane 1), U112 $\Delta relA::cam$ (lane 2) and U112 $\Delta relA::cam$ pRelA (lane 3). Cultures were passaged daily in CDM[−]S for 3 days, prior to overnight culture in CDM[−]S (2 mM PO_4). Bacteria were adjusted to OD_{600} 0.1 in CDM[−]S (0.4 mM PO_4) and grown for 3 h with shaking at 37 °C. $[^{32}\text{P}]\text{H}_3\text{PO}_4$ was added to each culture to a final concentration of 10 $\mu\text{Ci ml}^{-1}$ (0.37 MBq ml^{-1}), and incubated for a further 2 h. To induce (p)ppGpp production, 2.5 mg serine hydroxamate ml^{-1} was added and cultures were incubated for 30 min. Radionucleotides were detected by phosphorimaging following separation by TLC. Positive controls of GTP and (p)ppGpp were included (not shown).

of deletion of the *relA* gene on the ability of *Francisella* to grow in liquid media was evaluated. During exponential phase, U112 $\Delta relA::cam$ had a similar growth rate to the wild-type. However, the mutant entered stationary phase later and grew to significantly higher cell densities than the wild-type ($P < 0.001$) (Fig. 2a). The wild-type phenotype was partially restored when the mutation was complemented (Fig. 2b). Using a repeated measures ANOVA with Bonferroni's multiple comparisons test, the density of the mutant was found to be significantly different from that of the wild-type and complemented strain ($P < 0.01$).

To further investigate this growth phenomenon, the ability of U112 $\Delta relA::cam$ to form biofilms was examined. RelA has been demonstrated to be necessary for efficient biofilm formation in several bacteria (Lemos *et al.*, 2004; Balzer & Mclean, 2002), possibly by reducing the nutrient levels surrounding microcolonies as the biofilm matures. Interestingly in our studies, the rate and quantity of biofilm production by U112 $\Delta relA::cam$ were greater than those of the wild-type strain, as indicated by CV staining ($P < 0.01$) (Fig. 3).

Induction of RelA is involved in resistance of *Francisella* to stress

In order to survive the different conditions experienced both in the environment and in the host, bacteria must tightly regulate their gene expression. RelA has been reported to be involved in resistance to different stresses

Fig. 2. Growth of *F. novicida* in CDM in shaking cultures. (a) *F. novicida* U112 (●) or U112 $\Delta relA::cam$ (■) were grown in CDM at 37 °C with shaking at 150 r.p.m. Samples were taken at various time points and bacteria enumerated on BCGA plates. Data points represent means and SEM of triplicate experiments (*** P <0.001). (b) *F. novicida* U112 (black bar), U112 $\Delta relA::cam$ (chequered bar) or U112 $\Delta relA::cam$ pRelA (white bar) were grown at 37 °C with shaking at 150 r.p.m. for 24 h. Bacteria were harvested and enumerated on BCGA plates. Data are presented as means and SEM of four experiments (** P <0.01).

in many bacteria, including tolerance of heat stress of *Enterococcus faecalis* (Abranches *et al.*, 2009). Using heat stress as an indicator of stress survival, the ability of the U112 $\Delta relA::cam$ mutant to survive exposure to heat stress was investigated. The survival of cultures after incubation at 48 °C for 1 h was determined by viable counts, and compared with that at 37 °C (Fig. 4). At 48 °C, survival of both the wild-type and U112 $\Delta relA::cam$ was reduced compared with survival at 37 °C. To test whether inducing the stringent response would increase thermotolerance, strains were exposed to serine hydroxamate to mimic serine starvation prior to incubation at 37 and 48 °C (Fig. 4). Wild-type bacteria demonstrated similar levels of survival at 48 and 37 °C, indicating increased survival in serine-starved cultures; however, U112 $\Delta relA::cam$ counts were significantly reduced at 48 °C compared with 37 °C

Fig. 3. Determination of biofilm formation by *F. novicida* U112 (●) or U112 $\Delta relA::cam$ (■) in CDM in static cultures. Data are presented as means and SEM of 24 cultures (** P <0.01, *** P <0.001).

(P =0.03). These data suggest that increased survival at 48 °C requires prior induction of the stringent response in a RelA-dependent manner.

F. novicida U112 $\Delta relA::cam$ has reduced ability to replicate in macrophages *in vitro*

The stringent response has been reported to be involved in intracellular survival in many pathogens, such as *Campylobacter jejuni* (Gaynor *et al.*, 2005), *L. monocytogenes* (Taylor *et al.*, 2002) and *Brucella abortus* (Kim *et al.*, 2005). To examine whether the attenuation observed *in vivo* is due to a defect in intracellular survival, the ability of U112 $\Delta relA::cam$ to infect and replicate within murine

Fig. 4. Effect of RelA on thermotolerance of *Francisella*. Survival of *F. novicida* U112 (black bars) or U112 $\Delta relA::cam$ (chequered bars) at 48 °C before and after serine starvation [induced by the addition of serine hydroxamate (SH)]. Data are presented as means and SEM of three experiments (* P =0.03).

macrophages was assessed (Fig. 5). The uptake of wild-type and U112 $\Delta relA::cam$ was similar at 0 h post-infection. A mild reduction in replication at 24 and 48 h post-infection was observed in U112 $\Delta relA::cam$ cultures compared with the wild-type, yet was statistically significant ($P<0.05$). Survival of the complemented strain U112 $\Delta relA::cam$ pRelA was similar to that of the wild-type at 0, 24 and 48 h post-infection.

***F. novicida* U112 $\Delta relA::cam$ is attenuated *in vivo* and is able to induce a protective immune response**

RelA has been suggested to be involved in virulence of several pathogens; therefore, the virulence of U112 $\Delta relA::cam$ in a BALB/c mouse model of infection was assessed. Groups of mice were challenged subcutaneously with 8.4×10^3 , 8.4×10^4 and 8.4×10^5 c.f.u. of wild-type strain U112 or U112 $\Delta relA::cam$ and observed for 14 days (Fig. 6a). All animals survived challenge with U112 $\Delta relA::cam$, with the exception of one mouse from the highest dose group. Animals challenged with the wild-type strain had succumbed to infection by day 4 post-challenge. The median lethal dose (MLD) for *F. novicida* U112 has been previously calculated as 3.4×10^2 c.f.u. by the subcutaneous route (Milne *et al.*, 2007). These results demonstrate a significant increase in survival ($P=0.001$) when mice were challenged with U112 $\Delta relA::cam$, demonstrating that this mutant was attenuated >100 fold. To test whether U112 $\Delta relA::cam$ was able to stimulate a protective immune response, the surviving animals were challenged 56 days later with 6×10^3 c.f.u. (equivalent to 600 MLD) of wild-type *F. novicida* U112 by the intraperitoneal route and observed for 14 days (Fig. 6b).

Fig. 5. Survival of *F. novicida* U112 (black bars), U112 $\Delta relA::cam$ (chequered bars) or U112 $\Delta relA::cam$ pRelA (white bars) in J774A cells. Cells were infected at m.o.i. 5, and intracellular bacteria were harvested and enumerated at 0, 24 and 48 h post-infection (p. i.). Data are presented as means and SEM of triplicate wells, performed on three separate occasions (* $P<0.05$).

Fig. 6. Effects of inactivation of *relA* on virulence of *F. novicida* U112 in BALB/c mice. (a) Groups of six mice were challenged by the subcutaneous route with 8.4×10^3 (▼), 8.4×10^4 (●) or 8.4×10^5 (■) c.f.u. U112 $\Delta relA::cam$ or 1×10^4 c.f.u. wild-type strain U112 (▲). (b) Surviving animals and six naïve animals (▲) were challenged intraperitoneally 56 days later with 600 MLD wild-type *F. novicida* U112.

Survival of mice immunized with the lowest U112 $\Delta relA::cam$ dose was significantly increased compared with that of naïve animals ($P=0.02$). These results demonstrated that vaccination with *F. novicida* U112 $\Delta relA::cam$ induced high levels of protective immunity against wild-type *F. novicida* infection *in vivo*.

DISCUSSION

F. tularensis must closely regulate gene expression to maximize survival in response to changes in environmental conditions or varying niches. However, few regulators, such as two-component regulatory systems, have been identified (Mohapatra *et al.*, 2007). Other regulatory systems may therefore be important for *F. tularensis* virulence and survival. For example, polyphosphate has been shown to be important in the stress responses of a range of pathogens, including *F. tularensis* (Richards *et al.*,

2008). Polyphosphate has also been shown to be required for virulence for a range of pathogens, possibly by contributing to (p)ppGpp (Manganelli, 2007).

Two proteins well characterized for their role in (p)ppGpp metabolism are RelA and SpoT. RelA catalyses the production of (p)ppGpp, which is subsequently converted to ppGpp, while SpoT is a bifunctional enzyme capable of both synthesis and degradation of the signal molecule (Jain *et al.*, 2006). Both *relA* and *spoT* homologues are present in the *Francisella* genome. In some bacteria, inactivation of both *relA* and *spoT* is required to completely abolish (p)ppGpp synthesis, while in other bacteria, RelA is dominant in (p)ppGpp synthesis. In this study, we have confirmed the role of FTN_1518 in *F. novicida* U112 as a (p)ppGpp synthetase, and demonstrated that disruption of this gene results in abolition of ppGpp production under conditions of amino acid starvation. This is similar to the situation in *Pseudomonas aeruginosa* (Erickson *et al.*, 2004) and *V. cholerae* (Haralalka *et al.*, 2003), in which mutation of the *relA* gene alone results in reduced (p)ppGpp accumulation. Unlike other organisms such as *E. coli*, in which both *relA* and *spoT* genes need to be inactivated (Xiao *et al.*, 1991), RelA alone appears to be required for (p)ppGpp synthesis in *F. novicida* during amino acid starvation.

The role of (p)ppGpp in *Francisella* appears to be complex, as disruption of the *relA* gene did not result in a reduced growth rate, a stationary phase defect or reduced biofilm formation, as reported in other bacteria (Fisher *et al.*, 2005; Lemos *et al.*, 2004; Okada *et al.*, 2002). Many basic questions remain unanswered as to how (p)ppGpp exerts its effects, but it is thought that (p)ppGpp acts at the level of transcription, targeting RNA polymerase (Potrykus & Cashel, 2008). It has been proposed that growth defects observed in *relA* mutants can be attributed to the lack of (p)ppGpp-dependent expression of the *rpoS* gene, which encodes σ^S (Gentry *et al.*, 1993). This alternative sigma factor controls expression of genes or operons involved in protecting cells from stress, including stationary phase survival, high osmolarity, oxidative damage and acidic pH (Kazmierczak *et al.*, 2005). It is thought that (p)ppGpp increases the ability of both σ^S and σ^{32} to compete with the vegetative sigma factor σ^{70} for RNA polymerase (Jishage *et al.*, 2002), presumably resulting in increased expression of σ^S -induced general stress response and σ^{32} -dependent heat-shock response genes. Interestingly, *in silico* studies have only identified the σ^{32} and σ^{70} subunits in *Francisella*, and the other subunits are absent (Meibom *et al.*, 2008). The decreased ability of *F. novicida* to survive heat shock suggests a role for RelA in combating stress encountered during the infectious process, perhaps in a σ^{32} -dependent manner (Meibom *et al.*, 2008), where (p)ppGpp is the trigger. A similar role for RelA in resistance to heat stress has been reported in *Enterococcus faecalis*, where RelA is responsible for resistance to stress, whereas an alternative synthase RelQ is responsible for maintaining basal levels of (p)ppGpp (Abranches *et al.*, 2009).

The ability of U112 $\Delta relA::cam$ to grow to higher cell densities as planktonic and biofilm cultures is unusual, but similar effects have been observed in $\Delta relA\Delta spoT$ mutants of *E. coli*, which demonstrate more rapid growth (Xiao *et al.*, 1991) and increased biofilm formation in nutrient-rich media (Balzer & Mclean, 2002). It appears that *F. novicida* U112 $\Delta relA::cam$ is not able to respond to nutrient depletion, as a result of being unable to invoke the RelA-dependent stringent response. Bacterial cells in stationary phase are very different from exponentially growing bacteria. For example, stationary phase cells are smaller than those in exponential phase, have more robust membranes due to changes in lipid composition, and more highly cross-linked peptidoglycan (Davis *et al.*, 1980; Ingraham *et al.*, 1983). By monitoring nutrient levels in the surrounding environment and adjusting gene expression, for example by the stringent response, bacteria can prepare for stationary phase to optimize their survival when nutrients are depleted. The U112 $\Delta relA::cam$ mutant does not appear to modulate its growth rate in response to declining nutrient levels when it should be preparing for stationary phase. It is not known whether growth is finally arrested as a result of nutrient limitation or whether another regulatory pathway subsequently arrests growth.

Bacterial intracellular accumulation of (p)ppGpp has a direct effect on virulence, invasion, pathogenesis and host immune system evasion. Many pathogens have evolved survival strategies triggered by (p)ppGpp (Braeken *et al.*, 2006). For example, accumulation of (p)ppGpp in *Salmonella* appears to induce HilA expression, which in turn regulates expression of SPI 1 and SPI 2 virulence genes (Thompson *et al.*, 2006; Song *et al.*, 2004). The SPI 1 genes are involved in host cell invasion and the SPI 2 genes are required for replication within the host cell. Similar to our findings for *F. novicida*, *Salmonella* (p)ppGpp-deficient mutants are attenuated in mice but able to induce a protective immune response (Pizarro-Cerda & Tedin, 2004; Thompson *et al.*, 2006; Song *et al.*, 2004). In *Legionella pneumophila*, (p)ppGpp has been shown to trigger a cascade that leads to differentiation of replicating bacteria to a transmissible, non-replicating form, with the induction of virulence traits such as motility (Swanson & Hammer, 2000). Stress-induced (p)ppGpp production in *Helicobacter pylori* is required for survival during acid exposure and aerobic shock (Mouery *et al.*, 2006; Wells & Gaynor, 2006), conditions typically encountered during the course of infection. In *Francisella*, intracellular replication and virulence are dependent on MglA and SspA, which regulates the FPI (Guina *et al.*, 2007; Nano & Schmerk, 2007; Charity *et al.*, 2007). In this study, we have identified a global regulatory system that plays a role in *F. novicida* virulence, where a *relA* mutant had reduced survival in macrophage-like cells, and was attenuated more than 100-fold in a BALB/c mouse model. We also confirmed that the reduced virulence observed was not a result of a growth defect, as has been described for other (p)ppGpp-deficient

mutants (Fisher *et al.*, 2005; Lemos *et al.*, 2004; Okada *et al.*, 2002). Uptake of *F. novicida* U112 $\Delta relA::cam$ into murine macrophages was similar to that of the wild-type and indicates that RelA is not involved in immune cell invasion. However, intracellular replication was impaired, suggesting that RelA has a regulatory role in *Francisella*, affecting the expression of genes whose products contribute to survival in nutrient-poor and hostile environments found within host cells. In addition, though unable to cause lethality in mice, U112 $\Delta relA::cam$ was able to stimulate a protective immune response affording greater than 60% survival against a homologous wild-type challenge.

In conclusion, we have demonstrated that *F. novicida* U112 responds to amino acid starvation in a RelA-dependent manner, resulting in elevated intracellular (p)ppGpp levels, and that RelA is required for virulence and resistance to stress. This study provides further insight into the understanding of gene regulation in *F. tularensis*.

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REFERENCES

- Abranches, J., Martinez, A. R., Kajfasz, J. K., Chavez, V., Garsin, D. A. & Lemos, J. A. (2009). The molecular alarmone (p)ppGpp mediates stress responses, vancomycin tolerance and virulence in *Enterococcus faecalis*. *J Bacteriol* **191**, 2248–2256.
- Balzer, G. J. & Mclean, R. J. C. (2002). The stringent response genes *relA* and *spoT* are important for *Escherichia coli* biofilms under slow-growth conditions. *Can J Microbiol* **48**, 675–680.
- Baron, G. S. & Nano, F. E. (1998). MglA and MglB are required for the intramacrophage growth of *Francisella novicida*. *Mol Microbiol* **29**, 247–259.
- Battesti, A. & Bouveret, E. (2009). Bacteria possessing two RelA/SpoT-like proteins have evolved a specific stringent response involving the acyl carrier protein–SpoT interaction. *J Bacteriol* **191**, 616–624.
- Braeken, K., Moris, M., Daniels, R., Vanderleyden, J. & Michiels, J. (2006). New horizons for (p)ppGpp in bacterial and plant physiology. *Trends Microbiol* **14**, 45–54.
- Bugrysheva, J., Dobrikova, E. Y., Sartakova, M. L., Caimano, M. J., Daniels, T. J., Radolf, J. D., Godfrey, H. P. & Cabello, F. C. (2003a). Characterization of the stringent response and *relBbu* expression in *Borrelia burgdorferi*. *J Bacteriol* **185**, 957–965.
- Bugrysheva, J., Dobrikova, E. Y., Sartakova, M. L., Caimano, M. J., Daniels, T. J., Radolf, J. D., Godfrey, H. P. & Cabello, F. C. (2003b). Characterization of the stringent response and *relBbu* expression in *Borrelia burgdorferi*. *J Bacteriol* **185**, 957–965.
- Cashel, M. (1994). Detection of (p)ppGpp accumulation patterns in *Escherichia coli* mutants. In *Methods in Molecular Genetics*, vol. 3, *Molecular Microbiology. Techniques, part A*, pp. 341–356. Edited by K. W. Adolph. New York: Academic Press.
- Chamberlain, R. E. (1965). Evaluation of live tularemia vaccine prepared in a chemically defined medium. *Appl Microbiol* **13**, 232–235.
- Charity, J. C., Costante-Hamm, M. M., Balon, E. L., Boyd, D. H., Rubin, E. J. & Dove, S. L. (2007). Twin RNA polymerase-associated proteins control virulence gene expression in *Francisella tularensis*. *PLoS Pathog* **3**, e84.
- Dalebroux, Z. D., Edwards, R. L. & Swanson, M. S. (2009). SpoT governs *Legionella pneumophila* differentiation in host macrophages. *Mol Microbiol* **71**, 640–658.
- Davis, B. D., Dulbecco, R., Eisen, H. N. & Ginsberg, H. S. (1980). *Microbiology*. Hagerstown, MD: Harper and Row.
- Ellis, J., Oyston, P. C. F., Green, M. & Titball, R. W. (2002). Tularemia. *Clin Microbiol Rev* **15**, 631–646.
- Erickson, D. L., Lines, J. L., Pesci, E. C., Venturi, V. & Storey, D. G. (2004). *Pseudomonas aeruginosa relA* contributes to virulence in *Drosophila melanogaster*. *Infect Immun* **72**, 5638–5645.
- Fisher, S. D., Reger, A. D., Baum, A. & Hill, S. A. (2005). RelA alone appears essential for (p)ppGpp production when *Neisseria gonorrhoeae* encounters nutritional stress. *FEMS Microbiol Lett* **248**, 1–8.
- Gaynor, E. C., Wells, D. H., MacKichan, J. K. & Falkow, S. (2005). The *Campylobacter jejuni* stringent response controls specific stress survival and virulence-associated phenotypes. *Mol Microbiol* **56**, 8–27.
- Gentry, D. R., Hernandez, V. J., Nguyen, L. H., Jensen, D. B. & Cashel, M. (1993). Synthesis of the stationary-phase sigma-factor σ^S is positively regulated by ppGpp. *J Bacteriol* **175**, 7982–7989.
- Golovliov, I., Sjostedt, A., Mokrievech, A. & Pavlov, V. (2003). A method for allelic replacement in *Francisella tularensis*. *FEMS Microbiol Lett* **222**, 273–280.
- Gray, C. G., Cowley, S. C., Cheung, K. K. M. & Nano, F. E. (2002). The identification of five genetic loci of *Francisella novicida* associated with intracellular growth. *FEMS Microbiol Lett* **215**, 53–56.
- Guina, T., Radulovic, D., Bahrami, A. J., Bolton, D. L., Rohmer, L., Jones-Isaac, K. A., Chen, J., Gallagher, L. A., Gallis, B. & other authors (2007). MglA regulates *Francisella tularensis* subsp. *novicida* (*Francisella novicida*) response to starvation and oxidative stress. *J Bacteriol* **189**, 6580–6586.
- Hammer, B. K. & Swanson, M. S. (1999). Co-ordination of *Legionella pneumophila* virulence with entry into stationary phase by ppGpp. *Mol Microbiol* **33**, 721–731.
- Haralalka, S., Nandi, S. & Bhadra, R. K. (2003). Mutation in the *relA* gene of *Vibrio cholerae* affects in vitro and in vivo expression of virulence factors. *J Bacteriol* **185**, 4672–4682.
- Ingraham, J. L., Maaloe, O. & Neidhardt, F. C. (1983). *Growth of the Bacterial Cell*. Sunderland, MA: Sinauer Associates.
- Jain, V., Kumar, M. & Chatterji, D. (2006). ppGpp: stringent response and survival. *J Microbiol* **44**, 1–10.
- Jishage, M., Kvint, K., Shingler, V. & Nystrom, T. (2002). Regulation of σ factor competition by the alarmone ppGpp. *Genes Dev* **16**, 1260–1270.
- Kazmierczak, M. J., Wiedmann, M. & Boor, K. J. (2005). Alternative sigma factors and their roles in bacterial virulence. *Microbiol Mol Biol Rev* **69**, 527.
- Kim, S., Watanabe, K., Suzuki, H. & Watarai, M. (2005). Roles of *Brucella abortus* SpoT in morphological differentiation and intramacrophagic replication. *Microbiology* **151**, 1607–1617.
- Larsson, P., Oyston, P. C. F., Chain, P., Chu, M., Duffield, M. L., Gabbert, N. M., Fuxelius, H. H., Garcia, E., Halltorp, G. & other authors (2005). The complete genome sequence of *Francisella tularensis*, the causative agent of tularemia. *Nat Genet* **37**, 153–159.
- Lauriano, C. M., Barker, J. R., Yoon, S. S., Nano, F. E., Arulanandam, B. P., Hasset, D. J. & Klose, K. E. (2004). MglA regulates transcription of virulence factors necessary for *Francisella tularensis*

- intraamoebae and intramacrophage survival. *Proc Natl Acad Sci U S A* **101**, 4246–4249.
- Lemos, J. A. C., Brown, T. A. & Burne, R. A. (2004). Effects of RelA on key virulence properties of planktonic and biofilm populations of *Streptococcus mutans*. *Infect Immun* **72**, 1431–1440.
- Li, W., Liu, L., Chen, H. & Zhou, R. (2009). Identification of *Streptococcus suis* genes preferentially expressed under iron starvation by selective capture of transcribed sequences. *FEMS Microbiol Lett* **292**, 123–133.
- Lindgren, H., Golovliov, I., Baranov, V., Ernst, R. K., Telepnev, M. & Sjostedt, A. (2004). Factors affecting the escape of *Francisella tularensis* from the phagolysosome. *J Med Microbiol* **53**, 953–958.
- Magnusson, L. U., Farewell, A. & Nystrom, T. (2005). ppGpp: a global regulator in *Escherichia coli*. *Trends Microbiol* **13**, 236–242.
- Manganelli, R. (2007). Polyphosphate and stress response in mycobacteria. *Mol Microbiol* **65**, 258–260.
- Meibom, K. L., Dubail, I., Dupuis, M., Barel, M., Lenco, J., Stulik, J., Golovliov, I., Sjostedt, A. & Charbit, A. (2008). The heat-shock protein ClpB of *Francisella tularensis* is involved in stress tolerance and is required for multiplication in target organs of infected mice. *Mol Microbiol* **67**, 1384–1401.
- Milne, T. S., Michell, S. L., Diaper, H., Wikstrom, P., Svensson, K., Oyston, P. C. F. & Titball, R. W. (2007). A 55 kDa hypothetical membrane protein is an iron-regulated virulence factor of *Francisella tularensis* subsp. *novicida* U112. *J Med Microbiol* **56**, 1268–1276.
- Milton, D. L., O'Toole, R., Horstedt, P. & Wolf-Watz, H. (1996). Flagellin A is essential for the virulence of *Vibrio anguillarum*. *J Bacteriol* **178**, 1310–1319.
- Mittenhuber, G. (2001). Comparative genomics and evolution of genes encoding bacterial (p)ppGpp synthetases/hydrolases (the Rel, RelA and SpoT proteins). *J Mol Microbiol Biotechnol* **3**, 585–600.
- Mohapatra, N. P., Soni, S., Bell, B. L., Warren, R., Ernst, R. K., Muszynski, A., Carlson, R. W. & Gunn, J. S. (2007). Identification of an orphan response regulator required for the virulence of *Francisella* spp. and transcription of pathogenicity island genes. *Infect Immun* **75**, 3305–3314.
- Mouery, K., Rader, B. A., Gaynor, E. C. & Guillemin, K. (2006). The stringent response is required for *Helicobacter pylori* survival of stationary phase, exposure to acid, and aerobic shock. *J Bacteriol* **188**, 5494–5500.
- Nano, F. E. & Schmerk, C. (2007). The *Francisella* pathogenicity island. *Ann N Y Acad Sci* **1105**, 122–137.
- Nano, F. E., Zhang, N., Cowley, S. C., Klose, K. E., Cheung, K. K. M., Roberts, M. J., Ludu, J. S., Letendre, G. W., Meierovics, A. I. & other authors (2004). A *Francisella tularensis* pathogenicity island required for intramacrophage growth. *J Bacteriol* **186**, 6430–6436.
- Nylund, A., Ottem, K. F., Watanabe, K., Karlsbakk, E. & Krossoy, B. (2006). *Francisella* sp (Family Francisellaceae) causing mortality in Norwegian cod (*Gadus morhua*) farming. *Arch Microbiol* **185**, 383–392.
- Ojha, A. K., Mukherjee, T. K. & Chatterji, D. (2000). High intracellular level of guanosine tetraphosphate in *Mycobacterium smegmatis* changes the morphology of the bacterium. *Infect Immun* **68**, 4084–4091.
- Okada, Y., Makino, S., Tobe, T., Okada, N. & Yamazaki, S. (2002). Cloning of *rel* from *Listeria monocytogenes* as an osmotolerance involvement gene. *Appl Environ Microbiol* **68**, 1541–1547.
- Ostland, V. E., Stannard, J. A., Creek, J. J., Hedrick, R. P., Ferguson, H. W., Carlberg, J. M. & Westerman, M. E. (2006). Aquatic *Francisella*-like bacterium associated with mortality of intensively cultured hybrid striped bass *Morone chrysops* × *M. saxatilis*. *Dis Aquat Organ* **72**, 135–145.
- Oyston, P. C. F. (2008). *Francisella tularensis*: unravelling the secrets of an intracellular pathogen. *J Med Microbiol* **57**, 921–930.
- Parker, R. R., Steinhaus, E. A., Kohls, G. M. & Jellison, W. L. (1951). Contamination of natural waters and mud with *Pasteurella tularensis* and tularemia in beavers and muskrats in the northwestern United States. *Bull Natl Inst Health* **193**, 1–161.
- Parkhill, J., Achtman, M., James, K. D., Bentley, S. D., Churcher, C., Klee, S. R., Morelli, G., Basham, D., Brown, D. & other authors (2000). Complete DNA sequence of a serogroup A strain of *Neisseria meningitidis* Z2491. *Nature* **404**, 502–506.
- Pizarro-Cerda, J. & Tedin, K. (2004). The bacterial signal molecule, ppGpp, regulates *Salmonella* virulence gene expression. *Mol Microbiol* **52**, 1827–1844.
- Potrykus, K. & Cashel, M. (2008). (p)ppGpp: still magical? *Annu Rev Microbiol* **62**, 35–51.
- Primm, T. P., Andersen, S. J., Mizrahi, V., Avarbock, D., Rubin, H. & Barry, C. E. (2000). The stringent response of *Mycobacterium tuberculosis* is required for long-term survival. *J Bacteriol* **182**, 4889–4898.
- Quarry, J. E., Isherwood, K. E., Michell, S. L., Diaper, H., Titball, R. W. & Oyston, P. C. F. (2007). A *Francisella tularensis* subspecies *novicida* *purF* mutant, but not a *purA* mutant, induces protective immunity to tularemia in mice. *Vaccine* **25**, 2011–2018.
- Richards, M. I., Michell, S. L. & Oyston, P. C. F. (2008). An intracellularly inducible gene involved in virulence and polyphosphate production in *Francisella*. *J Med Microbiol* **57**, 1183–1192.
- Rohmer, L., Brittnacher, M., Svensson, K., Buckley, D., Haugen, E., Zhou, Y., Chang, J., Levy, R., Hayden, H. & other authors (2006). Potential source of *Francisella tularensis* live vaccine strain attenuation determined by genome comparison. *Infect Immun* **74**, 6895–6906.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Simon, R., Priefer, U. & Puhler, A. (1983). A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram-negative bacteria. *Biotechnology (N Y)* **1**, 784–791.
- Sjostedt, A. (2006). Intracellular survival mechanisms of *Francisella tularensis*, a stealth pathogen. *Microbes Infect* **8**, 561–567.
- Song, M. Y., Kim, H. J., Kim, E. Y., Shin, M. S., Lee, H. C., Hong, Y. J., Rhee, J. H., Yoon, H., Ryu, S. & other authors (2004). ppGpp-dependent stationary phase induction of genes on *Salmonella* pathogenicity island 1. *J Biol Chem* **279**, 34183–34190.
- Stepanovic, S., Vukovic, D., Dakic, I., Savic, B. & Svabic-Vlahovic, M. (2000). A modified microtiter-plate test for quantification of staphylococcal biofilm formation. *J Microbiol Methods* **40**, 175–179.
- Sureka, K., Dey, S., Datta, P., Singh, A. K., Dasgupta, A., Rodrigue, S., Basu, J. & Kundu, M. (2007). Polyphosphate kinase is involved in stress-induced *mprAB-sigE-rel* signalling in mycobacteria. *Mol Microbiol* **65**, 261–276.
- Swanson, M. S. & Hammer, B. K. (2000). *Legionella pneumophila* pathogenesis: a fateful journey from amoebae to macrophages. *Annu Rev Microbiol* **54**, 567–613.
- Taylor, C. M., Beresford, M., Epton, H. A. S., Sigee, D. C., Shama, G., Andrew, P. W. & Roberts, I. S. (2002). *Listeria monocytogenes relA* and *hpt* mutants are impaired in surface-attached growth and virulence. *J Bacteriol* **184**, 621–628.

Tettelin, H., Saunders, N. J., Heidelberg, J., Jeffries, A. C., Nelson, K. E., Eisen, J. A., Ketchum, K. A., Hood, D. W., Peden, J. F. & other authors (2000). Complete genome sequence of *Neisseria meningitidis* serogroup B strain MC58. *Science* **287**, 1809–1815.

Thompson, A., Rolfe, M. D., Lucchini, S., Schwerk, P., Hinton, J. C. D. & Tedin, K. (2006). The bacterial signal molecule, ppGpp, mediates the environmental regulation of both the invasion and intracellular virulence gene programs of *Salmonella*. *J Biol Chem* **281**, 30112–30121.

Wells, D. H. & Gaynor, E. C. (2006). *Helicobacter pylori* initiates the stringent response upon nutrient and pH downshift. *J Bacteriol* **188**, 3726–3729.

Williams, M. D., Ouyang, T. X. & Flickinger, M. C. (1994). Starvation-induced expression of SspA and SspB – the effects of a null mutation in *sspA* on *Escherichia coli* protein synthesis and survival during growth and prolonged starvation. *Mol Microbiol* **11**, 1029–1043.

Xiao, H., Kalman, M., Ikehara, K., Zemel, S., Glaser, G. & Cashel, M. (1991). Residual guanosine 3',5'-bispyrophosphate synthetic activity of *relA* null mutants can be eliminated by *spoT* null mutations. *J Biol Chem* **266**, 5980–5990.

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