Pivotal role of the *Francisella tularensis* heat-shock sigma factor RpoH

Nathalie Grall,^{1,2} Jonathan Livny,³ Matthew Waldor,^{3,4} Monique Barel,^{1,2} Alain Charbit^{1,2} and Karin L. Meibom^{1,2}

Correspondence

Karin L. Meibom
karin.meibom@inserm.fr

¹Université Paris Descartes, Faculté de Médecine Necker-Enfants Malades, F-75015 Paris, France

²INSERM, U570, Unit of Pathogenesis of Systemic Infections, F-75015 Paris, France

³Channing Laboratories, Brigham and Women's Hospital, Harvard Medical School, 181 Longwood Avenue, Boston, MA 02115, USA

⁴Howard Hughes Medical Institute, 4000 Jones Bridge Road, Chevy Chase, MD 20815-6789, USA

Francisella tularensis is a highly infectious pathogen that infects animals and humans to cause the disease tularemia. The primary targets of this bacterium are macrophages, in which it replicates in the cytoplasm after escaping the initial phagosomal compartment. The ability to replicate within macrophages relies on the tightly regulated expression of a series of genes. One of the most commonly used means of coordinating the regulation of multiple genes in bacteria consists of the association of dedicated alternative sigma factors with the core of the RNA polymerase (RNAP). *In silico* analysis of the *F. tularensis* LVS genome led us to identify, in addition to the genes encoding the RNAP core (comprising the $\alpha 1$, $\alpha 2$, β , β' and ω subunits), one gene (designated *rpoD*) encoding the major sigma factor σ^{70} , and a unique gene (*FTL_0851*) encoding a putative alternative sigma factor homologue of the σ^{32} heat-shock family (designated *rpoH*). Hence, *F. tularensis* represents one of the minority of bacterial species that possess only one or no alternative sigma factor in addition to the main factor σ^{70} . In the present work, we show that *FTL_0851* encodes a genuine σ^{32} factor. Transcriptomic analyses of the *F. tularensis* LVS heat-stress response allowed the identification of a series of orthologues of known heat-shock genes (including those for Hsp40, GroEL, GroES, DnaK, DnaJ, GrpE, ClpB and ClpP) and a number of genes implicated in *Francisella* virulence. A bioinformatic analysis was used to identify genes preceded by a putative σ^{32} -binding site, revealing both similarities to and differences from RpoH-mediated gene expression in *Escherichia coli*. Our results suggest that RpoH is an essential protein of *F. tularensis*, and positively regulates a subset of genes involved in the heat-shock response.

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INTRODUCTION

Francisella tularensis is a Gram-negative, facultative intracellular pathogen that causes tularemia in humans and a large number of animal species. Human infections can occur after ingestion of contaminated water or food,

Abbreviations: FDR, false discovery rate; FPI, *Francisella* pathogenicity island, MFS, major facilitator superfamily; PFM, position frequency matrix; qRT-PCR, quantitative RT-PCR; RNAP, RNA polymerase; TIGR, The Institute for Genomic Research.

The microarray data discussed in this paper have been deposited in ArrayExpress under the accession numbers E-MEXP-2048 and E-MEXP-2037.

Five supplementary tables, listing primers used in this study, genes significantly decreased in expression after temperature upshift, genes that were used in BioProspector analysis, putative binding sites found by BioProspector and used for building of matrices, and genes with a potential σ^{32} -binding sequence in their promoter region, are available with the online version of this paper.

through arthropod bites, from contaminated animal carcasses, or by inhalation of bacteria. Due to its high infectivity and the severity of disease, *F. tularensis* has been classified by the US Centers for Disease Control as a potential biological weapon (for reviews, see McLendon *et al.*, 2006; Sjostedt, 2006). The pathogenicity of *F. tularensis* is largely dependent on its ability to replicate inside host cells, and in particular in macrophages *in vivo*. Several factors important for virulence have been identified, and in particular genes located in the *Francisella* pathogenicity island (FPI) (de Bruin *et al.*, 2007; Golovliov *et al.*, 2003; Lai *et al.*, 2004; Ludu *et al.*, 2008; Nano *et al.*, 2004; Nano & Schmerk, 2007; Santic *et al.*, 2007; Tempel *et al.*, 2006; Weiss *et al.*, 2007), but the molecular mechanisms underlying disease are not yet fully understood.

Regulation of gene expression in bacteria occurs primarily at the transcriptional level. The association of dedicated alternative sigma factors to the core of the RNA polymerase

(RNAP) provides a simple and efficient way for bacteria to rapidly adapt to various environmental changes. The RNAP holoenzyme contains the subunits of the core molecule (comprising two α subunits, the β , β' and ω subunits) and a sigma factor enabling the holoenzyme to specifically recognize promoter elements and initiate transcription at these sites. The regulon of a single sigma factor can comprise hundreds of genes. Many sigma factors are induced in response to environmental changes and are often controlled by anti-sigma factors (for reviews see Gourse *et al.*, 2006; Gruber & Gross, 2003). The number of sigma factors encoded by different bacterial species varies considerably. For example, seven sigma factors have been identified in *Escherichia coli* to date, whereas *Streptomyces coelicolor* is thought to encode at least 61. Some bacteria contain only a single sigma factor, but the majority express several, and the number generally relates to the size of the genome and the environmental variation that allows growth of the organism (Kill *et al.*, 2005; Rodrigue *et al.*, 2006).

Another ubiquitous mode of bacterial response to environmental changes involves the function of two-component signal transduction systems (TCS). Each TCS consists of a membrane-bound sensor that monitors environmental variations and a dedicated cytoplasmic response regulator that mediates an adaptive response, i.e. a transcriptional regulation of gene expression. Strikingly, *Francisella* subspecies do not possess a single complete TCS pair (Mohapatra *et al.*, 2007 and references therein), and analyses of the *F. tularensis* genomes have revealed the presence of only a single gene encoding a protein homologous to an alternative sigma factor, thereby placing *F. tularensis* within the relatively small group of bacterial species that express only a single or no alternative sigma factor. The absence of any complete TCS and the presence of only one gene encoding an alternative sigma factor may reflect the fact that the *Francisella* genome is relatively small and/or is thought to be deteriorating, based on the high number of pseudogenes and incomplete biosynthetic pathways (Larsson *et al.*, 2005). The absence of multiple alternative sigma factors or TCS suggests that *F. tularensis* has a limited ability to respond to and proliferate in diverse environmental conditions.

Given the small number of putative regulatory proteins encoded by *F. tularensis*, we were interested in examining the function of the only alternative sigma factor that may help this organism in survival and in the stress response. In the present study, we show that the only alternative sigma factor encoded by the *F. tularensis* genome, RpoH, is a genuine σ^{32} unit, and our data suggest that *rpoH* is an essential gene of *F. tularensis*. To gain further insight into the RpoH-dependent mechanisms of gene expression in *F. tularensis* we performed a transcriptomic analysis of the heat-stress response of *F. tularensis* LVS and evaluated the role of RpoH in the regulation of the bacterial genome in an RpoH-overproducing strain. In addition, a bioinformatic analysis determined a consensus binding sequence

for RpoH and identified genes preceded by a putative RpoH-binding site. Our results demonstrate similarities between the heat-stress response in *F. tularensis* and the well-studied response in *E. coli*, but also illuminate differences between these two organisms. A number of heat-induced genes have previously been demonstrated to be of importance for the ability of *F. tularensis* to survive and/or multiply inside host cells, suggesting that stress responses are of significance for the virulence of *Francisella*.

METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1 and oligonucleotide sequences in Supplementary Table S1. *F. tularensis* LVS was grown on pre-made chocolate agar enriched with PolyViteX (bioMérieux), chocolate agar plates prepared from GC medium base, IsoVitalex vitamins and haemoglobin (BD Biosciences), or in Schaedler + vitamin K3 broth (Schaedler-K3, bioMérieux) at 37 °C or room temperature. *E. coli* strains were grown on Luria–Bertani (LB) plates or in LB broth (Difco) at 37 °C or room temperature aerobically.

For *F. tularensis*, antibiotics were used at 5 µg ml⁻¹ (kanamycin) and 1.75 µg ml⁻¹ (chloramphenicol). For *E. coli*, antibiotics were used at the following concentrations: 25 µg ml⁻¹ (kanamycin), 2.5 µg ml⁻¹ (chloramphenicol) and 50 µg ml⁻¹ (ampicillin).

The plasmid used for overexpression of *rpoH*, pFNLTP6gro-0851, was constructed by amplifying a 957 bp fragment (corresponding to the sequence 78 bp upstream of the *rpoH* gene and the entire gene) using primers 0851_F and 0851_R (Supplementary Table S1), followed by digestion with *NdeI* and *NotI* and cloning into plasmid pFNLTP6gro (Maier *et al.*, 2004; Meibom *et al.*, 2009) downstream of the *groE* operon promoter of *F. tularensis*. pFNLTP6gro-0851 was introduced into the LVS strain by electroporation.

The plasmid pBAD*rpoH* was constructed by amplifying the LVS *rpoH* gene using primers 0851_F2 and 0851_R2, followed by digestion with *EcoRI* and cloning into plasmid pBAD downstream of the arabinose-inducible pBAD promoter. The *rpoH* gene was verified to be in the right orientation by PCR using primers 5'pBAD and 0851_R2.

Functional complementation. *E. coli* K-12 strain MG1655 was transformed with either pRB11 (Yura *et al.*, 2007) or pBAD*rpoH*. Phage P1 was used to transduce the $\Delta rpoH::Kan\ zhf50::Tn10$ mutation from strain CAG48315 (Zhou *et al.*, 1988) into MG1655/pRB11 and MG1655/pBAD*rpoH*. For the transduction lysate preparation, an overnight culture of CAG48315 was diluted 100-fold in fresh LB supplemented with 5 mM CaCl₂ and 0.2 % glucose, and grown at 37 °C with shaking for 1 h before adding 200 µl P1 phage lysate into 5 ml culture. When the culture had lysed completely, several drops of chloroform were added and the transduction lysate was kept at 4 °C. For the transduction, the recipient strains (MG1655/pRB11 and MG1655/pBAD*rpoH*) were grown overnight in LB supplemented with 50 µg ampicillin ml⁻¹ and 0.2 % arabinose. The cells were then harvested and resuspended in LB supplemented with 50 µg ampicillin ml⁻¹, 0.2 % arabinose, 100 mM MgSO₄ and 5 mM CaCl₂. A 100 µl volume of bacterial cells and 100 µl transduction lysate were mixed and incubated at 37 °C with shaking for 30 min. After addition of 200 µl 1 M sodium citrate (pH 5.5), cells were incubated at 37 °C with shaking in 1 ml LB containing 50 µg ampicillin ml⁻¹ and 0.2 % arabinose for 1 h, and plated on LB agar supplemented with 50 µg ampicillin ml⁻¹, 25 µg kanamycin ml⁻¹ and 0.2 % arabinose. Colonies appearing after overnight incubation were verified to have the $\Delta rpoH::Kan\ zhf50::Tn10$ mutation by

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description or genotype	Source
<i>E. coli</i> strains		
DH5 α	F ⁻ ϕ 80 <i>lacZ</i> Δ M15 <i>recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1</i> (<i>lacZYA-argF</i>) U169	Laboratory strain collection
CAG48315	λ^- phtpg- <i>lacZ</i> Δ <i>rpoH</i> ::Kan <i>zhf50</i> ::Tn10, pRB11	Zhou <i>et al.</i> (1988)
MG1655	K-12	Arnaud Gutierrez
MG1655/pRB11	MG1655 containing plasmid pRB11 expressing <i>E. coli</i> <i>rpoH</i>	This study
MG1655/pBAD <i>rpoH</i>	MG1655 containing plasmid pBAD expressing <i>F. tularensis</i> <i>rpoH</i>	This study
MG1655 Δ <i>rpoH</i> /pRB11	MG1655/pRB11 transduced with P1 lysate from CAG48315	This study
MG1655 Δ <i>rpoH</i> /pBAD <i>rpoH</i>	MG1655/pBAD <i>rpoH</i> transduced with P1 lysate from CAG48315	This study
<i>F. tularensis</i> strains		
LVS	<i>F. tularensis</i> subspecies <i>holarctica</i> live vaccine strain	Laboratory strain collection
LVS/pFNLTP6 <i>gro</i> -0851	LVS with plasmid pFNLTP6 <i>gro</i> -0851 expressing <i>rpoH</i> from the <i>groES</i> promoter	This study
LVS/pFNLTP6 <i>gro</i>	LVS with plasmid pFNLTP6 <i>gro</i> with no insert	This study
Plasmids		
pRB11	pBR322 derivative with <i>E. coli</i> <i>rpoH</i> gene downstream of IPTG-inducible <i>tac</i> promoter	Yura <i>et al.</i> (2007)
pBAD	<i>araBAD</i> promoter, <i>araC</i> , pBR322 origin, Amp ^R	Invitrogen
pBAD <i>rpoH</i>	<i>F. tularensis</i> <i>rpoH</i> gene cloned downstream of arabinose-inducible promoter of pBAD	This study
pFNLTP6 <i>gro</i>	<i>E. coli</i> - <i>F. tularensis</i> shuttle vector with <i>groE</i> operon promoter, Km ^R Amp ^R , constructed from pFNLTP6 <i>gro-gfp</i> by deleting <i>gfp</i> gene	Meibom <i>et al.</i> (2009); Maier <i>et al.</i> (2006)
pFNLTP6 <i>gro</i> -0851	<i>E. coli</i> - <i>F. tularensis</i> shuttle vector with <i>F. tularensis</i> <i>rpoH</i> downstream of <i>groE</i> operon promoter, Km ^R Amp ^R	This study

PCR using primers KanaRtn903_F and KanaRtn903_R. The first strain obtained after transduction, MG1655 Δ *rpoH*/pRB11, was then grown on LB agar supplemented with 50 μ g ampicillin ml⁻¹ and 25 μ g kanamycin ml⁻¹ at room temperature, 30 and 37 °C. The second strain, MG1655 Δ *rpoH*/pBAD*rpoH*, was grown on LB agar supplemented with 50 μ g ampicillin ml⁻¹ and 25 μ g kanamycin ml⁻¹ in the presence and absence of 0.2 % arabinose at room temperature, 30 and 37 °C.

RNA extraction. RNA was extracted from exponential-phase bacteria with TRIzol reagent (Invitrogen). After extraction with chloroform, the aqueous phase was purified using the RNeasy Clean-up protocol (Qiagen). Contaminating DNA was removed from RNA preparations with RNA-free DNase I (Ambion).

Microarray experiments and data analyses. To prepare RNA for microarray analyses, *F. tularensis* LVS, *F. tularensis* LVS + pFNLTP6*gro*, and *F. tularensis* LVS + pFNLTP6*gro*-0851 were grown at 37 °C with shaking to OD₆₀₀ ~0.3–0.4. For *rpoH* overexpression experiments, bacterial cells were then harvested and RNA was extracted. For heat-shock-response experiments, the culture of *F. tularensis* LVS was split into two: one part of the culture was placed at 37 °C with shaking, whereas the other was incubated at 44 °C with shaking. After 30 min incubation, bacterial cells were harvested and RNA was extracted.

Reverse transcription and cDNA fluorescent labelling were performed for each sample on 3 μ g of RNA according to the protocol provided by The Institute for Genomic Research (TIGR; <http://pfgcr.jcvi.org>). Briefly, the RNA was reverse-transcribed using Superscript II reverse transcriptase in the presence of amino-allyl-dUTP, dNTP and random primers, followed by coupling of cyanine dye (Cy3 or Cy5). Microarray hybridization and post-hybridization washings were performed according to the TIGR protocol. Briefly, after pre-hybridization of microarray slides, hybridization was performed in

hybridization buffer (40 % formamide, 5 $\times$ saline sodium citrate (SSC), 0.1 % SDS, 0.6 μ g salmon sperm DNA μ l⁻¹) at 42 °C for 18 h in a hybridization chamber (Corning).

The *F. tularensis* microarrays, provided by the Pathogen Functional Genomics Resource Center (PFGRC; Rockville, MD, USA), contain 2331 oligonucleotides (70-mer) with four replicates per oligonucleotide, representing all the ORFs of *F. tularensis* Schu S4 and *F. tularensis* LVS.

Microarrays were scanned with a Genepix 4000B scanner (Molecular Devices). To determine signal fluorescence intensities for each spot, TIFF images were analysed using the Spotfinder and Midas software (TIGR). Statistical analyses were performed using the Significance Analysis of Microarrays (SAM) software (Tusher *et al.*, 2001). The number of genes with significantly modified expression was defined by performing a one-class analysis using a false discovery rate (FDR) of 0 %.

Two RNA extractions from independent experiments were done for each condition and each sample set was hybridized to two microarrays.

Real-time quantitative RT-PCR (qRT-PCR). To validate the microarray results, four ORFs were selected for qRT-PCR analysis with the same RNA samples used in the microarray hybridizations. For the heat-shock time-course experiment, RNA samples were isolated before temperature shift from 37 to 44 °C and after 10, 20, 30, 45 and 60 min incubation at 44 °C. For the analyses, 1 μ g RNA was reverse-transcribed using random hexamers and Superscript II reverse transcriptase (Invitrogen) according to the protocol provided by the manufacturer. Real-time PCR was performed with gene-specific primers using an ABI PRISM 7700 and SYBR green PCR master mix (Applied Biosystems). To calculate the amount of gene-specific transcript, a standard curve was plotted for each primer set using a series of diluted genomic DNA from LVS. To compare the

transcript amounts for the different conditions, the amounts of each gene transcript were normalized to DNA helicase (FTL_1656), as this gene has been shown to change little in expression during growth (Brotcke *et al.*, 2006). The expression of each gene was determined from three replicates in a single qRT-PCR experiment.

Bioinformatics analyses. The RpoH-binding site consensus sequence was identified as described previously (Slamti *et al.*, 2007) using BioProspector (Liu *et al.*, 2001) to search for conserved motifs with a structure similar to those of the RpoH-binding sites in *E. coli* and *Vibrio cholerae*. The search parameters and input sequences were adjusted until the motif identified corresponded to the previously predicted RpoH-binding sites upstream of *F. tularensis* *grpE* and *groES* (Meibom *et al.*, 2008) (Supplementary Tables S3 and S4). The sequences 200 bp upstream of the first 400 annotated *F. tularensis* ORFs were used as the background model. The final search parameters were as follows: motif, 2 blocks; width of the first motif block, 7; width of the second motif block, 7; minimum gap between the blocks, 13; maximum gap between the blocks, 16. Identification of putative RpoH-binding sites was accomplished in two steps. First, four position frequency matrices (PFMs) were constructed. These were composed of the PFMs of blocks 1 and 2 identified by BioProspector separated by 13–16 columns, respectively, in which the frequencies of all four nucleotides were set to be identical. Second, the program PATSER (van Helden, 2003) was used to search the 200 bp upstream of all *F. tularensis* genes for sequences corresponding to each of these four matrices. The minimum PATSER score threshold was set to 9. In cases where two putative binding sites had the same block 1 or block 2 sequences but different gap lengths, the lower-scoring site was discarded.

RESULTS

Identification of a functional *rpoH* gene of *F. tularensis* LVS

The *in silico* analysis of the LVS genome allowed us to identify all the genes involved in the synthesis of core RNAP. As reported by Charity *et al.* (2007), the *F. tularensis* genome contains two genes, designated *rpoA1* (FTL_0261) and *rpoA2* (FTL_0616), which encode two different α subunits of RNAP that differ from one another in regions that are predicted to be critical for dimer formation, promoter recognition, and activator interaction. FTL_1744 and FTL_1743 (*rpoB* and *rpoC*) encode the β and β' subunits, respectively, and FTL_1533 (*rpoZ*) the ω subunit.

The major sigma factor (σ^{70}) is encoded by FTL_1050 (*rpoD*). Strikingly, we identified only one gene, FTL_0851, encoding a putative alternative sigma factor sharing significant sequence similarity with other members of the family of σ^{32} heat-shock factors (hence designated *rpoH*). The *rpoH* sequence of LVS encodes a protein of 292 aa, with 53.4 % amino acid identity with σ^{32} of *E. coli* K-12, and is highly conserved among the *F. tularensis* subspecies. Multiple sequence alignments of σ^{32} family members have led to the identification of a highly conserved stretch of nine amino acids Q(R/K)(K/R)FFNLR that is designated the RpoH box (Nakahigashi *et al.*, 1995). It is of note that this box is perfectly conserved in the *F. tularensis* σ^{32} sequences, strongly suggesting that it encodes a functional σ^{32} factor.

Functional complementation of *E. coli* *rpoH*

We first tested the ability of the LVS *rpoH* gene to functionally complement the temperature-sensitive growth of an *E. coli* $\Delta rpoH$ mutant lacking σ^{32} , which can grow only at or below 20 °C (Zhou *et al.*, 1988). For this, we introduced the $\Delta rpoH$ mutation from strain CAG48315 (Zhou *et al.*, 1988; Table 1) into *E. coli* strain MG1655 by transduction. The recipient strain contains a plasmid encoding the *F. tularensis* *rpoH* gene (pBAD*rpoH*) under the control of an arabinose-inducible promoter. The resulting strain did not grow on agar plates without supplement at any temperature (room temperature, 30 or 37 °C; Fig. 1, middle row). However, the *F. tularensis* *rpoH* gene promoted growth of the mutant strain when arabinose was added to the medium (Fig. 1, top row), showing that *F. tularensis* *rpoH* can functionally complement the lack of σ^{32} in *E. coli*. As a control, we transduced the $\Delta rpoH$ mutation into a recipient strain containing a plasmid encoding the *E. coli* *rpoH* gene (pRB11; Yura *et al.*, 2007) (Fig. 1, bottom row), and this strain exhibited the same growth pattern as the MG1655 $\Delta rpoH$ /pBAD*rpoH* strain grown in the presence of arabinose. These results show that *F. tularensis* *rpoH* encodes a genuine σ^{32} unit of RNAP.

F. tularensis transcriptomic response to heat stress

We next studied gene expression changes in *F. tularensis* LVS in response to heat shock. For this, we extracted RNA from bacterial cultures grown in Schaedler medium (supplemented with vitamin K3) after a temperature upshift for 30 min at 44 °C. After cDNA synthesis and labelling, control (the same culture kept at 37 °C) and heat-shocked samples were paired and hybridized on the microarrays. Statistical analysis using SAM software (Tusher *et al.*, 2001) indicated that 181 genes exhibited significant (FDR 0 %) differential expression at a twofold or greater level in response to a temperature upshift from 37 to 44 °C (Table 2 and Supplementary Table S2). The wide distribution of putative functional roles attributed to the differentially expressed genes indicates the extent of the molecular response that enables *F. tularensis* LVS to survive and eventually adapt to thermal stress. The transcripts of 112 genes were more abundant after the temperature upshift (Table 2). Among these, 15 genes had annotated functions in protein fate. Well-known heat-shock proteins, such as Hsp40, GroEL, GroES, DnaK, DnaJ, GrpE, ClpB, ClpX, ClpP and HtpG, form the major part of this group. The major sigma factor RpoD (σ^{70}) was found to be upregulated, with a fold-induction of 4.6. In contrast, *rpoH* did not appear to be upregulated after a temperature upshift. About 40 % of the upregulated genes were genes encoding proteins of unknown function or pseudogenes. Transposases composed 21 % of the upregulated genes, but this could reflect cross-hybridizations on the microarrays as these transposases are highly homologous. Interestingly,

Fig. 1. The *rpoH* gene from *F. tularensis* functionally complements an *E. coli* $\Delta rpoH$ mutant. *E. coli* strain MG1655 $\Delta rpoH$ harbouring plasmid pBAD*rpoH* (encoding *rpoH* from *F. tularensis*) or pRB11 (encoding *rpoH* from *E. coli*) was streaked on LB agar containing ampicillin and 0.2 % arabinose, where indicated (+ara), and incubated for 24 h at 30 and 37 °C, or for 48 h at room temperature (RT).

five genes of the *F. tularensis* FPI were found to be downregulated: FTL_0115/1161, FTL_0116/1162 and FTL_0124/1170, encoding proteins of unknown function, and *pdpB1* and *iglB1* (intracellular growth locus B) (Supplementary Table S2). One third of the downregulated genes had annotated functions in metabolism, biosynthesis of cofactors or ribonucleotide biosynthesis. As for the upregulated genes, genes encoding proteins of unknown function or pseudogenes represented a significant portion of the downregulated genes. Furthermore, several genes encoding proteins with transport functions were downregulated after heat shock.

To verify the accuracy of the microarray data, we performed qRT-PCR analyses on transcripts of four chosen genes. For these experiments, we used one of the two RNA sample sets that were used for the microarray experiments. As seen in Fig. 2(a), the relative levels of *rpoH*, *dnaK*, *groEL* and *hsp40* transcripts fully supported the microarray data, with *dnaK*, *groEL* and *hsp40* expressed at higher levels at 44 °C than at 37 °C and the expression of *rpoH* not changed by the temperature upshift. To investigate the heat-shock response in a temporal manner, we performed

qRT-PCR analyses on four heat-shock-induced genes (*groEL*, *dnaK*, *grpE* and *hsp40*) over a period of 60 min incubation at 44 °C (Fig. 2b). For all four genes we observed increased expression at all times (10–60 min) compared with before heat shock. However, the transcript levels appeared to be less elevated at the later time points (45 and 60 min).

Role of *rpoH* in *F. tularensis* transcriptional regulation

In spite of our repeated efforts, we were unable to inactivate the *rpoH* gene of LVS. We tried to inactivate *rpoH* either by insertional mutagenesis or by allelic exchange at temperatures ranging from 20 to 37 °C, but were unsuccessful. It is thus likely (although not formally proven) that *rpoH* is essential in *F. tularensis*, as it is thought to be in other bacteria, such as *V. cholerae* and *Neisseria gonorrhoeae* (Laskos *et al.*, 2004; Slamti *et al.*, 2007).

To overcome the lack of an *rpoH* mutant, we evaluated the effect of overproduction of RpoH on the LVS transcrip-

Table 2. Genes significantly increased in expression (at least twofold) after temperature upshift

Locus*	Gene product	Gene	Cellular role category†	Fold change‡
FTL_0034	Hypothetical protein		Hypothetical proteins: conserved	2.02
FTL_0088	Acetyltransferase protein		Unknown function	2.38
FTL_0093	Chitinase		Metabolism	10.24
FTL_0094	ClpB protein	<i>clpB</i>	Degradation of proteins, peptides and glycopeptides	5.51
FTL_0144	Hypothetical protein		Unknown function: general	2.77
FTL_0163	MFS transporter		Transport and binding proteins	4.31
FTL_0180	Acyltransferase	<i>htrB</i>	Metabolism	2.25
FTL_0186	MFS transport protein, fragment		Transport and binding proteins	4.01
FTL_0201	Hypothetical protein		Unknown function	2.15
FTL_0202	Conserved hypothetical membrane protein		Cell envelope: other	3.29
FTL_0203	Hypothetical membrane protein		Unknown function: general	2.00
FTL_0219	Hypothetical protein		Unknown function: general	2.86
FTL_0267	Heat-shock protein 90	<i>htpG</i>	Protein folding and stabilization	3.01
FTL_0281	Heat-shock protein, hsp40		Protein folding and stabilization	7.13
FTL_0359	Type IV pili fibre building block protein		Unknown function	2.55
FTL_0365	—		Pseudogene	2.92
FTL_0390	Type IV pili fibre building block protein		Unknown function: general	1.99
FTL_0645	Hypothetical lipoprotein		Cell envelope	3.20
FTL_0662	Prophage repressor protein		Mobile and extrachromosomal element functions	3.31
FTL_0663	Hypothetical protein		Hypothetical proteins: conserved	4.02
FTL_0758	—		Pseudogene	2.24
FTL_0826	—		Pseudogene	2.09
FTL_0844	LysR transcriptional regulator family protein		Regulatory functions	2.35
FTL_0867	Hypothetical protein		Hypothetical proteins: conserved	2.78
FTL_0891	Trigger factor	<i>tig</i>	Protein folding and stabilization	4.38
FTL_0892	ATP-dependent Clp protease subunit P	<i>clpP</i>	Degradation of proteins, peptides and glycopeptides	3.70
FTL_0893	ATP-dependent protease ATP-binding subunit	<i>clpX</i>	Degradation of proteins, peptides and glycopeptides	2.87
FTL_0894	DNA-binding, ATP-dependent protease La	<i>lon</i>	Degradation of proteins, peptides and glycopeptides	2.53
FTL_0908	—		Pseudogene	2.07
FTL_0909	Hypothetical protein		Hypothetical proteins: conserved	2.29
FTL_0911	—		Pseudogene	2.34
FTL_0912	—		Pseudogene	1.97
FTL_0964	ATP-dependent protease ATP-binding subunit	<i>hslU</i>	Protein folding and stabilization	4.33
FTL_0965	ATP-dependent protease peptidase subunit	<i>hslV</i>	Degradation of proteins, peptides and glycopeptides	5.13
FTL_0996	AhpC/Tsa family protein		Detoxification	2.01
FTL_0997	Hypothetical protein		Hypothetical proteins: conserved	3.13
FTL_1008	—		Pseudogene	2.01
FTL_1048	Hypothetical protein		Unknown function: general	2.23
FTL_1049	DNA primase	<i>dnaG</i>	DNA replication, recombination and repair	2.21
FTL_1050	RNAP sigma-70 factor	<i>rpoD</i>	Transcription	4.58
FTL_1125	Transcription regulator	<i>hipA</i>	Regulatory functions	2.75
FTL_1126	Transcription regulator		Regulatory functions	2.26
FTL_1190	Chaperone protein GrpE (heat-shock protein family 70 cofactor)	<i>grpE</i>	Protein folding and stabilization	6.32
FTL_1191	Chaperone protein DnaK (heat-shock protein family 70 protein)	<i>dnaK</i>	Protein folding and stabilization	2.23
FTL_1192	Chaperone protein DnaJ (heat-shock protein 70 family cofactor)	<i>dnaJ</i>	Protein folding and stabilization	6.19
FTL_1194	Pirin family protein		Unknown function: general	4.17
FTL_1221	Hypothetical membrane protein		Cell envelope	5.11
FTL_1222	Conserved hypothetical protein		Hypothetical proteins: conserved	4.58
FTL_1226	—		Pseudogene	2.65
FTL_1257	—		Pseudogene	2.31
FTL_1259	—		Pseudogene	3.41
FTL_1260	—		Pseudogene	2.27

Table 2. cont.

Locus*	Gene product	Gene	Cellular role category†	Fold change‡
FTL_1265	2-Amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase/dihydropteroate synthase	<i>folK</i>	Biosynthesis of cofactors, prosthetic groups and carriers: folic acid	5.01
<i>FTL_1266</i>	Lipase/esterase		Metabolism	5.82
<i>FTL_1267</i>	Hypothetical protein		Hypothetical proteins: conserved	4.01
<i>FTL_1269</i>	—		Pseudogene	2.07
<i>FTL_1270</i>	—		Pseudogene	2.68
<i>FTL_1361</i>	Cold-shock protein	<i>cspA</i>	Hypothetical proteins	2.30
<i>FTL_1365</i>	Hypothetical protein		Hypothetical proteins: conserved	2.49
<i>FTL_1366</i>	Membrane fusion protein, putative		Transport and binding proteins	2.13
<i>FTL_1403</i>	Conserved hypothetical membrane protein		Cell envelope	2.90
<i>FTL_1411</i>	Hypothetical protein	<i>ybaB</i>	Hypothetical proteins: conserved	2.31
<i>FTL_1412</i>	Recombination protein RecR	<i>recR</i>	DNA replication, recombination and repair	2.07
<i>FTL_1517</i>	Hypothetical protein		Hypothetical proteins: conserved	2.09
<i>FTL_1535</i>	2-Dehydro-3-deoxyphosphooctonate aldolase		Cell envelope	1.99
<i>FTL_1555</i>	Hypothetical membrane protein		Cell envelope	4.32
<i>FTL_1575</i>	Hypothetical membrane protein		Cell envelope	4.09
<i>FTL_1600</i>	Periplasmic L-asparaginase II precursor	<i>ansB</i>	Metabolism	2.04
<i>FTL_1691</i>	Hypothetical protein		Hypothetical proteins: conserved	2.02
<i>FTL_1692</i>	Hypothetical protein		Cell envelope: surface structures	2.13
<i>FTL_1714</i>	Chaperone protein, GroEL	<i>groL</i>	Protein folding and stabilization	3.05
FTL_1715	Chaperonin protein, GroES	<i>groS</i>	Protein folding and stabilization	3.12
<i>FTL_1770</i>	Type IV pili nucleotide-binding protein		Transport and binding proteins	3.01
<i>FTL_1771</i>	Type IV pili nucleotide-binding protein	<i>pilT</i>	Transport and binding proteins	3.03
<i>FTL_1776</i>	Hypothetical membrane protein		Cell envelope	3.01
<i>FTL_1777</i>	—		Pseudogene	2.13
<i>FTL_1778</i>	—		Pseudogene	3.19
<i>FTL_1793</i>	Hypothetical protein		Hypothetical proteins: conserved	2.06
FTL_1805	ATPase		Unknown function	2.92
<i>FTL_1806</i>	MFS transport protein		Transport and binding proteins	2.91
<i>FTL_1807</i>	Histidyl-tRNA synthetase	<i>hisS</i>	tRNA aminoacylation	3.12
<i>FTL_1808</i>	Ribosome-binding factor A		Transcription	2.96
<i>FTL_1810</i>	Transcription elongation factor NusA	<i>nusA</i>	Transcription	4.04
<i>FTL_1811</i>	Hypothetical protein		Unknown function: general	2.74
<i>FTL_1814</i>	—		Pseudogene	2.76
<i>FTL_1878</i>	Two-component sensor protein KdpD	<i>kdpD</i>	Regulatory functions: other	2.14
<i>FTL_1894</i>	Transposase, fragment		Mobile and extrachromosomal element functions	4.16
<i>FTL_1913</i>	Sua5/YciO/YrdC family protein		Unknown function	2.06
<i>FTL_1945</i>	Hypothetical protein		Unknown function: general	1.95
FTL_1957	Heat-shock protein		Protein folding and stabilization	8.57
<i>FTL_XXXX§</i>	Transposase	<i>isftu2</i>	Mobile and extrachromosomal element functions	2.0–6.9
<i>FTL_YYYY </i>	Transposase	<i>isftu1</i>	Mobile and extrachromosomal element functions	2.3–3.2

*Genes in bold type have a putative RpoH-binding sequence located before the start codon (see Supplementary Table S3 for detailed information). Gene numbers marked in italic type have been identified in screens or selections for determinants required for intracellular survival and/or virulence (Kraemer *et al.*, 2009; Maier *et al.*, 2007; Su *et al.*, 2007; Tempel *et al.*, 2006; Weiss *et al.*, 2007).

†Cellular role category according to TIGR.

‡Fold change calculated as the average expression at 44 °C relative to 37 °C calculated from two independent experiments with duplicate hybridizations.

§A number of *isftu2*-type transposases: FTL_0079, 0220, 0270, 0367, 0441, 0468, 0568, 0575, 0591, 0622, 0861, 0868, 1301, 1368, 1471, 1516, 1655, 1680 and 1885.

||Three *isftu1*-type transposases: FTL_0128, 0417 and 0607.

Fig. 2. Quantification by RT-PCR of transcript levels of selected genes after heat shock. (a) Validation of microarray experiment. Transcript levels were normalized to that of DNA helicase (FTL_1656) and the fold difference (relative to 37 °C) and SDs are shown for the *dnaK*, *groEL*, *hsp40* and *rpoH* genes. (b) Change in expression of four heat-shock genes during 60 min incubation at 44 °C. Transcript levels were normalized to that of DNA helicase (FTL_1656) and the fold difference (relative to sample taken before temperature shift) and SDs are shown for the *dnaK*, *groEL*, *hsp40* and *grpE* genes. Data are from triplicate samples.

tome. For this, we compared the transcriptomes of LVS (transformed with the plasmid pFNLTP6gro without inserted gene) to that of an LVS overexpressing RpoH (i.e. transformed with the plasmid pFNLTP6gro_0851, expressing *FTL_0851* from the *groES* promoter). We extracted RNA from exponentially grown bacterial cultures, and after cDNA synthesis and labelling, control and RpoH-overexpressing samples were paired and hybridized to the microarrays. Statistical analysis indicated that only seven genes exhibited significant increased expression (FDR 0%) in response to overexpression of RpoH (Table 3). As expected, *rpoH* itself was upregulated 21-fold. The six other upregulated genes all encoded well-

known heat-shock proteins (Hsp40, Hsp90, GroES, GroEL, DnaK and DnaJ). The transcriptional control by RpoH therefore seems to be very specific to heat-shock proteins.

Consensus sequence for *Francisella* RpoH-dependent promoter

To identify an RpoH-binding site consensus sequence, we analysed the 200 bp sequence upstream of all 112 heat-upregulated genes listed in Table 2 using Bioprosearcher (Liu *et al.*, 2001). These analyses failed to identify an RpoH consensus binding sequence, which we reasoned was likely due to the fact that a relatively small proportion of heat-

Table 3. Genes showing a change in expression in the presence of a plasmid overexpressing RpoH

Locus	Gene product*	Gene	Cellular role category†	Fold change‡
FTL_0851	RNAP sigma factor	<i>rpoH</i>	Transcription: transcription factors	20.6
FTL_0281	Heat-shock protein, Hsp40		Protein fate: protein folding and stabilization	2.9
FTL_0267	Heat-shock protein 90	<i>hspG</i>	Protein fate: protein folding and stabilization	2.9
FTL_1191	Chaperone protein DnaK (heat-shock protein family 70 protein)	<i>dnaK</i>	Protein fate: protein folding and stabilization	2.6
FTL_1192	Chaperone protein DnaJ (heat-shock protein 70 family cofactor)	<i>dnaJ</i>	Protein fate: protein folding and stabilization	2.0
FTL_1714	Chaperone protein, GroEL	<i>groEL</i>	Protein fate: protein folding and stabilization	2.2
FTL_1715	Chaperone protein, GroES	<i>groES</i>	Protein fate: protein folding and stabilization	2.3

*Genes with significantly changed expression, upregulated at least twofold, are included in the table and displayed according to their functional category.

†Cellular role category according to TIGR.

‡Values are average fold change between LVS/pFNLTP6gro-0851 and LVS/pFNLTP6gro from two independent experiments with duplicate hybridization. Each microarray contains four spots for each gene.

Fig. 3. Consensus sequence of the *F. tularensis* σ^{32} -binding site displayed with WebLogo (<http://weblogo.berkeley.edu>). The height of each column indicates the sequence conservation at that position, while the height of symbols within the stack indicates the relative frequency of each nucleic acid at that position. N_(13–16) indicates the gap between the two blocks.

regulated genes identified in our microarray analyses are directly regulated by RpoH, making it difficult for Bioprosector to identify conserved binding sites. To address this problem, we repeated our Bioprosector analyses using only those upregulated genes annotated by NCBI as chaperones, chaperonins, proteases or heat-shock proteins, classes of genes known to be directly regulated by RpoH in other bacteria (Supplementary Tables S3 and S4). This yielded several putative binding sites, several of which corresponded to previously predicted *F. tularensis* RpoH-binding sites (Meibom *et al.*, 2008). A PFM representing the sites identified by Bioprosector was constructed and the program PATSER (van Helden, 2003) was used to interrogate the upstream regions of all 1754 ORFs in the NCBI annotation of LVS for putative RpoH-binding sites. A total of 72 non-overlapping putative RpoH-binding sites with a score of 9 or above were identified upstream of 71 genes (Supplementary Table S5), including 13 of the genes upregulated by heat shock (Table 4 and marked in bold type in Table 2). From these 13 putative sites a consensus binding sequence was determined (Fig. 3) that shares

significant homology with the RpoH-binding site consensus sequences in *E. coli* and *V. cholerae* (Slamti *et al.*, 2007).

DISCUSSION

The bacterial heat-shock response results in a transient induction of a set of highly conserved heat-shock proteins, including chaperones and proteases. The alternative σ -factor σ^{32} (RpoH) is the primary regulator that controls the transcription of genes during heat shock and some other general stress conditions. The activity of the heat-shock proteins ensures the correct folding of proteins during normal growth, and is essential for resistance to stress by minimizing the damage caused by the accumulation of misfolded and aggregated proteins. Our examination of the heat-shock response in *F. tularensis* resulted in the identification of many bacterial heat-shock proteins, some of which have been previously reported to increase in amount after heat or oxidative stress (Ericsson *et al.*, 1994; Lenco *et al.*, 2005) and in response to mammalian body

Table 4. Heat-induced genes with a potential σ^{32} -binding sequence in the promoter region

Distance*	Length†	Binding site sequence	Locus	Gene product‡
34	27	CTTGAAAAACAGCAAAGTATCCTTAAG	FTL_0094	ClpB protein
81	30	CTTGAAAAAACTAACTTCCACTCTCATATA	FTL_0267	Heat-shock protein 90, HtpG
84	27	CTTGTATTTATATAAAAAGACCTTATA	FTL_0281	Heat-shock protein, Hsp40
7	28	CTTGAAAATGATTAGAAAAAGTATTTTA	FTL_0663	Hypothetical protein
101	29	CTTGTCATATATTTAATAATAAACATATA	FTL_0891	Trigger factor
162	28	CTTGAAGTGATGTTTCATATACCTTCAT	FTL_0894	DNA-binding, ATP-dependent protease La
48	29	CTTGTAATAATTAATATCAATTCCATTTA	FTL_0965	ATP-dependent protease, peptidase subunit, HslV
82	29	CTTGAAAATACAGAATTAATCACTATCTA	FTL_1190	Chaperone protein GrpE (heat-shock protein family 70 cofactor)
92	29	CTTGAAAAGATTATAAATATGCCCATCTA	FTL_1191	Chaperone protein DnaK (heat-shock protein family 70 protein)
68	30	CTTGAAAATTAAAAAAAAGCCCCCAATTTC	FTL_1265	2-Amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase/dihydropteroate synthase
98	29	CTTGAAAATTTTTTTTTTGACTCAATATC	FTL_1715	Chaperonin protein, GroES
40	29	CTTGTATTTAGCATGATCGTATCATTCTT	FTL_1805	ATPase
75	27	CTTGAAATTTTAAAAACCGATCATATA	FTL_1957	Heat-shock protein

*Distance from ATG start codon.
†Length (nucleotides) of the putative σ^{32} -binding site.
‡Gene product according to TIGR.

temperature (Horzempa *et al.*, 2008), in addition to various other genes.

Role of σ^{32} in heat-stress response

Control of σ^{32} amount and activity in *E. coli* is complex and is performed at transcriptional, post-transcriptional and post-translational levels. The heat-shock response in *E. coli* is induced as a result of increased σ^{32} levels, primarily due to an increase in *rpoH* mRNA translation and increased stability and activity of σ^{32} (Arsène *et al.*, 2000), whereas control of *rpoH* transcription seems to be of only minor importance. Similarly, we did not observe an increase in transcription of *rpoH* in *F. tularensis* after temperature upshift. In contrast, transcription of σ^{70} was increased after heat shock, suggesting that σ^{70} -containing RNAPs are required for the heat-shock response in *Francisella*, perhaps in combination with gene-specific regulators.

To more directly define the genes dependent on RpoH, we performed transcriptional profiling of *F. tularensis* LVS harbouring a plasmid expressing RpoH from the *groE* promoter and compared it with bacteria carrying an empty plasmid. This allowed us to identify six genes (in addition to *rpoH* itself) that were expressed at increased levels in the strains overexpressing RpoH. These genes encode the heat-shock proteins Hsp40, HptG, DnaK, DnaJ, GroES and GroEL, which were all induced after heat stress as well. The previous studies of the σ^{32} regulon in *E. coli*, *V. cholerae* and *N. gonorrhoeae* identified these genes also (Gunesekere *et al.*, 2006; Nonaka *et al.*, 2006; Slamti *et al.*, 2007; Zhao *et al.*, 2005), demonstrating common features of RpoH regulation in different species. However, the number of genes identified in *F. tularensis* was unexpectedly low when compared with other bacterial species. To date, there is no promoter that allows the specific induction of transcription in *Francisella*, and our experiments were therefore performed under continuous overexpression of RpoH. Based on the knowledge of the regulation of σ^{32} in *E. coli*, we believe this could lead to underestimation of the genes directly controlled by RpoH. In the current model for regulation of σ^{32} in *E. coli*, called the ‘unfolded protein titration model’, the stability and activity of σ^{32} rely on the level of unfolded protein relative to the chaperone level (for review see Guisbert *et al.*, 2008). When unfolded protein levels are low compared with chaperones, σ^{32} is predominantly bound to the chaperones and is inactive, whereas a high level of unfolded proteins will titrate the chaperones away from σ^{32} , thereby rendering σ^{32} active. In our experimental set-up, unfolded protein levels are expected to be low (since no stress is imposed on the bacteria), whereas the chaperones will be expressed as a result of RpoH overexpression. If the same mechanism of σ^{32} regulation exists in *F. tularensis* as in *E. coli*, our set-up will lead to chaperone-mediated inactivation of σ^{32} , and this could be responsible for the low number of genes we observe to be induced in the RpoH-overexpressing strain.

Defining an RpoH consensus box

Using the data we obtained from the transcriptional profiling, we performed a bioinformatics analysis to determine a consensus sequence for binding of σ^{32} (Fig. 3). We identified a putative σ^{32} -binding site in the promoter region of 13 genes that were induced after heat shock. Among these are all the genes induced by RpoH overexpression, or the first gene in the presumed two-gene transcripts *dnaK-dnaJ* and *groES-groEL*, further suggesting that these genes are directly under σ^{32} control. In the remainder of the heat-induced genes, we did not observe any putative σ^{32} -binding sites, indicating that some of these are not controlled directly by σ^{32} . The consensus binding sequence for σ^{32} resembles the consensus sequence from *V. cholerae* and *E. coli* (Nonaka *et al.*, 2006; Slamti *et al.*, 2007), and several of the genes and putative operons that are preceded by a σ^{32} -binding site in *F. tularensis* are also found in *E. coli*, although small variations are often observed (Nonaka *et al.*, 2006) (Fig. 4). We observed heat-mediated upregulation of the four consecutive genes *tig-clpP-clpX-lon*, and bioinformatics analysis identified a σ^{32} -binding site upstream of the *tig* and *lon* genes (Fig. 4a). However, in *E. coli*, only *clpP*, *clpX* and *lon* are induced by heat shock, and a σ^{32} -binding site is found upstream of *clpP* and *lon*. In both *F. tularensis* and *E. coli*, the transcription unit *dnaK-dnaJ* is induced by heat shock and preceded by a σ^{32} -binding site. In *F. tularensis*, these genes are adjacent to *grpE*, which is also heat-induced and preceded by a σ^{32} -binding site, although in *E. coli* *grpE* is located elsewhere on the genome (Fig. 4b). The organization of the two-gene transcriptional unit *groES-groEL* seems to be identical in the two species (Fig. 4d), as is the case with the *hslV-hslU* genes, with the exception that the *E. coli* genes are followed by a third heat-induced gene, *menA* (Fig. 4c). The protease FtsH carries out degradation of σ^{32} in *E. coli* and is itself induced by heat shock, and the transcription unit contains a σ^{32} -binding site (Fig. 4e). Interestingly, in *F. tularensis* we did not observe increased expression of the *ftsH* gene after heat shock, and the genetic region encoding FtsH is not predicted to contain a σ^{32} -binding site (Fig. 4e). The region containing *ftsH* seems to have been duplicated in the LVS strain, whereas it is only found in one copy in the other strains of *F. tularensis* for which sequence information is available. Currently, we do not know whether the activity of σ^{32} in *F. tularensis* is controlled by a mechanism similar to the ‘unfolded protein titration model’ and whether degradation of σ^{32} is performed by the FtsH protease. In any case, the regulatory mechanism must differ from the one in *E. coli*, since FtsH is not part of the heat-shock response in *F. tularensis*.

We also observed σ^{32} -binding sites next to a number of genes that we did not find to be upregulated by RpoH overexpression or after heat shock. It is possible that some of these were missed in the transcriptomic analysis of heat stress or were not induced under constant overexpression of RpoH. We performed transcriptomic analysis after 30 min of heat exposure, and it is probable that some genes

Fig. 4. Schematic representations of the organization of selected genes containing putative σ^{32} -binding sequences in *F. tularensis* and *E. coli*. Black arrows represent genes that are induced by heat stress. Grey arrows represent genes that are not induced by heat stress. Gene symbols are indicated above arrows and locus numbers to the left in each panel (numbers preceded by FTL_ designate the loci in *F. tularensis* LVS and numbers preceded by b the loci in *E. coli* MG1655). *isftu*, transposase; H, hypothetical protein; *mfs*, major facilitator transporter. The locations of σ^{32} -binding sites in *E. coli* are from Nonaka *et al.* (2006).

are induced only early in the heat-shock response. Our qRT-PCR analysis of the temporal expression of four heat-shock genes showed an increased expression at all times from 10 to 60 min of heat exposure, although with less elevated transcript levels at the later time points. These results indicate that the heat-shock response is initiated promptly and continues for up to 1 h, but do not rule out the possibility that some genes could have increased expression only early in the heat-shock response. Interestingly, a number of the genes containing putative σ^{32} -binding sites (that are not induced by heat stress) are induced by oxidative stress (N. Grall, unpublished results), indicating that not only the heat-shock response is mediated by σ^{32} . Finally, we anticipate that some of the putative sites do not represent true σ^{32} -binding sites, although they were identified in our bioinformatics analysis.

A relationship between heat-stress response and virulence

Remarkably, a considerable number of the genes that were upregulated by heat stress (20 genes, see Table 2) have been identified in screens or selections for genes required for

intracellular multiplication and/or survival. These genes include *clpB* (Gray *et al.*, 2002; Maier *et al.*, 2007; Meibom *et al.*, 2008; Su *et al.*, 2007; Tempel *et al.*, 2006; Weiss *et al.*, 2007), *hspG* (Weiss *et al.*, 2007), *FTL_0663* (Kraemer *et al.*, 2009; Su *et al.*, 2007), *dnaK* (Tempel *et al.*, 2006) and *FTL_1806* [encoding major facilitator superfamily (MFS) transport protein] (Maier *et al.*, 2007; Weiss *et al.*, 2007). The identification of these genes, which are important for intracellular survival, may indicate that stress responses play a central role in the pathogenesis of *F. tularensis* and its ability to survive inside host cells. The intracellular environment is hostile to the bacterium, and induction of stress responses may be imperative to its survival within host cells.

Given the significant number of genes (18%) that are induced under heat stress and are implicated in the virulence of *Francisella*, it will be interesting to further study the molecular mechanisms underlying the stress response. In particular, an understanding of how the activity of σ^{32} is controlled, including determining which proteins and signals mediate this control, will lead to a much better understanding of how σ^{32} -mediated stress responses contribute to survival within the mammalian host.

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