RaoN, a small RNA encoded within *Salmonella* pathogenicity island-11, confers resistance to macrophage-induced stress

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Bacterial small non-coding RNAs act as important regulators that control numerous cellular processes. Here we identified RaoN, a novel small RNA encoded in the *cspH-envE* intergenic region on *Salmonella* pathogenicity island-11 (SPI-11). RaoN contributes to survival under conditions of acid and oxidative stress combined with nutrient limitation, which partially mimic the intramacrophage environment. Indeed, inactivation of *raoN* reduces the intramacrophage replication of *Salmonella enterica* serovar Typhimurium. Genome-wide transcriptome analysis revealed that the lactate dehydrogenase gene *ldhA* is upregulated in the *raoN* knockout mutant. Notably, both inactivation and overexpression of *ldhA* in the WT strain render *Salmonella* more sensitive to oxidative stress, particularly when combined with nutrient limitation. However, *ldhA* is not the sole determinant of RaoN function in facilitating intramacrophage survival of *Salmonella*. Together, our data suggest that balanced regulation of *ldhA* expression by RaoN is necessary for survival under *in vitro* stress conditions and contributes to the intramacrophage growth of *Salmonella*.

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INTRODUCTION

Salmonella enterica serovar Typhimurium is a facultative intracellular pathogen that causes gastroenteritis in humans and a systemic disease in mice (Haraga *et al.*, 2008; Monack *et al.*, 2004). In the systemic typhoid-like disease, ingested *S. enterica* serovar Typhimurium cells must first survive the acid pH of the stomach and then penetrate the gut barrier via M cells in the Peyer's patches of the intestine (Jones *et al.*, 1994). From there, *Salmonella*-infected phagocytes enter the lymphatic system and bloodstream, which enables the bacteria to disseminate to the spleen and liver (Vazquez-Torres *et al.*, 1999).

Survival and replication of *S. enterica* serovar Typhimurium within macrophages is essential for its ability to cause systemic disease in mice. Bacteria within the *Salmonella*-containing vacuole (SCV) encounter

numerous stresses, such as nutrient limitation, acidic pH and reactive oxygen and nitrogen species (Kingsley & Bäumlér, 2000). An SCV acidifies to between pH 4.0 and 5.0 within 60 min after formation (Rathman *et al.*, 1996) and limits the availability of essential cations and other nutrients required by *Salmonella* (Kingsley & Bäumlér, 2000). Macrophages are potent generators of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which are effective antimicrobial agents and become more potent at an acidic pH (Fang, 2004; Jackett *et al.*, 1978; Stuehr & Nathan, 1989). ROS generation by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is crucial for the control of *Salmonella*, especially during the early stages of infection, while RNS generation by the inducible nitric oxide synthase controls the replication of the internalized bacteria later in the infection (Mastroeni *et al.*, 2000; Underhill & Ozinsky, 2002; Vazquez-Torres *et al.*, 2000a). *Salmonella* and other intracellular bacteria have acid resistance mechanisms, which can also provide cross protection against other stresses, including heat, oxidative and osmotic stress (Foster & Spector, 1995; Vandal *et al.*, 2009).

SPIs contain large clusters of virulence genes related to host cell invasion and intracellular pathogenesis (Mills *et al.*,

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Abbreviations: DIG, digoxigenin; mROS, mitochondrial ROS; NADPH, nicotinamide adenine dinucleotide phosphate; RACE, rapid amplification of cDNA ends; RNS, reactive nitrogen species; ROS, reactive oxygen species; RT, reverse transcription; SCV, *Salmonella*-containing vacuole; SPI, *Salmonella* pathogenicity island; sRNA, small non-coding RNA; TTSS, type III secretion system.

1995). Of the 21 SPIs known to date, the genome of *S. enterica* serovar Typhimurium has 11 SPIs (SPIs 1–6, 9, 11–13 and 16), including SPI-1 and SPI-2 that have been most extensively studied (Sabbagh *et al.*, 2010). SPI-1 encodes a type III secretion system (TTSS) involved in the invasion of intestinal epithelial cells (Galán & Collmer, 1999; Mills *et al.*, 1995). SPI-2 encodes a second TTSS that has a central role in systemic infections and intracellular pathogenesis (Hensel, 2000). In addition, SPI-11 includes genes, such as *pagC*, *pagD* and *msgA*, important for survival of *S. enterica* serovar Typhimurium in macrophages (Gunn *et al.*, 1995; Miller *et al.*, 1989).

In bacteria, small non-coding RNAs (sRNAs) are an important part of environmental response pathways (Gottesman *et al.*, 2006). They are approximately 50–500 nt in length and are often encoded in intergenic regions (Altuvia, 2007) and bounded by a Rho-independent terminator (Vogel & Sharma, 2005). Most sRNAs serve as regulators by base pairing with target mRNAs as *trans*-acting factors and either inhibiting or promoting gene expression by regulating mRNA translation or stability (Storz *et al.*, 2011; Waters & Storz, 2009). Some sRNAs modulate the expression of target mRNAs and are *cis*-encoded factors transcribed convergently to the targeted mRNA (Brantl, 2007).

Many sRNAs have been identified in *S. enterica* serovar Typhimurium (Kröger *et al.*, 2012; Padalon-Brauch *et al.*, 2008; Papenfort *et al.*, 2008; Vogel, 2009), and recent findings suggest that this organism expresses 140 sRNAs at the early stationary phase (Kröger *et al.*, 2012). In addition, several sRNAs have been described that affect *Salmonella* virulence. *InvR* sRNA acts as a repressor of *OmpD* protein synthesis (Pfeiffer *et al.*, 2007). *IsrM* sRNA, which is encoded by a *Salmonella* pathogenicity island, targets the mRNAs coding for *SopA*, a SPI-1 effector, and *HilE*, a global regulator of the expression of SPI-1 proteins (Gong *et al.*, 2011). *SgrS* sRNA post-transcriptionally represses the expression of the secreted virulence factor *SopD* (Papenfort *et al.*, 2012).

In this study, we identified a novel sRNA, RaoN (RNA involved in response to acid and oxidative stress during nutrient limitation), which contributes to intramacrophage replication of *Salmonella*. Our results demonstrate that the stress-inducible RaoN sRNA suppresses the transcription of *ldhA*, thereby allowing *Salmonella* to survive under *in vitro* growth conditions that partially mimic the host environment. This regulatory strategy functions to enhance intramacrophage survival, but other RaoN-regulated functions are also likely to be important.

METHODS

Bacterial strains, media and growth conditions. The bacterial strains used in this study are listed in Table 1. Cells were routinely cultured at 37 °C in Luria-Bertani (LB) medium or Vogel and Bonner E minimal medium supplemented with 0.4 % glucose (Maloy & Roth,

1983; Vogel & Bonner, 1956). For growth analysis, overnight cultures of the *S. enterica* serovar Typhimurium strains were diluted 100-fold into E glucose medium (pH 5.0) or LB medium containing 5 mM hydrogen peroxide. The cultures were grown with constant shaking at 37 °C, and the optical density at 600 nm (OD₆₀₀) values were determined hourly using a spectrophotometer (Spectronic 20D+, Thermo Spectronic). The following antibiotics were used for selection: ampicillin (Ap, 60 µg ml⁻¹), chloramphenicol (Cm, 30 µg ml⁻¹), kanamycin (Km, 50 µg ml⁻¹) or tetracycline (Tc, 10 or 20 µg ml⁻¹ for minimal or rich media, respectively).

Construction of *S. enterica* serovar Typhimurium strains. The *raoN* knockout mutant was constructed using suicide vector-mediated gene replacement as described previously (Edwards *et al.*, 1998; Lee *et al.*, 2007). Recombinant suicide vector pDMS197-*raoN* was transferred from *Escherichia coli* χ 7213 to *S. enterica* serovar Typhimurium UK1 WT via conjugation. Diaminopimelic acid (13 µg ml⁻¹) was added to media for the growth of *E. coli* χ 7213. *Salmonella* transconjugants containing single-crossover plasmid insertions were selected on LB agar containing Tc (20 µg ml⁻¹). Subsequently, loss of the suicide vector through a second homologous recombination was selected on LB agar containing 5 % sucrose by using *sacB*-based counter-selection (Gay *et al.*, 1985). The resulting deletion mutant was verified by the presence of PCR products of the expected size. The *hfq* and *ldhA* knockout strains were constructed using the lambda red recombinase system (Datsenko & Wanner, 2000). The Km^r cassette was amplified from pKD4 using the two primer pairs *hfq*-Mu-F/*hfq*-Mu-R and *ldhA*-Mu-F/*ldhA*-Mu-R, and the resulting PCR products were electroporated into the UK1 WT strain carrying plasmid pKD46. The genotype of the generated mutants was verified using PCR and DNA sequencing, and the Km^r cassette was then removed using plasmid pCP20. The chromosomal epitope-tagged allele of *ldhA* (*ldhA*-3 × FLAG::Km^r) was constructed as previously described (Uzzau *et al.*, 2001). The Km^r cassette was amplified from pSUB11 using the primers *ldhA*-3 × FLAG-F and *ldhA*-3 × FLAG-R. *Salmonella* strains YK5104 (Δ *raoN* Δ *ldhA*::Km^r), YK5106 (Δ *raoN* *ldhA*-3 × FLAG::Km^r) and YK5107 (*rpoS1071*::Tn10dCm) were constructed using phage P22-mediated generalized transduction as previously described (Davis *et al.*, 1980).

Plasmid construction. Gene cloning and molecular biology procedures were performed as described by Sambrook & Russell (2001). The plasmids used in this study are listed in Table 1. To construct pDMS197-*raoN* that contained the sequences immediately upstream and downstream of the deleted region, PCR was performed using the primer pairs *raoN*-up-F/*raoN*-up-R and *raoN*-do-F/*raoN*-do-R, respectively. pACYC184-*raoN* was constructed by PCR amplifying the *raoN* gene and its promoter from *S. enterica* serovar Typhimurium chromosomal DNA using the primers *raoN*-Comp-F and *raoN*-Comp-R. pACYC184-*ldhA* was constructed in a similar manner using the primer pair *ldhA*-Comp-F/*ldhA*-Comp-R. Construct integrity was verified by DNA sequencing.

Random transposon mutagenesis and screening. The Tn10dTc transposon was mobilized into the *S. enterica* serovar Typhimurium UK1 WT strain under conditions of nutrient limitation (E glucose minimal medium) and acid stress (pH 5.0), and insertion mutants that exhibited a growth defect in acidified E glucose medium (pH 5.0) were identified as candidate genes related to survival in the macrophage. The phenotype was confirmed by moving the mutation into the parent *S. enterica* serovar Typhimurium strain using P22-mediated transduction (Davis *et al.*, 1980). Bacteriophage P22HT int 105 was propagated in a donor strain (Tn10dTc insertion mutant) and used to infect the recipient strain (UK1 WT). The transductants were selected on LB agar containing Tc (20 µg ml⁻¹). P22 H5 was used to confirm that the transductants were phage-free and not P22 lysogens (Maloy *et al.*, 1996). The site of the Tn10dTc insertion in the

Table 1. Bacterial strains, bacteriophages and plasmids used in this study

Strain, phage or plasmid	Relevant characteristics	Source or reference
<i>S. enterica</i> serovar Typhimurium strains		
SF530 (χ 3761)	WT UK1	Curtiss & Hassan (1996)
YK5100	UK1 Tn10 <i>dTc</i> insertion in the <i>cspH-envE</i> intergenic region	This study
YK5101	UK1 Δ <i>raoN</i>	This study
YK5102	UK1 Δ <i>hfq</i>	This study
YK5103	UK1 Δ <i>ldhA</i>	This study
YK5104	UK1 Δ <i>raoN</i> Δ <i>ldhA</i> ::Km ^r	This study
YK5105	UK1 <i>ldhA</i> -3 $\times$ FLAG::Km ^r	This study
YK5106	UK1 Δ <i>raoN</i> <i>ldhA</i> -3 $\times$ FLAG::Km ^r	This study
TE6133	LT2 <i>rpoS1071</i> ::Tn10 <i>dCm</i>	Brown & Elliott (1996)
YK5107	UK1 <i>rpoS1071</i> ::Tn10 <i>dCm</i>	This study
<i>E. coli</i> strains		
DH5 α	F ⁻ <i>endA1 recA1 hsdR17(rK⁻ mK⁺) supE44 thi-1 gvrA96 relA1</i> Φ 80 <i>dlacZ</i> M15 Δ (<i>lacZYA-argF</i>) <i>U169</i>	RBC
χ 7232	pDMS197, (-)DAP ^a , Tet ^r	Edwards <i>et al.</i> (1998)
χ 7213	(-)DAP	Edwards <i>et al.</i> (1998)
Phages		
P22HT int 105	Used for generalized transduction	Sanderson <i>et al.</i> (1995)
H5	P22 <i>c2</i> mutant	Sanderson <i>et al.</i> (1995)
Plasmids		
pGEM-T Easy	Multicopy vector for cloning PCR products, Amp ^r	Promega
pDMS197	Suicide vector; <i>oriT oriV sacB</i> , Tet ^r	Edwards <i>et al.</i> (1998)
pDMS197- <i>raoN</i>	pDMS197 containing the <i>raoN</i> gene, Tet ^r	This study
pACYC184	Low-copy-number cloning vector, Tet ^r Cm ^r	NEB
pACYC184- <i>raoN</i>	pACYC184 with native promoter and <i>raoN</i> gene, Cm ^r	This study
pACYC184- <i>ldhA</i>	pACYC184 with native promoter and <i>ldhA</i> gene, Cm ^r	This study

chromosome was amplified using arbitrarily primed PCR with the primer pairs Tn10-3L/Arb-1b and IS10-L/Arb-1, and was sequenced using the primer IS10-L (O'Toole & Kolter, 1998; Welsh & McClelland, 1990).

RNA isolation and Northern blot analysis. Total RNA was isolated using the hot-phenol method (Laoide & Ullmann, 1990). Briefly, cultured cells were collected and resuspended in lysis solution (20 mM sodium acetate, pH 5.5, 1 mM EDTA, 0.5% SDS). The cell lysates were incubated with hot (65 °C) 20 mM sodium acetate (pH 4.8)-saturated phenol for 5 min at 65 °C. Phenol extraction was performed until no residue was apparent at the interface. RNA was precipitated with 0.1 M KCl and three volumes of ethanol at -20 °C and, following centrifugation, the pellet was resuspended in RNase-free water. The quantity and purity of the RNA were determined using a NanoDrop spectrophotometer (NanoDrop Tech. Inc.). The *raoN*, *msgA*, *hfq*, *envE*, *ldhA* and 5S rRNA probes were amplified from *S. enterica* serovar Typhimurium UK1 chromosomal DNA using the primer pairs listed in Table 2. The purified PCR products were labelled using a digoxigenin (DIG) DNA labelling kit. RNA was separated using denaturing formaldehyde-agarose gel electrophoresis and was transferred to a GeneScreen Plus nylon membrane (Perkin-Elmer). Following overnight hybridization with DIG-labelled probes at 55 °C, blots were soaked in blocking reagent for 1 h and incubated with alkaline phosphatase-conjugated anti-DIG Fab fragments (Roche). Signals were visualized using CDP-Star (Roche).

5'-RACE. 5'-RACE (rapid amplification of cDNA ends) assays were performed as described previously (Argaman *et al.*, 2001). The total RNA (15 μ g) was untreated or treated with 25 units of tobacco acid

pyrophosphatase (TAP; Epicentre Technologies) to convert 5'-triphosphates into monophosphates at 37 °C for 60 min. An adaptor RNA (Argaman *et al.*, 2001) was then ligated to the 5'-ends at 17 °C for 16 h with 50 units of T4 RNA ligase (Promega), and reverse transcription (RT) was performed using the primer *raoN*-GSP-RT. cDNAs were amplified with the adaptor-specific primer adaptor-F and the gene-specific primer *raoN*-GSP-R at the following conditions: 95 °C for 10 min; 30 cycles of 95 °C for 30 s, 55 °C for 1 min, 72 °C for 1 min; 72 °C for 10 min. PCR products were separated on a 2% agarose gel, and bands of interest were cloned into pGEM-T easy vector (Promega) and sequenced.

Disk diffusion assays. Disk diffusion assays were performed as described previously (Lee *et al.*, 2012). Cells were grown in LB medium to an optical density at OD₆₀₀ of 0.4. Aliquots (100 μ l) of these cultures were mixed with 4 ml of 0.7% LB or minimal E glucose soft agar (kept at 40 °C) and were directly poured onto LB or minimal E glucose agar plates (containing 15 ml of 1.5% LB or minimal E glucose agar). After 30 min at room temperature, the plates were dried for 20 min in a laminar airflow hood, and then used for the assays. Whatman filter paper disks containing 10 μ l of 2 M hydrogen peroxide were placed on top of the agar, and the plates were incubated at 37 °C overnight. The diameters of the inhibition zones were measured after subtraction of the diameter of the filter paper disk (8 mm).

Macrophage infection and survival assay. The murine macrophage cell line J774A.1 was grown to confluence in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C in a 5% CO₂ incubator.

Table 2. Primer sequences used in this study

Primer name	Oligonucleotide sequence, 5'→3'	Use
raoN-Comp-F	<u>CCCAAGCTT</u> ATGACGCAAAGATGTTTCAG	Complementation
raoN-Comp-R	GCTCTAGAACCGGAATTAATCTGGTTTT	
ldhA-Comp-F	<u>CGCGGATCCTT</u> TGTTCCAGAGCGATACGG	
ldhA-Comp-R	<u>CCCAAGCTT</u> AGGGGAGCGACAGGATTAGA	Northern blot
raoN-Northern-F	ACGTGAAGAAATGTACAGCCGT	
raoN-Northern-R	TATTGGCGATTATATACCCAC	
msgA-Northern-F	TCCTCAATGAACTCACAAAACG	
msgA-Northern-R	GACAGGCCAGTGAAGCGAT	
hfq-Northern-F	TGCAAGGTCAAATCGAGTCC	
hfq-Northern-R	TCTCTTCGCTGTCCTGTTGC	
envE-Northern-F	AACCACACTGGTTCTCTGCCT	
envE-Northern-R	ACAGGGTCGCCTTTTCTGAC	
ldhA-qRT-F	TTGCGCCGGATTATAATAATG	
ldhA-qRT-R	AAATGGTAGTTTTCCGGCGT	
raoN-up-F	<u>CGGAGCTCGGTT</u> AACTCTTCGTGAATAAAA	Δ raoN mutant
raoN-up-R	<u>CGGGATCCCGT</u> ACTCGCTAAATTTTCTG	
raoN-do-F	<u>CGGGATCCCGT</u> GCTACGTCATATTCAGTGG	
raoN-do-R	<u>GCTCTAGAGCT</u> GACCAATTTAAGCTGGG	Δ hfq mutant
hfq-Mu-F	ATGGCTAAGGGCAATCTTTACAAGATCCGTTCTGAACGGTGTAGGCTGGAGCTGCTTC	
hfq-Mu-R	TGTTGCTGTGATGGGAAACCGGGCGAGACGGGACAACAGTCATATGAATATCCTCCTTAG	
ldhA-Mu-F	ATGGATGTGAAGCAGTTTGTATCTTCGTGAATGACGACGGGTGTAGGCTGGAGCTGCTTC	Δ ldhA mutant
ldhA-Mu-R	TCCTGGATAACATCGTTTGACTTATCTTCAAAGAACAGGTCATATGAATATCCTCCTTAG	
ldhA-3 × FLAG-F	CCAGCTTGAGAAGGGCGAAGCCTGCCCGAACGCGCTGTTCTGACTACAAAAGACCATGACG	
ldhA-3 × FLAG-R	CCGTCATGTCCTCGCCAAAGCTGAGCTCCGGGCGGTGACCATATGAATATCCTCCTTA	3 × FLAG tagging
5S rRNA-F	GGTGGTCCCACCTGACCC	
5S rRNA-R	ATGCCTGGCAGTTCCCTACT	
β -Actin-F	GAGATGGCCACGGCTGCT	Normalization control
β -Actin-R	TTCTGCATCCTGTCGGCA	
raoN-GSP-RT	TGGCGATTATACCCACTGAA	
adaptor-F	GATATGCGCGAATTCCTGTAGA	5'-RACE
raoN-GSP-R	ACCCACTGAATATGACGTAGCA	
Tn10-3L	TCCATTGCTGTTGACAAAGGGAAT	
Arb-1b	GGCCAGCGAGCTAACGAGACNNNNGATAT	Mapping Tn10dTc insertion
IS10-L	CAAGATGTGTATCTACCTTAAC	
Arb-1	GGCCAGCGAGCTAACGAGAC	

*Restriction enzyme recognition sequences are underlined.

The J774A.1 cells were seeded onto 24-well tissue culture plates at a density of 2×10^5 macrophages per well. After 3 h, the cells were treated with LPS ($1 \mu\text{g ml}^{-1}$) for 18 h and then challenged with *S. enterica* serovar Typhimurium strains at an m.o.i. of 10:1. The plates were centrifuged for 5 min at 500 g to enhance bacteria-macrophage contact and then further incubated for 30 min at 37 °C to permit phagocytosis. To remove the extracellular bacteria, cells were washed with PBS three times, followed by incubation with DMEM containing gentamicin ($100 \mu\text{g ml}^{-1}$) for 1.5 h. The cells were washed with PBS three times and then incubated with DMEM containing 10% FBS and $10 \mu\text{g ml}^{-1}$ gentamicin at 37 °C. Macrophage cells were lysed with 1% Triton X-100 for 15 min, after which the number of bacteria present were enumerated by counting the c.f.u.

Microarray analysis. Two microarrays were performed in biological triplicates. Total RNA was extracted from UK1 WT and Δ raoN strains grown in LB to an OD₆₀₀ of 0.6 and then subjected to oxidative stress (5 mM hydrogen peroxide) for 30 min. Each RNA sample was labelled with Cy3- or Cy5-conjugated dCTP by a RT reaction using SuperScript II (Invitrogen) and hybridized to a NimbleGen 4-plex array that was based on *S. enterica* serovar Typhimurium LT2 (Roche NimbleGen, Inc.). Arrays were scanned using an Axon GenePix 4000B scanner (Molecular Devices). Gene expression levels were calculated with NimbleScan software, version 2.4 (Roche NimbleGen, Inc.), and the relative signal intensities were generated using the robust multiarray average algorithm. Data analyses were performed using GeneSpring GX, version 7.3.1 (Agilent Technologies). The fold change was calculated using the

average signal intensities for the $\Delta raoN$ mutant divided by those from the WT.

Quantitative real-time RT-PCR. The relative abundance of the *ldhA* transcript was measured by quantitative real-time RT-PCR (qRT-PCR). cDNA was synthesized using random hexamer primers and the Taqman RT kit (Roche). qRT-PCR was performed using SYBR Green (Bio-Rad) and the gene-specific primer pair *ldhA*-qRT-F/*ldhA*-qRT-R. Expression of *ldhA* was calculated as the fold change based on the cycles-to-threshold values as described previously (Talaat *et al.*, 2002). 5S rRNA was used as a normalization control.

Immunoblot analysis. Cells were harvested in SDS sample buffer (60 mM Tris/HCl, pH 6.8, 30 % glycerol, 2 % SDS, 0.1 % bromophenol blue and 14.4 mM 2-mercaptoethanol) and boiled for 10 min to prepare the protein lysates. Proteins were resolved by electrophoresis in 12 % SDS-PAGE and transferred onto a nitrocellulose membrane. Membranes were blocked with blocking solution (Tris-buffered saline with Tween-20 containing 5 % skimmed milk) and immunoblotted with mouse monoclonal antibodies against the FLAG tag (Sigma) or DnaK (Stressgen). Horseradish peroxidase-conjugated goat anti-mouse IgG was used as the secondary antibody. Immunoreactive bands were visualized using a BM chemiluminescence blotting substrate (POD) (Roche).

Microarray data accession number. The microarray data were deposited in the GEO NCBI database under the accession number GSE37626.

RESULTS AND DISCUSSION

Identification of a novel sRNA that contributes to survival under conditions of nutrient limitation and acid stress

The SCV in macrophages undergoes acidification and restricts nutrients that are required by *Salmonella* (Kingsley & Bäumlér, 2000; Rathman *et al.*, 1996). To identify candidate genes that contribute to survival in the macrophage, we performed a transposon (Tn10dTc)-based random mutagenesis under conditions of nutrient limitation (minimal medium) and acid stress (pH 5.0). Of the approximately 6000 random transposon insertions screened, we first selected relatively small colonies on acidified E glucose agar plates (pH 5.0), although their differences were slight. We then monitored their growth rates in broth culture and finally isolated a mutant that exhibited a growth defect in acidified E glucose medium (pH 5.0). However, its growth was comparable to that of the WT in LB medium (pH 7.0) (Fig. 1a). By sequencing the genomic DNA flanking the transposon (O'Toole & Kolter, 1998; Welsh & McClelland, 1990), the Tn10dTc-inserted region was determined to be located between the *cspH* and *envE* genes on SPI-11 (Fig. 1b).

To test the existence of a novel sRNA-encoding gene in the *cspH-envE* intergenic region, Northern blot analysis was conducted on total RNA isolated from UK1 WT and Tn10dTc-insertion mutant cells that were collected 30 min after transfer to the acidified E glucose medium (pH 5.0). When the intergenic region was divided into two smaller probes named P1 and P2 (Fig. 1b), a positive hybridization

signal was detected only with the P2 probe, indicating that a transcript was expressed from the second half of the intergenic region. The expression of P2 region was induced by acid and nutrient stress, but little if any expression was detected in the transposon-insertion mutant (Fig. 1c), implying that the growth defect of the mutant is due to the lack of the expression of P2 region. These results suggest that the *cspH-envE* intergenic region encodes a novel sRNA, which we have named RaoN.

To date, no sRNA-encoding genes have been reported in the *cspH-envE* intergenic region. One likely reason is that this intergenic region contains no Rho-independent transcription terminators, which are often used for the computational prediction of sRNAs (Argaman *et al.*, 2001; Sridhar *et al.*, 2010). However, some sRNAs were found to be terminated by Rho, and identification of Rho-dependent terminators with associated promoters was suggested as an additional method for finding novel sRNAs (Peters *et al.*, 2009). It is also possible that *raoN* was not identified by previous experimental screens because its expression is induced only under specific stress conditions.

RaoN is strongly induced by combined treatment with nutrient and oxidative stress

To examine whether *raoN* is also induced in response to oxidative stress, we first constructed a *raoN* null mutant that lacked the promoter region. Cells were grown in LB medium to an OD₆₀₀ of 0.6 and exposed to hydrogen peroxide (5 mM) for 30 min. Northern blot analysis indicated that *raoN* expression was induced by hydrogen peroxide treatment in the WT strain, but no corresponding band was detected in the *raoN* mutant (Fig. 2a). We further tested the effect of combined treatment of nutrient and oxidative stress on *raoN* expression. Cells were grown in LB medium to an OD₆₀₀ of 0.6 and, after transfer to fresh E glucose medium (nutrient stress) containing 5 mM hydrogen peroxide, were incubated for an additional 30 min. Notably, the expression of *raoN* was strongly induced under the combined condition (Fig. 2a), which partially mimics the conditions within macrophage phagosomes (Kingsley & Bäumlér, 2000).

It has been shown that a large number of sRNAs associate with the RNA chaperone Hfq, which has a stabilizing effect on sRNAs and promotes pairing between sRNAs and their target mRNAs (Otaka *et al.*, 2011; Valentin-Hansen *et al.*, 2004; Waters & Storz, 2009). Since the RaoN region is not present in the available datasets of Hfq-bound sRNAs in *Salmonella* (Chao *et al.*, 2012; Sittka *et al.*, 2008), we examined whether Hfq is required for the induction of RaoN. Indeed, *raoN* expression was not detected in the Δhfq mutant (Fig. 2a). Hfq is known to be required for efficient translation of the alternative sigma factor RpoS (σ^S) (Brown & Elliott, 1996), which plays an important role in response to various stresses, including acid shock, starvation and oxidative stress (Hengge-Aronis, 2002; Loewen & Hengge-Aronis, 1994; O'Neal *et al.*, 1994). We

Fig. 1. Isolation of a growth-defective mutant under conditions of nutrient limitation and acid stress. (a) Growth comparison of the *S. enterica* serovar Typhimurium UK1 WT and the Tn10dTc-insertion mutant strains. Cells were grown in acidified E glucose medium (pH 5.0) and LB medium (pH 7.0) with constant shaking at 37 °C, and the OD₆₀₀ values were determined at the indicated times. Three independent experiments were performed for each strain, and the error bars indicate the SD. (b) Schematic diagram showing the genetic organization of the novel sRNA gene, *raoN*. The Tn10dTc insertion site was determined by sequencing the chromosomal DNA flanking the transposon. The black dotted lines represent the P1 and P2 probes used for Northern blot analyses. The transcription start site (+1) of *raoN* is in bold. Putative -35 and -10 promoter elements, in bold type and underlined, were predicted using BPRM software (<http://www.softberry.com/>). (c) Northern blot analysis was performed to identify a novel intergenic (*cspH*-*envE*) transcript. Cells were grown to an OD₆₀₀ of 0.6 in LB medium and then transferred to the acidified E glucose medium (pH 5.0). Samples were collected after an additional 30 min incubation at 37 °C. 5S rRNA was used as a loading control.

therefore hypothesized that *raoN* transcription might be not directly dependent on Hfq but instead depend on RpoS. However, the transcription levels of *raoN* were almost the same in the WT and *rpoS* mutant strains under combined stress conditions. (Fig. 2b). Thus, the mechanisms as to how Hfq regulates *raoN* expression are yet to be determined. We next determined whether mutation of

Fig. 2. Transcription of *raoN* is strongly induced by combined treatment of nutrient and oxidative stress. (a) Effect of oxidative and nutrient stress on *raoN* expression. (b) Effect of *rpoS* gene knockout on *raoN* transcription under combined stress conditions. (c) Effect of *raoN* gene deletion on *envE* transcription under combined stress conditions. Northern blots were performed using total RNA extracted from *S. enterica* serovar Typhimurium UK1 WT, Δ *raoN*, Δ *hfq* and *rpoS*::Tn10dCm strains. Cells were grown to an OD₆₀₀ of 0.6 in LB medium and then transferred to LB medium supplemented with 5 mM hydrogen peroxide (oxidative stress) or E glucose medium supplemented with 5 mM hydrogen peroxide (oxidative and nutrient stress). Samples were collected after an additional 30 min incubation at 37 °C. 5S rRNA was used as a loading control. (d) 5'-RACE analysis of *raoN* transcripts. RT-PCR products of TAP-treated or untreated RNA were separated on a 2% agarose gel. The control PCR was performed with chromosomal DNA as template. The black arrow indicates the enhanced RT-PCR signal upon TAP treatment. (e) Determination of the approximate size of sRNA RaoN by Northern blot analysis. After transfer of RNA to a nylon membrane, the membrane was cut into strips each corresponding to one lane, and each strip was hybridized with a different DIG-labelled probe.

raoN affects expression of the neighbouring downstream gene, *envE*, which encodes an envelope protein of unknown function. As shown in Fig. 2(c), deletion of the *raoN* promoter region had no effect on *envE* transcription. Interestingly, the expression of *envE* was completely shut off in response to hydrogen peroxide, while it was constitutively expressed under normal conditions. We therefore speculate that *envE* expression may be deleterious under oxidative stress conditions.

To further identify the transcriptional start site of *raoN*, we performed 5'-RACE analysis on the WT strain following

Fig. 3. A mutation in *raoN* confers sensitivity to oxidative stress, particularly when combined with nutrient limitation. (a) Schematic representation of the relevant genetic organization of the strains used in this work. (b) The effect of hydrogen peroxide on the growth of the *S. enterica* serovar Typhimurium UK1 WT, Tn10dTc insertion mutant, $\Delta raoN$ and complemented strains. Fresh stationary phase starter cultures were subcultured at a concentration of 1 : 100 in LB medium containing 5 mM hydrogen peroxide. The cultures were grown with constant shaking at 37 °C, and the OD₆₀₀ values were determined at the indicated times. (c) Determination of the hydrogen peroxide susceptibilities of several *S. enterica* serovar Typhimurium strains under conditions of nutrient limitation. Disk diffusion assays were performed on minimal E glucose agar plates with a filter paper disk containing 10 μ l of 2 M hydrogen peroxide. Each bar represents the average zone of inhibition, expressed as the total diameter minus the diameter of the filter paper disk (8 mm). Three independent experiments were performed for each strain. Error bars indicate the SD.

30 min of oxidative stress. Fig. 2(d) shows that specific PCR amplification was detected only in the TAP-treated RNA sample. Cloning and sequencing of this 5'-RACE product revealed that the 5'-end of RaoN is the indicated G residue (Fig. 1b). The length of *raoN* was also determined to be approximately 200 bp, as judged from the size of the RNA band detected by Northern blot analysis (Fig. 2e). In this analysis, the membrane with the bound RNA was cut into strips each corresponding to one lane, and each strip was then hybridized with a different probe.

RaoN contributes to resistance to oxidative stress, particularly when combined with nutrient limitation

To determine if RaoN confers resistance to oxidative stress, we compared the growth rate of several *S. enterica* serovar Typhimurium UK1 strains in LB medium containing 5 mM hydrogen peroxide. Although 5 mM exceeds the concentration of hydrogen peroxide encountered within phagocytic cells, no significant differences in growth rate were observed between strains in LB medium containing lower

Fig. 4. RaoN confers a growth advantage to *S. enterica* serovar Typhimurium within macrophages. Murine J774A.1 macrophages were infected with *S. enterica* serovar Typhimurium UK1 WT, Δ *raoN* and complemented strains and the numbers of viable intracellular bacteria were determined at 4 h and 20 h after infection. The data are presented as the average of at least three independent experiments that were performed in duplicate, and the SD is indicated by the error bars.

concentrations of hydrogen peroxide (0.5–1 mM). The relevant genetic organizations are schematically represented in Fig. 3(a). Both the Δ *raoN* mutant and the transposon-insertion mutant grew significantly more slowly than the WT in the presence of hydrogen peroxide (Fig. 3b), but they eventually almost caught up with the WT, indicating a significant growth delay under oxidative stress. When *raoN* was expressed from its own promoter in the pACYC184 plasmid, it restored growth to rates similar to those of the WT (Fig. 3b). However, no significant differences were observed between WT and mutant strains when disk diffusion assays were performed on LB agar plates with a filter paper disk containing hydrogen peroxide (data not shown).

To further examine whether the *raoN* mutant shows enhanced sensitivity to oxidative stress during nutrient limitation, disk diffusion assays were performed on minimal E glucose agar plates with hydrogen peroxide, conditions that led to high RaoN levels, as seen by Northern blot analysis (Fig. 2a). Disruption of *raoN* resulted in significantly increased susceptibility to these combined stress conditions (Fig. 3c). These results suggest a pivotal role for RaoN in resistance to oxidative stress during nutrient limitation.

RaoN is required for *S. enterica* serovar Typhimurium replication within murine macrophages

As *Salmonella*-infected macrophages generate acid, nutrient, oxidative and nitrosative stresses as defences against

bacterial pathogens (Kingsley & Bäuml, 2000), we hypothesized that the *raoN* mutant would show reduced replication within murine macrophages. To evaluate this hypothesis, the murine macrophage cell line J774A.1 was infected with the WT, the Δ *raoN* mutant or the Δ *raoN* mutant complemented with an intact *raoN* gene. After 4 h and 20 h of infection, the intracellular survival of bacterial cells was monitored by counting the c.f.u. As shown in Fig. 4, the *S. enterica* serovar Typhimurium *raoN* mutant had reduced net intracellular replication compared to the WT at 20 h post-infection, while a slight difference was observed between the WT and mutant strains at 4 h post-infection. Complementation with a plasmid-borne *raoN* gene restored survival of the *raoN* mutant to the levels of the WT. These results suggest that RaoN plays an important role during *Salmonella* infection in macrophages.

ROS produced by NADPH oxidase are known to be important for antibacterial responses during the early stages of infection (Mastroeni *et al.*, 2000; Vazquez-Torres *et al.*, 2000a), but it was also reported that TTSS encoded by SPI-2 allows *Salmonella* to avoid NADPH oxidase-dependent killing by interfering with trafficking of oxidase-containing vesicles to the phagosome (Vazquez-Torres *et al.*, 2000b). Therefore, we can speculate that the WT and the *raoN* mutant strains may survive similarly in early stages of infection due to the role of SPI-2 TTSS. Recent studies revealed that, in addition to NADPH oxidase-derived ROS, mitochondrial ROS (mROS) also contribute to murine macrophage bactericidal activity (West *et al.*, 2011). Since the production of mROS plays a critical role in the control of *Salmonella* replication between 8 and 24 h after infection (West *et al.*, 2011), the reduced survival of the *raoN* mutant within murine macrophages at 20 h post-infection may be related to the activity of mROS.

A *raoN* null mutant displays increased expression of the *ldhA* gene under oxidative stress conditions

To identify putative target genes regulated by RaoN, we used microarray-based gene expression profiling. Under conditions of oxidative stress (5 mM hydrogen peroxide), the transcriptome of the *raoN* mutant was relatively unperturbed with only two genes upregulated >1.75-fold (Fig. 5a). These results indicate that only small transcriptomic changes are correlated with the distinct phenotype shown in Figs 3 and 4. The observed change in the transcript abundance of *ldhA*, which encodes lactate dehydrogenase A, was confirmed by qRT-PCR analysis (fold change $\pm$ SD = 1.72 ± 0.073). In addition, we found that transcription of *ldhA* itself is induced by hydrogen peroxide in the WT and mutant strains (data not shown). Thus, the modest induction of *ldhA* led us to further investigate the role of LdhA in the RaoN-mediated response to hydrogen peroxide. Using Northern blot and immunoblot analyses, we showed that *trans*-complementation of the *raoN* mutant with a plasmid-encoded *raoN* reduced *ldhA* transcription and protein levels, respectively, to levels at least as low as those of WT (Fig. 5b, c). These

Fig. 5. Inactivation of *raoN* leads to increased expression of the *ldhA* gene under oxidative stress conditions. (a) Microarray transcriptional analysis of *S. enterica* serovar Typhimurium UK1 WT and Δ *raoN* strains. Black diamonds indicate genes whose expression changed at least 1.75-fold. Data shown are mean fluorescence intensities from two independent experiments. (b, c) Complementation of the Δ *raoN* mutant with a plasmid-borne *raoN* restores WT expression levels of *ldhA*. Total RNA was used to check the transcription of *ldhA* by Northern blot analysis (b). Whole-cell lysates were used for immunoblot analysis with specific antibodies against the FLAG tag (c). To determine LdhA protein levels, the 3 $\times$ FLAG epitope was inserted at the C terminus of LdhA. Cells were grown in LB medium to an OD₆₀₀ of 0.6 and then subjected to 5 mM hydrogen peroxide for 30 min. 5S rRNA and DnaK were used as loading controls. Representative results of at least three independent experiments are shown.

raoN mutant irrespective of oxidative stress (data not shown). However, this striking phenotype could not be complemented *in trans* by a plasmid-borne *raoN* (the reason is unknown). Thus, it remains elusive whether inactivation of *raoN* affects flagellar phase variation in *S. enterica* serovar Typhimurium.

Balanced expression of *ldhA* is necessary for survival under conditions of oxidative stress, particularly combined with nutrient limitation

To test the involvement of the *ldhA* gene in oxidative stress response, we compared the growth rate of *S. enterica* serovar Typhimurium strains in LB medium containing 5 mM hydrogen peroxide (Fig. 6a). The *ldhA* mutant exhibited a clear growth defect. In particular, consistent with the microarray data, the increased expression of *ldhA* (pACYC184-*ldhA*) in the WT strain made cells more sensitive to oxidative stress, suggesting that LdhA confers protection against oxidative stress but may be toxic when overexpressed. To further examine the role of *ldhA* in resistance to oxidative stress during nutrient limitation, disk diffusion assays were performed on minimal E glucose agar plates with hydrogen peroxide (Fig. 6b). The results reveal a similar pattern of hydrogen peroxide sensitivity as shown in Fig. 6(a), suggesting that the maintenance of optimal cellular levels of LdhA is critical for growth under conditions of oxidative stress, particularly when combined with nutrient limitation.

We next examined the involvement of *ldhA* in *Salmonella* replication within murine macrophages. To determine the relative expression levels of *ldhA* from bacteria recovered from macrophages infected with WT and *raoN* mutant strains, RT-PCR analysis was conducted on total RNA isolated from macrophage lysates at 20 h post-infection. As predicted, the *raoN* mutant displayed increased expression of the *ldhA* gene within murine macrophages (Fig. 6c). However, as shown in Fig. 6(d), the expression level of *ldhA* is less important in protection against macrophage

results suggest that inactivation of *raoN* upregulates the synthesis of lactate dehydrogenase A in response to oxidative stress. However, no evidence has yet been found for direct regulation of *ldhA* expression by RaoN. Since transcription of *ldhA* is known to be indirectly induced in response to pyruvate (Jiang *et al.*, 2001), we can speculate that the sRNA RaoN may affect some aspects of glucose metabolism, causing an increase in pyruvate concentration.

The expression of the *fljB* gene, which encodes phase-2 flagellin, also increased in the *raoN* mutant (Fig. 5a). This finding has been further confirmed and extended by demonstrating that *fljB* expression was increased in the

Fig. 6. RaoN-mediated regulation of *ldhA* expression confers resistance to oxidative stress, particularly when combined with nutrient limitation. (a) The effect of hydrogen peroxide on the growth of several *S. enterica* serovar Typhimurium strains. Fresh stationary phase starter cultures were subcultured at a concentration of 1:100 in LB medium containing 5 mM hydrogen peroxide. The cultures were grown with constant shaking at 37 °C, and the OD₆₀₀ values were determined at the indicated times. A representative dataset is shown. (b) Disk diffusion assays were performed on minimal E glucose agar plates with hydrogen peroxide. Three independent experiments were performed for each strain. Error bars indicate the SD. (c) RT-PCR analysis of *Salmonella* *ldhA* transcription within murine macrophages at 20 h post-infection. Total RNA was extracted from lysates of murine macrophages infected with WT and *raoN* mutant strains. 5S rRNA and β -actin were used as loading controls. (d) Effects of deletion or overexpression of the *ldhA* gene on *Salmonella* growth within macrophages. Murine J774A.1 macrophages were infected with various *S. enterica* serovar Typhimurium strains and the numbers of viable intracellular bacteria were determined at 20 h after infection. Data are presented as the average of three independent experiments performed in duplicate, and the error bars indicate the SD.

intracellular killing, suggesting that RaoN may control other genes that are important for intramacrophage replication of *Salmonella*. We therefore conclude that the RaoN-mediated fine-tuning of *ldhA* expression is necessary for resistance to oxidative stress *in vitro*, but is not absolutely required for intramacrophage survival of *Salmonella*.

Lactate dehydrogenase converts pyruvate, the final product of glycolysis, to lactate via the oxidation of NADH to NAD⁺, thus maintaining glycolytic activity when oxygen is absent or in short supply (Pedersen *et al.*, 2012). It has also been reported that glycolysis is required for intramacrophage replication of *S. enterica* serovar Typhimurium (Bowden *et al.*, 2009). Thus, the role of *Salmonella* *ldhA*

in macrophages may be to regenerate NAD⁺ for maintenance of glycolysis. We found that in *S. enterica* serovar Typhimurium hydrogen peroxide treatment induces the expression of *ldhA* (data not shown). However, *S. enterica* is known to be very sensitive to the antimicrobial activity of lactate (De Keersmaecker *et al.*, 2006; Marianelli *et al.*, 2010). Therefore, we can speculate that during conditions leading to excretion of lactate, *Salmonella* may have a feedback-inhibition system to prevent overexpression of *LdhA*. The data presented here clearly show RaoN acts as a negative regulator of *ldhA* expression under conditions of oxidative stress.

Concluding remarks

Our data demonstrate that a novel sRNA RaoN is required for survival under oxidative stress and within murine macrophages. In addition, we show that RaoN negatively regulates the expression of *ldhA* under oxidative stress conditions, by mechanisms not yet resolved (Fig. 5). Since *LdhA* confers resistance to oxidative stress but increases sensitivity when overexpressed (Fig. 6a, b), the balanced expression of *ldhA* by RaoN is critical under stress conditions. However, the regulation of *ldhA* expression is only partially responsible for the contributions of RaoN to intramacrophage survival and replication of *S. enterica* serovar Typhimurium (Fig. 6d). We speculate that the RaoN sRNA likely regulates the expression of other genes important for growth in the macrophage. No obvious candidates, apart from *ldhA*, were revealed by our transcriptome analysis (Fig. 5a). The genes regulated by RaoN in macrophages may not be expressed under our *in vitro* conditions, or RaoN activity may affect gene expression without affecting the steady-state level of its target mRNA(s). Future studies will be required to identify these targets and to determine how RaoN confers a survival advantage to *Salmonella* within macrophages. In summary, these findings implicate the RaoN sRNA in the regulation of *LdhA* and, likely, other pathways under combined nutrient and oxidative stress conditions.

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REFERENCES

Altuvia, S. (2007). Identification of bacterial small non-coding RNAs: experimental approaches. *Curr Opin Microbiol* **10**, 257–261.

Argaman, L., Hershberg, R., Vogel, J., Bejerano, G., Wagner, E. G., Margalit, H. & Altuvia, S. (2001). Novel small RNA-encoding genes in the intergenic regions of *Escherichia coli*. *Curr Biol* **11**, 941–950.

Bowden, S. D., Rowley, G., Hinton, J. C. & Thompson, A. (2009). Glucose and glycolysis are required for the successful infection of macrophages and mice by *Salmonella enterica* serovar typhimurium. *Infect Immun* **77**, 3117–3126.

Brantl, S. (2007). Regulatory mechanisms employed by *cis*-encoded antisense RNAs. *Curr Opin Microbiol* **10**, 102–109.

Brown, L. & Elliott, T. (1996). Efficient translation of the RpoS sigma factor in *Salmonella typhimurium* requires host factor I, an RNA-binding protein encoded by the *hfq* gene. *J Bacteriol* **178**, 3763–3770.

Chao, Y., Papenfort, K., Reinhardt, R., Sharma, C. M. & Vogel, J. (2012). An atlas of Hfq-bound transcripts reveals 3' UTRs as a genomic reservoir of regulatory small RNAs. *EMBO J* **31**, 4005–4019.

Curtiss, R., III & Hassan, J. O. (1996). Nonrecombinant and recombinant avirulent *Salmonella* vaccines for poultry. *Vet Immunol Immunopathol* **54**, 365–372.

Datsenko, K. A. & Wanner, B. L. (2000). One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A* **97**, 6640–6645.

Davis, R. W., Botstein, D. & Roth, J. R. (1980). *Advanced Bacterial Genetics*. New York: Cold Spring Harbor Laboratory Press.

De Keersmaecker, S. C., Verhoeven, T. L., Desair, J., Marchal, K., Vanderleyden, J. & Nagy, I. (2006). Strong antimicrobial activity of *Lactobacillus rhamnosus* GG against *Salmonella typhimurium* is due to accumulation of lactic acid. *FEMS Microbiol Lett* **259**, 89–96.

Edwards, R. A., Keller, L. H. & Schifferli, D. M. (1998). Improved allelic exchange vectors and their use to analyze 987P fimbria gene expression. *Gene* **207**, 149–157.

Fang, F. C. (2004). Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. *Nat Rev Microbiol* **2**, 820–832.

Foster, J. W. & Spector, M. P. (1995). How *Salmonella* survive against the odds. *Annu Rev Microbiol* **49**, 145–174.

Galán, J. E. & Collmer, A. (1999). Type III secretion machines: bacterial devices for protein delivery into host cells. *Science* **284**, 1322–1328.

Gay, P., Le Coq, D., Steinmetz, M., Berkelman, T. & Kado, C. I. (1985). Positive selection procedure for entrapment of insertion sequence elements in gram-negative bacteria. *J Bacteriol* **164**, 918–921.

Gong, H., Vu, G. P., Bai, Y., Chan, E., Wu, R., Yang, E., Liu, F. & Lu, S. (2011). A *Salmonella* small non-coding RNA facilitates bacterial invasion and intracellular replication by modulating the expression of virulence factors. *PLoS Pathog* **7**, e1002120.

Gottesman, S., McCullen, C. A., Guillier, M., Vanderpool, C. K., Majdalani, N., Benhammou, J., Thompson, K. M., FitzGerald, P. C., Sowa, N. A. & FitzGerald, D. J. (2006). Small RNA regulators and the bacterial response to stress. *Cold Spring Harb Symp Quant Biol* **71**, 1–11.

Gunn, J. S., Alpuche-Aranda, C. M., Loomis, W. P., Belden, W. J. & Miller, S. I. (1995). Characterization of the *Salmonella typhimurium* pagC/pagD chromosomal region. *J Bacteriol* **177**, 5040–5047.

Haraga, A., Ohlson, M. B. & Miller, S. I. (2008). *Salmonellae* interplay with host cells. *Nat Rev Microbiol* **6**, 53–66.

Hengge-Aronis, R. (2002). Signal transduction and regulatory mechanisms involved in control of the σ^S (RpoS) subunit of RNA polymerase. *Microbiol Mol Biol Rev* **66**, 373–395.

Hensel, M. (2000). *Salmonella* pathogenicity island 2. *Mol Microbiol* **36**, 1015–1023.

Jackett, P. S., Aber, V. R. & Lowrie, D. B. (1978). Virulence and resistance to superoxide, low pH and hydrogen peroxide among strains of *Mycobacterium tuberculosis*. *J Gen Microbiol* **104**, 37–45.

Jiang, G. R., Nikolova, S. & Clark, D. P. (2001). Regulation of the *ldhA* gene, encoding the fermentative lactate dehydrogenase of *Escherichia coli*. *Microbiology* **147**, 2437–2446.

Jones, B. D., Ghorri, N. & Falkow, S. (1994). *Salmonella typhimurium* initiates murine infection by penetrating and destroying the

- specialized epithelial M cells of the Peyer's patches. *J Exp Med* **180**, 15–23.
- Kingsley, R. A. & Bäuml, A. J. (2000). Salmonella interactions with professional phagocytes. *Subcell Biochem* **33**, 321–342.
- Kröger, C., Dillon, S. C., Cameron, A. D., Papenfort, K., Sivasankaran, S. K., Hokamp, K., Chao, Y., Sittka, A., Hébrard, M. & other authors (2012). The transcriptional landscape and small RNAs of *Salmonella enterica* serovar Typhimurium. *Proc Natl Acad Sci U S A* **109**, E1277–E1286.
- Laoide, B. M. & Ullmann, A. (1990). Virulence dependent and independent regulation of the *Bordetella pertussis* cya operon. *EMBO J* **9**, 999–1005.
- Lee, Y. H., Kim, B. H., Kim, J. H., Yoon, W. S., Bang, S. H. & Park, Y. K. (2007). CadC has a global translational effect during acid adaptation in *Salmonella enterica* serovar Typhimurium. *J Bacteriol* **189**, 2417–2425.
- Lee, Y. H., Kingston, A. W. & Helmann, J. D. (2012). Glutamate dehydrogenase affects resistance to cell wall antibiotics in *Bacillus subtilis*. *J Bacteriol* **194**, 993–1001.
- Loewen, P. C. & Hengge-Aronis, R. (1994). The role of the sigma factor sigma S (KatF) in bacterial global regulation. *Annu Rev Microbiol* **48**, 53–80.
- Maloy, S. R. & Roth, J. R. (1983). Regulation of proline utilization in *Salmonella typhimurium*: characterization of put:Mu d(Ap, lac) operon fusions. *J Bacteriol* **154**, 561–568.
- Maloy, S. R., Stewart, V. J. & Taylor, R. K. (1996). *Genetic Analysis of Pathogenic Bacteria: a Laboratory Manual*. New York: Cold Spring Harbor Laboratory Press.
- Marianelli, C., Cifani, N. & Pasquali, P. (2010). Evaluation of antimicrobial activity of probiotic bacteria against *Salmonella enterica* subsp. *enterica* serovar typhimurium 1344 in a common medium under different environmental conditions. *Res Microbiol* **161**, 673–680.
- Mastroeni, P., Vazquez-Torres, A., Fang, F. C., Xu, Y., Khan, S., Hormaeche, C. E. & Dougan, G. (2000). Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. II. Effects on microbial proliferation and host survival *in vivo*. *J Exp Med* **192**, 237–248.
- Miller, S. I., Kukral, A. M. & Mekalanos, J. J. (1989). A two-component regulatory system (*phoP phoQ*) controls *Salmonella typhimurium* virulence. *Proc Natl Acad Sci U S A* **86**, 5054–5058.
- Mills, D. M., Bajaj, V. & Lee, C. A. (1995). A 40 kb chromosomal fragment encoding *Salmonella typhimurium* invasion genes is absent from the corresponding region of the *Escherichia coli* K-12 chromosome. *Mol Microbiol* **15**, 749–759.
- Monack, D. M., Mueller, A. & Falkow, S. (2004). Persistent bacterial infections: the interface of the pathogen and the host immune system. *Nat Rev Microbiol* **2**, 747–765.
- O'Neal, C. R., Gabriel, W. M., Turk, A. K., Libby, S. J., Fang, F. C. & Spector, M. P. (1994). RpoS is necessary for both the positive and negative regulation of starvation survival genes during phosphate, carbon, and nitrogen starvation in *Salmonella typhimurium*. *J Bacteriol* **176**, 4610–4616.
- O'Toole, G. A. & Kolter, R. (1998). Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. *Mol Microbiol* **28**, 449–461.
- Otaka, H., Ishikawa, H., Morita, T. & Aiba, H. (2011). PolyU tail of rho-independent terminator of bacterial small RNAs is essential for Hfq action. *Proc Natl Acad Sci U S A* **108**, 13059–13064.
- Padalon-Brauch, G., Hershberg, R., Elgrably-Weiss, M., Baruch, K., Rosenshine, I., Margalit, H. & Altuvia, S. (2008). Small RNAs encoded within genetic islands of *Salmonella typhimurium* show host-induced expression and role in virulence. *Nucleic Acids Res* **36**, 1913–1927.
- Papenfort, K., Pfeiffer, V., Lucchini, S., Sonawane, A., Hinton, J. C. & Vogel, J. (2008). Systematic deletion of *Salmonella* small RNA genes identifies CyaR, a conserved CRP-dependent riboregulator of OmpX synthesis. *Mol Microbiol* **68**, 890–906.
- Papenfort, K., Podkaminski, D., Hinton, J. C. & Vogel, J. (2012). The ancestral SgrS RNA discriminates horizontally acquired *Salmonella* mRNAs through a single G-U wobble pair. *Proc Natl Acad Sci U S A* **109**, E757–E764.
- Pedersen, M. B., Gaudu, P., Lechardeur, D., Petit, M. A. & Gruss, A. (2012). Aerobic respiration metabolism in lactic acid bacteria and uses in biotechnology. *Annu Rev Food Sci Technol* **3**, 37–58.
- Peters, J. M., Mooney, R. A., Kuan, P. F., Rowland, J. L., Keles, S. & Landick, R. (2009). Rho directs widespread termination of intragenic and stable RNA transcription. *Proc Natl Acad Sci U S A* **106**, 15406–15411.
- Pfeiffer, V., Sittka, A., Tomer, R., Tedin, K., Brinkmann, V. & Vogel, J. (2007). A small non-coding RNA of the invasion gene island (SPI-1) represses outer membrane protein synthesis from the *Salmonella* core genome. *Mol Microbiol* **66**, 1174–1191.
- Rathman, M., Sjaastad, M. D. & Falkow, S. (1996). Acidification of phagosomes containing *Salmonella typhimurium* in murine macrophages. *Infect Immun* **64**, 2765–2773.
- Sabbagh, S. C., Forest, C. G., Lepage, C., Leclerc, J. M. & Daigle, F. (2010). So similar, yet so different: uncovering distinctive features in the genomes of *Salmonella enterica* serovars Typhimurium and Typhi. *FEMS Microbiol Lett* **305**, 1–13.
- Sambrook, J. & Russell, D. W. (2001). *Molecular Cloning: a Laboratory Manual*, 3rd edn. New York: Cold Spring Harbor Laboratory Press.
- Sanderson, K. E., Hessel, A. & Rudd, K. E. (1995). Genetic map of *Salmonella typhimurium*, edition VIII. *Microbiol Rev* **59**, 241–303.
- Sittka, A., Lucchini, S., Papenfort, K., Sharma, C. M., Rolle, K., Binnewies, T. T., Hinton, J. C. & Vogel, J. (2008). Deep sequencing analysis of small noncoding RNA and mRNA targets of the global post-transcriptional regulator, Hfq. *PLoS Genet* **4**, e1000163.
- Sridhar, J., Sambaturu, N., Sabarinathan, R., Ou, H. Y., Deng, Z., Sekar, K., Rafi, Z. A. & Rajakumar, K. (2010). sRNAscaner: a computational tool for intergenic small RNA detection in bacterial genomes. *PLoS ONE* **5**, e11970.
- Storz, G., Vogel, J. & Wassarman, K. M. (2011). Regulation by small RNAs in bacteria: expanding frontiers. *Mol Cell* **43**, 880–891.
- Stuehr, D. J. & Nathan, C. F. (1989). Nitric oxide. A macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells. *J Exp Med* **169**, 1543–1555.
- Talaat, A. M., Howard, S. T., Hale, W., IV, Lyons, R., Garner, H. & Johnston, S. A. (2002). Genomic DNA standards for gene expression profiling in *Mycobacterium tuberculosis*. *Nucleic Acids Res* **30**, e104.
- Underhill, D. M. & Ozinsky, A. (2002). Phagocytosis of microbes: complexity in action. *Annu Rev Immunol* **20**, 825–852.
- Uzzau, S., Figueroa-Bossi, N., Rubino, S. & Bossi, L. (2001). Epitope tagging of chromosomal genes in *Salmonella*. *Proc Natl Acad Sci U S A* **98**, 15264–15269.
- Valentin-Hansen, P., Eriksen, M. & Udesen, C. (2004). The bacterial Sm-like protein Hfq: a key player in RNA transactions. *Mol Microbiol* **51**, 1525–1533.
- Vandal, O. H., Roberts, J. A., Odaira, T., Schnappinger, D., Nathan, C. F. & Ehrh, S. (2009). Acid-susceptible mutants of *Mycobacterium tuberculosis* share hypersusceptibility to cell wall and oxidative stress and to the host environment. *J Bacteriol* **191**, 625–631.

Vazquez-Torres, A., Jones-Carson, J., Bäuml, A. J., Falkow, S., Valdivia, R., Brown, W., Le, M., Berggren, R., Parks, W. T. & Fang, F. C. (1999). Extraintestinal dissemination of *Salmonella* by CD18-expressing phagocytes. *Nature* **401**, 804–808.

Vazquez-Torres, A., Jones-Carson, J., Mastroeni, P., Ischiropoulos, H. & Fang, F. C. (2000a). Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. I. Effects on microbial killing by activated peritoneal macrophages in vitro. *J Exp Med* **192**, 227–236.

Vazquez-Torres, A., Xu, Y., Jones-Carson, J., Holden, D. W., Lucia, S. M., Dinauer, M. C., Mastroeni, P. & Fang, F. C. (2000b). *Salmonella* pathogenicity island 2-dependent evasion of the phagocyte NADPH oxidase. *Science* **287**, 1655–1658.

Vogel, J. (2009). A rough guide to the non-coding RNA world of *Salmonella*. *Mol Microbiol* **71**, 1–11.

Vogel, H. J. & Bonner, D. M. (1956). Acetylornithinase of *Escherichia coli*: partial purification and some properties. *J Biol Chem* **218**, 97–106.

Vogel, J. & Sharma, C. M. (2005). How to find small non-coding RNAs in bacteria. *Biol Chem* **386**, 1219–1238.

Waters, L. S. & Storz, G. (2009). Regulatory RNAs in bacteria. *Cell* **136**, 615–628.

Welsh, J. & McClelland, M. (1990). Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res* **18**, 7213–7218.

West, A. P., Brodsky, I. E., Rahner, C., Woo, D. K., Erdjument-Bromage, H., Tempst, P., Walsh, M. C., Choi, Y., Shadel, G. S. & Ghosh, S. (2011). TLR signalling augments macrophage bactericidal activity through mitochondrial ROS. *Nature* **472**, 476–480.

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