RNA BIOLOGY
2019, VOL. 16, NO. 11, 1643–1657
https://doi.org/10.1080/15476286.2019.1653680

Taylor & Francis
Taylor & Francis Group

RESEARCH PAPER

Characterization of novel small RNAs (sRNAs) contributing to the desiccation response of *Salmonella enterica* serovar Typhimurium

Emmaline C. Barnhill[a*], Aline Crucello[a,b*], Dominika Houserova[c*], Valeria M. King[a,d], Shivam V. Amin [a,c], Justin T. Roberts [a,e], Michael E. Zambrano[a], Jeffrey D. DeMeis[a], Donavon J. Dahmer[f], Zara Ijaz[f], Addison A. Barchie[a], Brianna C. Watters[a], James E. Prusak[a], Meghan A. Dean[a], Nathaniel W. Holton[c], Jaire A. Ferreira-Filho [g], Anderson S. Sant'Ana [b], Michael P. Spector [f], and Glen M. Borchert [a,c]

[a]Department of Biology, University of South Alabama, Mobile, AL, USA; [b]Department of Food Science, University of Campinas, Campinas, Brazil; [c]Department of Pharmacology, USA College of Medicine, Mobile, AL, USA; [d]Department of Molecular and Cell Biology, University of California Berkeley, Berkeley, CA, USA; [e]Department of Biochemistry and Molecular Genetics, University of Colorado School of Medicine, Aurora, CO, USA; [f]Department of Biomedical Sciences, University of South Alabama, Mobile, AL, USA; [g]Center for Molecular Biology and Genetic Engineering, University of Campinas, Campinas, Brazil

ABSTRACT

Noncoding RNA (ncRNA) modulation of gene expression has now been ubiquitously observed across all domains of life. An increasingly apparent role of ncRNAs is to coordinate changes in gene expressions in response to environmental stress. *Salmonella enterica*, a common food-born pathogen, is known for its striking ability to survive, adapt, and thrive in various unfavourable environments which makes it a particularly difficult pathogen to eliminate as well as an interesting model in which to study ncRNA contributions to cellular stress response. Mounting evidence now suggests that small RNAs (sRNAs) represent key regulators of *Salmonella* stress adaptation. Approximately 50–500 nucleotides in length, sRNAs regulate gene expression through complementary base pairing with molecular targets and have recently been suggested to outnumber protein-coding genes in bacteria. In this work, we employ small RNA transcriptome sequencing to characterize changes in the sRNA profiles of *Salmonella* in response to desiccation. In all, we identify 102 previously annotated sRNAs significantly differentially expressed during desiccation; and excitingly, 71 novel sRNAs likewise differentially expressed. Small transcript northern blotting and qRT-PCRs confirm the identities and expressions of several of our novel sRNAs, and computational analyses indicate the majority are highly conserved and structurally related to characterized sRNAs. Predicted sRNA targets include several proteins necessary for desiccation survival and this, in part, suggests a role for desiccation-regulated sRNAs in this stress response. Furthermore, we find individual knock-outs of two of the novel sRNAs identified herein, either sRNA1320429 or sRNA3981754, significantly impairs the ability of *Salmonella* to survive desiccation, confirming their involvements (and suggesting the potential involvements of other sRNAs we identify in this work) in the *Salmonella* response to desiccation.

ARTICLE HISTORY
Received 18 March 2019
Revised 1 August 2019
Accepted 2 August 2019

KEYWORDS
Bacteria; desiccation; ncRNA; noncoding RNA; osmoprotectant; prokaryotic; ProP; *Salmonella*; sRNA; stress; transcriptome

Introduction

Novel noncoding RNA (ncRNA) discovery and the elucidation of defined roles for ncRNAs in gene coordination, transposable element silencing, and normal cellular metabolism continues to accelerate across bacteria and eukaryotes alike. Similar to transcription factors, the majority of ncRNAs are thought to be regulatory and frequently act as master gene regulators [1,2]. That said, cells and organisms are subjected to an array of challenges and perturbations, or stresses, in their environment throughout their life cycles, and the molecular responses to such changes involve the coordinated modulation of gene expressions. Although research into the cellular stress survival has classically focused on protein involvements, ncRNAs, which may ultimately be found to outnumber protein-coding genes in prokaryotes and especially eukaryotes, are increasingly implicated in the molecular mechanisms driving these responses [1,2]. Numerous forms of long and short ncRNAs (e.g. circRNAs [3], lncRNAs [4,5], and miRNAs [6,7] in eukaryotes and CRISPR [8], 6S [9], and sRNAs [10] in prokaryotes) now have well-characterized roles in immediate cellular and organismal stress protections as well as in more persistent adaptions.

The ability of certain species (e.g. nematodes [11]), vascular plants [12], and bacteria [13]) to survive desiccation (or extreme dehydration) for extended periods is a particularly striking example of the ability of cells to adapt to and survive even the most drastic environmental fluctuations. As bacterial small noncoding RNA (sRNA) expressions are frequently associated with stress [14–16], we recently hypothesized likely contribute to *Salmonella* survival during desiccation. When facing a low water environment, *Salmonella* adapt primarily by the intracellular accumulation of osmoprotectants (largely through the

CONTACT Glen M. Borchert borchert@southalabama.edu Department of Biology, University of South Alabama, Mobile, AL, USA
*These authors contributed equally to this work.
Supplemental data for this article can be accessed here.

action of proline channel ProP) preventing intracellular water loss. Interestingly, ProP is regulated by ProQ, an osmoregulatory protein that has been shown to be a global sRNA-binding molecule [17,18], suggesting the likely importance of sRNAs to the desiccation response and cell survival. Understanding the regulatory processes that lead to resistance and adaptation is essential to establishing new control strategies for this pathogen.

Bacterial sRNAs are typically 50 to 500 nucleotides in length, originate from 'empty' intergenic regions of chromosomes, and are often induced or repressed by specific stimuli during environmental stress or virulence [19]. SRNAs typically modulate metabolic functions and stress-related responses, such as nutrient deprivation, by binding target mRNAs and inhibiting their expression [14]. The majority of functionally characterized sRNAs are known to bind the 5′ UTRs of multiple mRNAs [20–22] and coordinate their expressions. As an example, the bacterial sRNA OxyS is markedly induced by oxidative stress during which it directly binds and regulates at least eight target mRNAs cumulatively altering the expression of ~40 genes [23].

Until quite recently, it was widely believed that the *S. enterica* genome was almost entirely annotated and that virtually all its genes had been identified. However, next generation transcriptome sequencing has quickly undermined this paradigm. In just the last 5 years, studies have identified over 350 previously undescribed ncRNA genes dynamically expressed in *S. enterica* [15,24,25]. That said, in this report, we describe 173 sRNAs significantly differentially expressed in response to desiccative stress (including 71 previously undescribed sRNAs) and outline a detailed methodology for their characterization. Excitingly, we find the loss of either sRNA1320429 or sRNA3981754, significantly impairs the ability of *Salmonella* to survive low water environments confirming their involvements (and suggesting the potential involvements of other sRNAs identified in this work) in the *Salmonella* response to desiccation.

Results

Salmonella sRNAs differentially expressed during desiccation

To identify and examine the expression patterns of *Salmonella* sRNAs during desiccation, *Salmonella enterica* serovar Typhimurium (strain SL1344) cultures were collected under three sample conditions (stationary phase (SP), 24 h desiccated, and 72 h desiccated), and their small RNA transcriptomes were subsequently isolated and sequenced, resulting in over 40 million raw sequencing reads for each. After mapping the RNA-seq reads to the intergenic regions of the SL1344 reference genome and all known *Salmonella* sRNAs using the CLC Genomics Workbench, we initially identified 149 chromosomal sequences differentially expressed (≥2.0x) between SP and either 24 h or 72-h desiccated samples (work flow summarized in Supplementary File 1). We found 102 of these differentially expressed sequences corresponded to previously characterized sRNAs (Table 1) and 47 mapped to broader 'gene-empty' or intergenic regions suggesting they contain novel sRNAs. In addition to 102 previously annotated sRNAs, we also identified 71 unique, novel sRNAs (Table 2) mapping to these 47 intergenic regions bringing the total number of sRNAs we find differentially expressed during desiccation to 173 (Tables 1 and 2, Supplementary Files 2,3).

102 differentially expressed sRNAs have been previously identified

In brief, we identified 173 putative sRNAs differentially expressed after desiccation for 24 and/or 72 h as compared to controls (fold change ≥ 2.0). Of these, we found 102 (~59%) correspond to previously annotated sRNAs (Table 1). Interestingly, most of the previously reported sRNAs we found under- or overexpressed during desiccation were also differentially expressed under nutrient limitation conditions (63%) (including 14 we previously identified as being involved in the carbon starvation-stress response [15]), followed by peroxide shock (41%), bile shock (33%), and NaCl shock (31%) [24,25] (Table 1). Notably, we find RyhB-1, an sRNA which acts on at least four genes related to iron homoeostasis (*sodB, acnA, acnB, ftn*) in *Salmonella*, significantly overexpressed after 72 h of desiccation. Previous experiments have shown that RyhB-mediated regulation is responsible for phenotypic changes related to acid and peroxide resistance, carbon source utilization, tricarboxylic acid cycle and sensitivity to antibiotics [26,27]. As such, our results further suggest the importance of RyhB as a general regulator of the cellular stress response system.

71 putative sRNAs are novel

While we found 102 of our differentially expressed sRNA sequences corresponded to known *Salmonella* sRNAs, we also identified differential expressions from 47 broader regions containing no annotated sRNAs or other known genes. To more precisely define sRNA sequences from these 47 intergenic regions, we employed a peak-calling strategy utilizing visualization of expression data on the SL1344 chromosome via the Integrative Genomics Viewer (IGV) and identified 71 distinct putative sRNA sequences expressed from the 47 broader intergenic regions (Table 2, Supplementary File 2). While we intially identified each of our novel sRNAs by examining intergenic regions, peak-calling of these broader regions along with their flanking sequences did result in the identification of 40 of our novel sRNAs partially overlapping the 5′ or 3′ ends of protein-coding genes. That said, overlaps were typically <25% of total sRNA lengths, and putative sRNA expressions were confirmed to significantly differ from those of size matched sequences selected from both a central internal location and the opposing terminus. Importantly, each of the 71 consensus sequences resulting from peak-calling within these broad regions were realigned to the full body of known SL1344 sRNAs and again confirmed to not correspond to any previously annotated sRNAs. Significant differential expression (>1.5-fold) for each of the 71 putative sRNAs was independently validated in our sequencing data with 65 of these shorter, better defined sequences exhibiting over a two-fold change in expression (Table 2, Supplementary Files 3,4). Unsupervised hierarchical clustering of the expressions of

Table 1. Known sRNAs implicated in desiccation stress response in *Salmonella enterica*. Previous characterisations and expressions in three sample conditions (stationary phase growth, 24 hr desiccated, and 72 hr desiccated) are indicated in transcripts per million.

sRNA	Previous Characterization	Exp. In Stationary	Exp. In 24-hr Desiccation	Exp. In 72-hr Desiccation
RyhB-1	Ellermeier & Slauch, 2008; Padalon-Brauch et al., 2008	27.9	0.0	110.3
STnc3830	Ramachandran et al., 2012	6.5	0.0	115.1
IsrQ	Padalon-Brauch et al., 2008	15.9	0.0	157.6
sRNA697722	Amin et al., 2016	15.9	505.4	282.4
sRNA1318601	Amin et al., 2016	69.0	1865.0	430.6
sRNA1170414	Amin et al., 2016	33.9	595.6	134.1
STnc3150	Ramachandran et al., 2012	137.0	1900.5	655.2
STnc3420	Kroger et al., 2013	80.6	923.8	265.5
tpke11	Rivas et al., 2001	63.0	707.4	103.8
IsrL	Padalon-Brauch et al., 2008	614.4	6679.4	3233.8
STnc3680	Kroger et al., 2013	74.4	10.9	117.7
STnc3240	Kroger et al., 2013	1001.6	9779.0	3160.0
MicC	Pfeiffer et al., 2009	13.7	83.9	129.6
C0664	Hershberg et al., 2003	24.8	12.1	114.4
STnc3500	Ramachandran et al., 2012	124.3	1159.3	368.4
STnc3920	Kroger et al., 2013	17.6	47.5	160.5
STnc380	Pfeiffer et al., 2007; Sittka et al., 2008	16.5	60.5	130.8
STnc790	Sittka et al., 2009	1964.0	15245.0	2846.0
SroC	Vogel et al., 2003	17043.7	129573.2	40843.5
STnc1590	Kröger et al., 2012	508.5	3833.2	1477.7
STnc1690	Kröger et al., 2012	579.2	4361.7	978.6
STnc200	Pfeiffer et al., 2007; Sittka et al., 2008	142.4	1022.9	368.4
sRNA294324	Amin et al., 2016	35.6	142.1	249.4
SraA	Argaman et al., 2001	102.9	720.7	231.0
STnc3310	Kroger et al., 2013	3845.6	26697.9	7451.2
STnc4120	Kroger et al., 2013	425.0	2814.5	569.4
STnc1540	Kröger et al., 2012	65.1	408.5	275.8
sRNA10	Sridhar et al. 2010	384.5	2314.6	643.6
STnc1460	Kröger et al., 2012	2580.2	15380.1	12827.6
STnc1420	Kröger et al., 2012	494.9	2674.8	1461.3
STnc1300	Kröger et al., 2012	47.1	28.2	152.4
STnc1400	Kröger et al., 2012	59.5	166.4	321.0
RyfA	Wassarman et al., 2001	3993.8	21215.8	20672.9
STnc980	Kroger et al., 2013	3168.7	15643.5	6274.0
STnc4160	Kroger et al., 2013	353.0	102.7	504.4
STnc1030	Kroger et al., 2013	23607.0	113313.5	47610.3
sRNA3405375	Amin et al., 2016	40.6	193.1	100.3
STnc4040	Kroger et al., 2013	31.2	84.3	142.4
sRNA3551252	Amin et al., 2016	961.8	4237.3	1241.5
STnc3110	Kroger et al., 2013	829.7	3632.0	1157.6
RyeF	Zhang et al., 2003; Chao et al., 2012, Kröger et al., 2012	227116.3	979720.1	306817.5
STnc3850	Kroger et al., 2013	24.7	61.9	104.5
STnc1650	Kröger et al., 2012	1422.4	6009.0	1826.6
STnc4220	Kroger et al., 2013	50.1	206.4	130.1
STnc4250	Kroger et al., 2013	228.3	933.6	530.9
STnc4190	Kroger et al., 2013	37.2	25.1	101.8
STnc3640	Ramachandran et al., 2012	243.7	116.1	463.5
SgrS	Wadler & Vanderpool, 2009; Papenfort et al., 2012; Papenfort et al., 2013	9469.1	35888.7	10789.4
STnc3180	Kroger et al., 2013	106.5	371.7	162.0
STnc3290	Kroger et al., 2013	733.9	2456.0	1394.2
STnc770	Sittka et al., 2009	688.8	2281.0	819.2
sRNA924744	Amin et al., 2016	11361.8	36145.1	12879.3
STnc3530	Ramachandran et al., 2012	451.8	1428.7	1046.8
STnc3220	Ramachandran et al., 2012	201.1	590.5	611.7
STnc1290	Kröger et al., 2012	372.1	556.2	1108.0
STnc3460	Kroger et al., 2013	92.7	203.6	274.9
CsrB	Fortune et al., 2006	500191.3	224632.9	653475.2
RygC	Zhang et al., 2003; Fozo et al., 2008; Rudd, 1999	4720.3	13314.3	5186.2
SraL	Argaman et al., 2001	17867.1	49604.7	46410.3
STnc2010	Chao et al., 2012; Kröger et al., 2012	569.8	1552.2	882.6
STnc3480	Kroger et al., 2013	101.5	275.4	182.6
STnc4080	Kroger et al., 2013	119.1	313.7	197.7
STnc1110	Kröger et al., 2012	6793.7	14600.4	7425.0
AmgR	Lee & Groisman, 2010	1171.8	771.2	559.0
STnc760	Sittka et al., 2009	391.4	603.3	283.9
FnrS	Boysen et al., 2010; Durand & Storz, 2010	1703.3	2314.2	1070.7
STnc1270	Kröger et al., 2012	1558.4	2263.2	1024.3
STnc1830	Kroger et al., 2013	286.9	427.8	192.6
STnc1760	Ramachandran et al., 2012	389.1	660.0	297.1
STnc1710	Ramachandran et al., 2012	795.3	1708.6	756.5
STnc780	Sittka et al., 2009	4740.7	5332.0	2319.6
GlmZ	Urban & Vogel, 2008	95171.3	92652.8	40338.1
t44	Rivas et al., 2001	1833.4	1908.9	685.8
GcvB	Sharma et al., 2007	51769.7	60666.1	21402.8
STnc3080	Kroger et al., 2013	180.7	396.6	133.9
STnc2090	Chao et al., 2012	56052.7	99725.5	32507.6
STnc1480	Kröger et al., 2012	14667.9	27422.5	8710.1

(*Continued*)

Table 1. (Continued).

sRNA	Previous Characterization	Exp. In Stationary	Exp. In 24-hr Desiccation	Exp. In 72-hr Desiccation
STnc1330	Kröger et al., 2012	3604.2	7379.1	2291.1
STnc1990	Ramachandran et al., 2012	424.4	643.8	197.6
sRNA176086	Amin et al., 2016	225.7	445.3	135.8
STnc3010	Ramachandran et al., 2012	233.9	70.0	115.6
STnc1130	Kröger et al., 2012	519.4	1195.9	350.5
RybA	Wassarman et al., 2001	1906.0	4460.3	1255.7
IsrI	Padalon-Brauch et al., 2008	13155.9	41853.4	11609.5
STnc3990	Ramachandran et al., 2012	676.9	350.1	178.6
STnc4150	Kroger et al., 2013	744.3	281.5	176.5
STnc700	Sittka et al., 2009	9605.0	3139.9	2238.6
sRNA4130247	Amin et al., 2016	365.5	82.7	134.1
STnc3760	Ramachandran et al., 2012	137.0	311.2	70.1
OxyS	Altuvia et al., 1997; Padalon-Brauch et al., 2008	97.6	208.7	44.5
SraB	Argaman et al., 2002	494.4	731.5	145.8
STnc4200	Ramachandran et al., 2012	411.8	1056.6	176.5
sRNA2759412	Amin et al., 2016	229.3	736.2	121.7
STnc3700	Ramachandran et al., 2012	595.4	1719.2	270.4
sRNA1799950	Amin et al., 2016	170.6	631.5	99.2
STnc1170	Kröger et al., 2012	626.1	1482.3	218.4
sRNA1548865	Amin et al., 2016	238.8	675.1	58.2
sRNA1253515	Amin et al., 2016	71.6	496.4	22.3
sRNA1888744	Amin et al., 2016	17.7	186.7	0.0
MicA	Papenfort et al., 2006; Bossi & Figueroa-Bossi, 2007	20.1	106.0	0.0
DsrA	Majdalani et al., 2001	37.6	135.2	0.0
STnc4070	Kroger et al., 2013	48.1	184.8	0.0

these 71 novel sRNAs identified four principle clusters. Nearly 2/3 of the putative sRNAs make up the largest cluster characterized by expression almost exclusively at 24 h of desiccation. In contrast, 13 genes are predominately expressed during normal conditions then turned off during desiccation whereas only 3 sRNAs are the most highly expressed after 72 h of desiccation (Figure 1).

Next, to determine similarities between our novel sequences and other known bacterial sRNAs, we aligned our putative sRNAs to the non-coding RNA databases Rfam and sRNATarbase [29,30] and also examined their probable secondary structures via Mfold [31]. Notably, we found 55 of our putative sRNA gene loci are well conserved (at least 80% identical over their entire length) in the genomes of related bacterial genera (Figure 2), and that 13 of our novel sRNAs correspond to annotated sRNAs or other noncoding RNA species in related bacteria (Table 2). In addition, we found another of our putative sRNAs (sRNA901248) shares an identical 23 base pair sequence with *RybA*, a known SL1344 sRNA we also find differentially expressed during desiccation [32] (Table 1). Furthermore, Mfold analyses indicate over 90% of our candidate sRNAs likely adopt complex secondary structures consisting of a series of short stem loops similar to structures of characterized bacterial sRNAs [33] (Figure 3, Table 2, Supplementary File 2).

Confirmation of sRNA expressions

Differential expressions of five annotated and five putative sRNAs were verified by qRT-PCR (Figure 4(a,b)). Importantly, all qPCR-based sRNA expression trends largely agreed with RNA-seq-based expressions (Tables 1 and 2, Figure 1, Supplementary File 3). The magnitude of changes in expression, however, did at times differ notably between techniques. For example, the RNA-seq-based expression of STnc3920 at 72 h was determined to be ~10x its expression in SP controls (Table 1) whereas its qRT-PCR -based expression at 72 h was calculated as a more modest 3x that of controls (Figure 4(a)). That said, the disparity between STnc3920 changes in expression was by far the most pronounced with sRNA relative expression changes typically agreeing within 50% between methods (Tables 1 and 2, Figure 4(a,b)).

Next, we elected to confirm the existence and the changes in expression of two of the sRNAs described here by small transcript northern blotting, a more direct and definitive method. This allowed us to confirm that proposed sRNAs form discrete species rather than being part of longer transcripts. We found northern analysis of the first of these, sRNA3981754, largely confirmed our predicted length of 87nts (Figure 4(c)) and in agreement with our expression analyses. That said, we found northern blotting more closely agreed with our qRT-PCR -based expression analyses than with our RNA-seq-based expressions. RNA-seq-based expressions for sRNA3981754 were 187.6, 75.8, and 21.6 transcripts per million during SP, 24 h, and 72 h of desiccation, respectively (Table 2). In contrast, while our qRT-PCR likewise found sRNA3981754 to be most highly expressed during SP, it determined expression decreased by 88% by 24 h of desiccation then decreased even further by 72 h (Figure 4(b)) closely paralleling our northern analyses. Similarly, northern analysis of sRNA1320429 generally confirmed our predicted length of 78 nts (Figure 4(c)) and in agreement with our expression analyses suggesting sRNA1320429 expression increases by 5 to 15 fold during desiccation (Figure 4(b), Table 2).

Deletion of novel sRNAs can significantly impair the Salmonella desiccation response

To explore potential functional roles of novel sRNAs during the desiccation response, we selected sRNA1320429 and sRNA3981754 for deletion analysis, as we found both significantly and dynamically expressed in response to desiccation and were able to successfully confirm their expressions and identitites by northern (Figure 4). The Lambda-Red

Table 2. Novel sRNAs implicated in the desiccation stress response in *Salmonella enterica*. Known sRNA similarities, sRNA conservation, predicted structural complexity, flanking/overlapping genes, putative targets, and expressions in stationary phase, 24 hr desiccated, and 72 hr desiccated cell samples are indicated.

sRNA[a]	Conservation, sRNA Similarity[b]	Structure[c]	Flanking Genes & Overlap[d]	Putative Targets – IntaRNA[e]	Exp. In Stationary[f]	Exp. In 24 hr D[f]	Exp. In 72 hr D[f]
sRNA294677	WC, RF00391	HS	rRNA; tRNA^{Asp}; dkgB-1	-	25	117.7	78.8
sRNA458139	WC	HS	tsaA-1(3′); yajB-1	-	16.2	31.4	13.6
sRNA466762	WC	HS	STnc1060(3′); tsx-1	-	29.2	33.7	18.5
sRNA539269	WC	HS	priC-1	-	3.7	6.4	3.6
sRNA539528	WC	HS	apt-1	-	3.9	4.4	1.5
sRNA898738	WC	HS	ybiF-1; ompX-1(5′)	-	45.6	56	19
sRNA901248	WC, RF00110	HS	ybiP-1; RybA(5′); CBW16908	-	154.6	210.1	90.7
sRNA978535	WC	HS	cspD-1(3′); clpS-1	cspD	32.3	19.1	7.6
sRNA982124	WC	HS	tnp-1(3′)	-	71.5	108.9	56.5
sRNA982481	WC, RF02902	HS	CBW16982	-	1.9	16.7	17.2
sRNA997533	WC	HS	ftsK-1(3′)	-	2.7	9.3	14.3
sRNA997685	WC	HS	ftsK-1(3′)	oadA1	5.6	15.8	7.9
sRNA998116	WC	HS	ftsK-1; lolA-1(5′)	-	9.1	10.1	3.6
sRNA1000215	-	HS	ycaJ-1; serS-1	-	9.8	9.8	4
sRNA1118334	WC	HS	ompA-1(3′); sulA-1	ompA	24.9	28.3	15.2
sRNA1227667	WC	HS	rne-1(3′)	ribonuclease E	7	6	2.7
sRNA1227716	WC, RF00040	HS	rne-1	ribonuclease E	16	13.5	6
sRNA1290392	WC, RF02076	HS	tRNA-Arg; STnc520	RatA	42	19.8	11.1
sRNA1291223	-	HS	pliC-1(3′)	lysozyme inhibitor	26.8	37.1	11.8
sRNA1291436	-	HS	pliC-1	-	27	25.3	10.6
sRNA1291652	-	HP	CBW17281	SsaE; Dcp	32.4	18	6.5
sRNA1320429	WC	HS	yeaG-1(3′)	-	38	581.9	582.9
sRNA1320654	WC	HS	yeaG-1	PrkA	43.7	250	82.1
sRNA1440272	WC, RF00391	HS	ssaE-1; sseA-1	CpxP-like	120.1	252.1	186.4
sRNA1440474	-	HP	ssaE-1; sseA-1(5′); sseBa-1	-	31.6	18.5	6.2
sRNA1448803	-	HS	ssaK-1(3′); ssaL-1; STnc1220	-	11.1	14	5.6
sRNA1866912	-	HS	CBW17833(3′); ycgN-1	-	547.2	1420.8	603
sRNA1886080	WC	HS	CBW17853(5′)	-	23.7	25.2	6.6
sRNA1922930	-	HS	CBW17896; CBW17897	endonuclease	1.7	6.5	2.4
sRNA1954465	WC	HS	aspS-1; yecD-1	aspS	8.7	11.5	4
sRNA1954754	-	HS	yecD-1(3′)	-	7.2	10.3	3.4
sRNA1996414	WC	HS	sirA-1 (3′)	dbrr	22.9	8.1	2.8
sRNA1996681	WC	HS	yecF-1; sirA-1	-	4.8	2.8	1.9
sRNA2111563	WC, RF00174	HS	cbiA-1; pag	ProP effector protein	10.9	3.6	1.8
sRNA2111772	-	HS	cbiA-1; pocR-1	-	7.2	1.3	0.9
sRNA2437186	WC	HS	nuoA-1(3′)	nuoA	15.8	17.6	6.1
sRNA2437382	WC	HP	nuoA-1; CBW184000	lytictransglycosylase; patatin family protein	26.3	14.8	7.6
sRNA2495587	WC	HS	sixA-1(3′); fadJ-1	sixA	100	162.4	82.9
sRNA2499697	WC	HS	yfcZ-1(3′)	-	24.9	21.3	7.9
sRNA2499837	WC	HS	fadL-1; yfcZ-1	-	5.6	18.3	9.6
sRNA2522653	WC	HS	nupC-1; mntH-1(3′)	mntH	4.7	24.3	7.4
sRNA2522758	-	HS	nupC-1; mntH-1	treR; CysZ	6.3	23.1	6.7
sRNA2522977	WC	HS	nupC-1(5′)	-	3.6	23.2	8
sRNA2828025	WC, RF00391	HS	rimM-1; rps16-1(3′); ffh-1	30S ribosomal protein S16	7.9	21.7	10.2
sRNA2828147	WC	HS	rps16-1; ffh-1	ABC transporter; RecF; pncA	3.9	13.7	5.5
sRNA2828280	WC	HS	rps16-1; ffh-1(5′)	-	8.6	26.1	10.3
sRNA2948961	-	HS	pipB2-1	glgX; Kdul; HyaE; nrdD	19.4	73.8	37.3
sRNA2950404	-	HP	pipB2-1(3′)	pipB2	7.3	9.6	3.3
sRNA2950578	WC	HP	pipB2-1	murC; SDR	20.6	7.6	4.4
sRNA2969551	WC	HS	ygaP-1; stpA-1	-	9.8	14	9.3
sRNA3393373	-	HS	ygiH-1; gcp-1	-	26	63.5	38.3
sRNA3395090	WC, RF02818	HS	rpsU-1; dnaG-1(5′)	-	7	25.3	9
sRNA3417448	WC	HS	yqjC-1(3′); yqjE-1	ferredoxin	80	405.6	151.6
sRNA3417670	WC	HS	yqjC-1; yqjE-1	ybbK; oadA1	64.7	484	218
sRNA3435592	WC, RF00010	HP	gark-1	-	4254.2	2399.9	1744.1
sRNA3435639	WC, RF00010	HS	gark-1	-	11178.2	7799	5212.6
sRNA3758077	WC	HS	rpoH-1(3′); ftsX-1	rpoH	123.6	132.5	58.9
sRNA3857685	WC, RF01766	HS	yiaG-1; cspA-1(5′)	-	452.4	1577	563.7
sRNA3981754	WC	HS	slsA-1(3′); cigR-1	-	187.6	75.8	21.6
sRNA3981945	WC, RF00391	HS	sciZ-1; yafV-1	-	106.6	250.6	166.6
sRNA4066303	WC	HS	dnaA-1(3′)	DnaA	12.6	19.9	7.7
sRNA4066490	-	HS	dnaA-1	UmuD	15.5	13.5	6.4
sRNA4318787	-	HS	yiiU-1(3′); menG-1	ilvD; NirC; kup; manY	64.7	114.2	50.4
sRNA4433610	WC	HS	metH-1; yjbB-1(5′)	-	21.7	72.7	34
sRNA4534936	WC	HS	yjcH-1; acs-1(5′)	rtcA	3.7	26.9	13.5

(*Continued*)

Table 2. (Continued).

sRNA[a]	Conservation, sRNA Similarity[b]	Structure[c]	Flanking Genes & Overlap[d]	Putative Targets – IntaRNA[e]	Exp. In Stationary[f]	Exp. In 24 hr D[f]	Exp. In 72 hr D[f]
sRNA4626941	WC	HS	hflX-1; hflK-1(5′)	CreC; rpiA	12.9	47.7	20.6
sRNA4662099	-	HS	cpdB-1; cysQ-1	cpdB	10.1	16.6	7.9
sRNA4662318	WC	HS	cpdB-1; cysQ-1(5′)	-	7.2	25.3	10.2
sRNA4663946	WC	HS	ytfK-1(5′); ytfL-1	-	83	98.2	50.6
sRNA4837237	WC	HS	osmY-1; yjjU-1	ndk; epmA	43.8	203.2	108.5
sRNA4876851	WC	HS	arcA-1; yjjY-1(3′)	-	19.7	29.6	11.2

[a]sRNA – name refers to the starting nucleotide position of the putative sRNA sequence on the SL1344 chromosome.
[b]Conservation, sRNA Similarity – (WC), 'well-conserved' sRNAs are at least 80% identical over their entire length to a genomic sequence found within at least one other distinct bacterial genus. RF number refer to RFAM sequence family significantly aligning to indicated sRNA.
[c]Structure – Hairpin (HP), simple sRNA secondary structure containing one hairpin predicted with Mfold; Highly structured (HS), possesses more than one hairpin or simple structural element within predicted secondary structure.
[d]Flanking genes and Overlap determined by alignment in Ensembl – (5′) indicates that sRNA overlaps 5′-end of CDS; (3′) indicates that sRNA overlaps 3′-end of CDS.
[e]Putative Targets determined by IntaRNA, assuming a fdr cut-off of 0.1.
[f]Exp. In Stationary – normalized number of transcripts per million (TPM) at sRNA location during normal stationary-phase growth; Exp. In 24 hr D – normalized TPM at sRNA location after 24 h of desiccation; Exp. In 72 hr D – normalized TPM at sRNA location after 72 h of desiccation.

recombinase method was employed to delete each of these sRNAs [34] resulting in the creation of two mutant *Salmonella* strains: *Δ13204 and Δ3981* for subsequent phenotypic testing. Deletions were verified by PCR confirmation of sRNA (~100–200 bp) replacement with a chloramphenicol resistance cassette (1,034 bp) (Figure 5(a)).

Since these sRNAs were identified by their dynamic expression in response to desiccation, we subjected deletion mutant strains to conditions identical to those used during our initial desiccation response sequencing analysis. When SP *Salmonella* were subjected to these same desiccation conditions, we found *Δ13204* survival was significantly lower, ~34% less than controls after 24 h of desiccation ($p = 0.0067$) and ~55% less than wild type *Salmonella* after 72 h ($p = 0.0017$) as compared to controls. Similarly, reduced but less pronounced effects on survivability were observed for *Δ3981* (Figure 5(b)). More strikingly, when log phase *Salmonella* mutants were subjected to these same desiccation conditions, both deletion mutants showed roughly a 70% to 80% lower survival rate than wild type *Salmonella* or a control sRNA deletion mutant (*Δ92*) [15] at 24 h ($p = 0.000297$ and $p = 0.000271$) and similarly significant lower survivals at 72 h (Figure 5(c)). This is expected as bacteria in an exponential phase of growth are generally thought to be more susceptible to various environmental stresses due to the inherent vulnerability of highly prolific and metabolically active systems [35–37]. Importantly, effects of sRNA overexpression on survival of log phase deletion mutants and wild type Salmonella subjected to desiccation for 24 and 72 h was also determined. We find transformation of sRNA deletion mutants with complementary sRNA expression vectors fully corrects impaired survivability during desiccation. In addition, we find transformation of a sRNA3981754 expression vector similarly conveys enhanced desiccation survivability to control strains (Supplementary File 5).

Deletion of novel sRNAs can significantly impair the Salmonella response to other stresses

To determine whether deletions of these sRNAs similarly affected survivability in response to other stresses, we next evaluated the effects of heat and peroxide exposure. While both desiccation sRNA deletion mutants did consistently exhibit approximately 20% to 40% lower survivability after hydrogen peroxide exposure depending on controls, observed decreases were not statistically significant (Figure 5(d)). In contrast, we found deletion of either sRNA conferred a marked effect on survivability during heat challenge. After exposure to 55°C for 45 min, we observed significantly enhanced survivability for both mutants (*Δ13204*, $p = 0.0282$ and *Δ3981*, $p = 0.0158$) with each surviving over five times greater than wild type *Salmonella* or a control sRNA mutant (*Δ92*) [15] (Figure 5(e)).

Prediction and analysis of potential targets

Many sRNAs have been found to regulate their neighbouring transcripts [38]. Therefore, as an initial means of identifying potential regulatory targets of our novel sRNAs, we determined the proximity of our sequences to neighbouring genes in the SL1344 annotated reference genome and found that all 71 of our sequences were located within 300 bp of an annotated SL1344 gene, including ribosomal RNA, transfer RNA, and protein-coding loci (Table 2, Supplementary File 2). However, while some sRNAs do target neighbouring genes, proximity is often not a requirement for regulation, and as the majority of sRNAs have been shown to regulate genes from distant loci *in trans*, IntaRNA [39] was principally employed to predict potential sRNA-target interactions based on the thermodynamic energy requirements for hybridization and site accessibility resulting in a total of 144 predicted targets (Table 2).

We next mapped 87 of 130 predicted target gene GIs (GenInfo Identifiers) to 1507 UniProtKB IDs, and mapped an additional 32 of the remaining GIs to the UniParc sequence archive. Target genes were then clustered according to gene ontology (molecular function) and enzyme class (Table 2, Supplementary File 6). Interestingly, ~89% of the predicted targets of sRNAs differentially expressed in desiccation are associated with catalytic, binding or transcription regulator activities, and ~75% of the predicted enzyme targets correspond to transferases, oxidoreductases and lyases (Supplementary File 6).

Discussion

Salmonella is one of the most challenging bacteria for food manufacturers and is a leading cause of enterocolitis

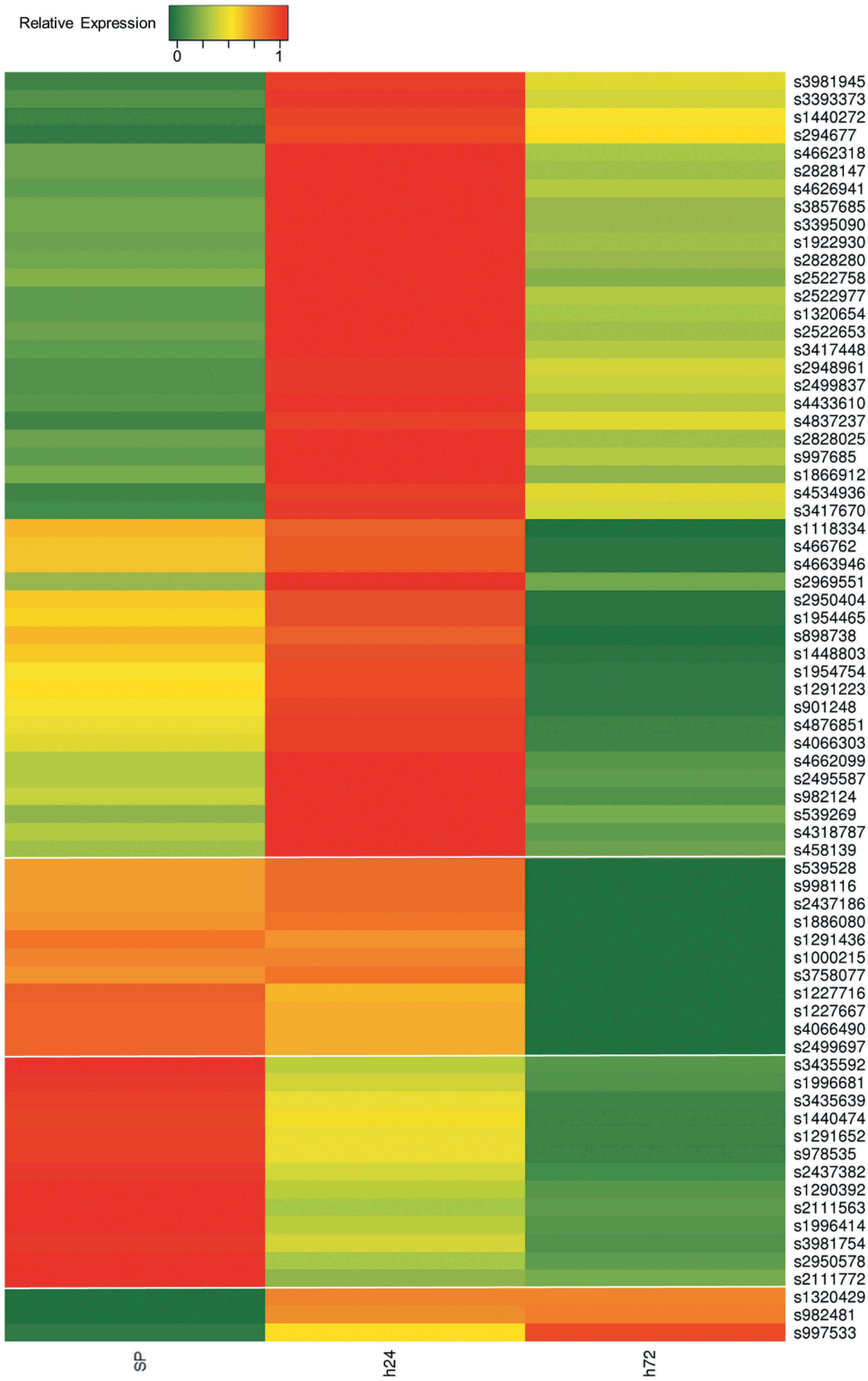

Figure 1. Gene expression analysis of novel desiccation-related sRNA genes. Heatmap showing relative expressions of novel desiccation-related sRNA genes. SP, stationary phase; h24, 24 h desiccated; h72, 72 h desiccated. Four gene clusters (demarcated by breaks) were defined in accordance with unsupervised hierarchical clustering [28].

worldwide. As the food processing chain commonly involves dehydration as a means of long-term food storage, desiccation is an important environmental stress encountered by *Salmonella* [40]. As such, a better understanding of the desiccation stress response of this pathogen is therefore of great practical importance. Such knowledge is critical for the development of effective strategies for commercial application in control of potential bacterial contaminants. Although low water activity has been associated with an increase of *Salmonella* resistance to heat and other severe conditions, often contributing to foodborne outbreaks, there is a lack of information regarding regulatory genes that may act on these

```
sRNA294677              AGAGAGTAGGACAAAAGCGAAAGCTTTTGAACGTTGCGAAGCAACGGCCCGAAGGGTGAATCACGAAGTGATTCATAAACTGCCA
Cedecea lapagei         AGAGAGTAGGACAAAAGCGAAAGCTTTTGAACGTTGCGAAGCAACGGCCCGAAGGGTGAATCACAAGGTGATTCATAAACTGCCA
Citrobacter freundii    AGAGAGTAGGACAAAAGCGAAAGCTTTTGAACGTTGCAAAGCAACGGCCCGAAGGGTGAATCACGTAGGGATTCATAAACTGCCA
Enterobacteriaceae      AGAGAGTAGGACAAAAGCGAAAGCTTTTGAACGTTGCGAAGCAACGGCCCGAAGGGTGAATCACGAAGTGATTCATAAACTGCCA
Escherichia coli        AGAGAGTAGGACAAAAGCGAAAGCTTTTGAACGTTGCGAAGCAACGGCCCGAAGGGTGAATCACGAAGTGATTCATAAACTGCCA
Kluyvera georgiana      AGAGAGTAGGACAAAAGCGAAAGCTTTTGAACGTTGCGAAGCAACGGCCCGAAGGGTGAATCACAAAGTGATTCATAAACTGCCA
Phytobacter sp.         AGAGAGTAGGACAAAAGCGAAAGCTTTTGAACGTTGCGCAGCAACGGCCCGAAGGGTGAATCACGAAGTGATTCATACACTGCCA
Shigella sonnei         AGAGAGTAGGACAAAAGCGAAAGCTTTTGAACGTTGCGAAGCAACGGCCCGAAGGGTGAATCACGAAGTGATTCATAAACTGCCA
                        ************************************  *************************   * ******** *******

sRNA1118334             TTTCATTTTTTGCGCCTCGTTATCATCCAAAATACGCCATGAATATCTCCAACGAGATAACACGGTTAAATCCTTCACCGGG
Citrobacter freundii    TTTCATTTTTTGCGCCTCGTTATCATCCAAAATACGCCATGAATATCTCCAACGAGATAACACGGTTAAATCCTTCACCGGG
Citrobacter koseri      TTTCATTTTTTGCGCCTCGTTATCATCCAAAATACGCCATGAATATCTCCAACGAGATAACACGGTTAAATCCTTCACCGGG
Escherichia albertii    TTTCATTTTTTGCGCCTCGTTATCATCCAAAATACGCCATGAATATCTCCAACGAGATAACACGGTTAAATCCTTCACCGGG
Escherichia coli        TTTCATTTTTTGCGCCTCGTTATCATCCAAAATACGCCATGAATATCTCCAACGAGATAACACGGTTAAATCCTTCACCGGG
Shigella dysenteriae    TTTCATTTTTTGCGCCTCGTTATCATCCAAAATACGCCATGAATATCTCCAACGAGATAACACGGTTAAATCCTTCACCGGG
Shigella flexneri       TTTCATTTTTTGCGCCTCGTTATCATCCAAAATACGCCATGAATATCTCCAACGAGATAACACGGTTAAATCCTTCACCGGG
Shigella sonnei         TTTCATTTTTTGCGCCTCGTTATCATCCAAAATACGCCATGAATATCTCCAACGAGATAACACGGTTAAATCCTTCACCGGG
                        ********************************************************************************

sRNA978535              AACCACTTTACAGTACCCGTTTCCATGCTTCGACATCCTTCGTAAATCTTATATAAGTAAGA
Citrobacter amalona     AACCACTTAACAGTACCCGTTTCCATGCTTCGACATCCTTCGTAAATCTTATATAAGTAAGA
Citrobacter braakii     AACCACTTAACAGTACCCGTTTCCATGCTTCGACATCCTTCGTAAATCTTATATAAGTAAGA
Enterobacter sp.        AACCACTTAACAGTACCCGTTTCCATGCTTCGACATCCTTCGTAAATCTTATTTAAGTAAGA
Escherichia alberti     AACCACTTAACAGTACCCTTTTCCATGCTTCGACATCCTTCGCAAATCTTATACAAGTAAGA
Escherichia coli        AACCACTTAACAGTACCCGTTTCCATACTTCGACATCCTTCGTAAATCTTATATAAGTAAGA
Metakosakonia sp.       AACCACTTAACAGTACCCGTTTCCATGCTTCGACATCCTTCGTAAATCTTATTTAAGTAAGA
Phytobacter ursingi     AACCACTTGACAGTACCCGTTTCCATGCTTCGACATCCTTCGTAAATCTTATTTAAGTAAGA
Shigella dysenteria     AACCACTTAACAGTACCCTTTTCCATGCTTCGACATCCTTCGCAAATCTTATACAAGTAAGA
Shigella flexneri       AACCACTTAACAGTACCCTTTTCCATGCTTCGACATCCTTCGCAAATCTTATACAAGTAAGA
Shigella sonnei         AACCACTTAACAGTACCCTTTTCCATGCTTCGACATCCTTCGCAAATCTTATACAAGTAAGA
                        ******** ********* ******* *************** *********  ********
```

Figure 2. Multiple alignments of candidate sRNAs with genomic sequences from other bacterial genera. (a) sRNA294677. (b) sRNA1118334. (c) sRNA978535. Genomic sequences were retrieved from NCBI using BLASTN [55] search for bacterial RNA. Alignments were generated using ClustalW [56]. *, 100% nucleotide identity.

specific pathways [40]. As such, to gain further insight into the mechanisms underlying *Salmonella* responses to desiccation, in this work we have identified 173 intergenic sRNAs differentially expressed during desiccation. While 102 of these sRNAs have been previously annotated, to our knowledge, this is the first work describing changes in their expressions during (and potential to act as regulators of) this pathogen's desiccation response system. Notably, in addition to these 102 previously annotated sRNAs our transcriptomic analyses have simultaneously identified 71 novel, putative sRNAs likewise differentially expressed in response to desiccation. While qRT-PCRs of five of these strongly agree with RNA-seq-based expressions, the lengths and identities of only two were directly confirmed by small transcript northern blotting (Figure 4), and similar experiments will ultimately be required to confirm that each of the remaining putative sRNAs truly represent bona fide sRNAs and definitively exclude the possibility that they represent RNA-seq/qRT-PCR bias artefacts.

That said, unsupervised hierarchical clustering of the expressions of the 71 novel sRNAs described here identified four principle clusters. Nearly 2/3 of the putative sRNAs make up the largest cluster characterized by expression almost exclusively at 24 h of desiccation (Figure 1). Notably, clustering of the expressions of the 102 annotated sRNAs we also find differentially expressed during desiccation (Table 1) closely resembles that of our novel sRNAs in that 72 of the 102 annotated sRNAs group together into a single predominant cluster likewise characterized by expression almost exclusively at 24 h of desiccation (Supplementary File 7). Of note, the two sRNAs examined in our deletion analyses were in large part selected based on their opposing expression profiles. We find sRNA1320429 levels to be negligible during SP and that it is primarily expressed during desiccation. In contrast, sRNA3981754 is most highly expressed during SP, and its expression is decreased by at least 60% after 24 h of desiccation (Figure 4). As such, we initially hypothesized that a loss of sRNA1320429 would impair desiccation survival whereas a loss of sRNA3981754 would likely have no effect. Much to our surprise, whereas we found deletions of other unrelated sRNA loci had little to no effect on desiccation survivability, we found knocking out either of these sRNAs significantly impairs the ability of cells to survive desiccation while conversely conferring an enhanced survivability during heat challenge (Figure 5). While the mechanisms responsible for these phenotypic observations remain unclear, we feel it suggests both of these sRNAs are necessary for initial survivability during desiccation and that these sRNAs are likely involved in regulating the same or related genetic pathways.

Importantly, recent reports have demonstrated the association of several distinct sRNAs in regulatory stress responses and virulence in *Salmonella*. *Salmonella* is quickly becoming a model organism for RNA-mediated regulation, with hundreds of novel sRNAs recently identified by transcriptomic studies [15,24,25]. Of note, our previous work identified uncharacterized sRNAs that are differentially expressed under starvation stress response (SSR). Remarkably, we found that 58 of the 63 sRNAs we identified under SSR conditions had not been previously characterized in *Salmonella* or other *Enterobacteriaceae* [15]. These results clearly suggest that sRNA regulation in *Salmonella* remains largely unexplored and that the discovery of novel sRNAs is an essential first step to understanding how sRNAs control pathogenesis and adaptation processes in response to environmental changes.

While *Salmonella* sRNA discovery is proceeding robustly, the identification of targets now becomes a critical bottleneck for further progress in this field. In this report, we used

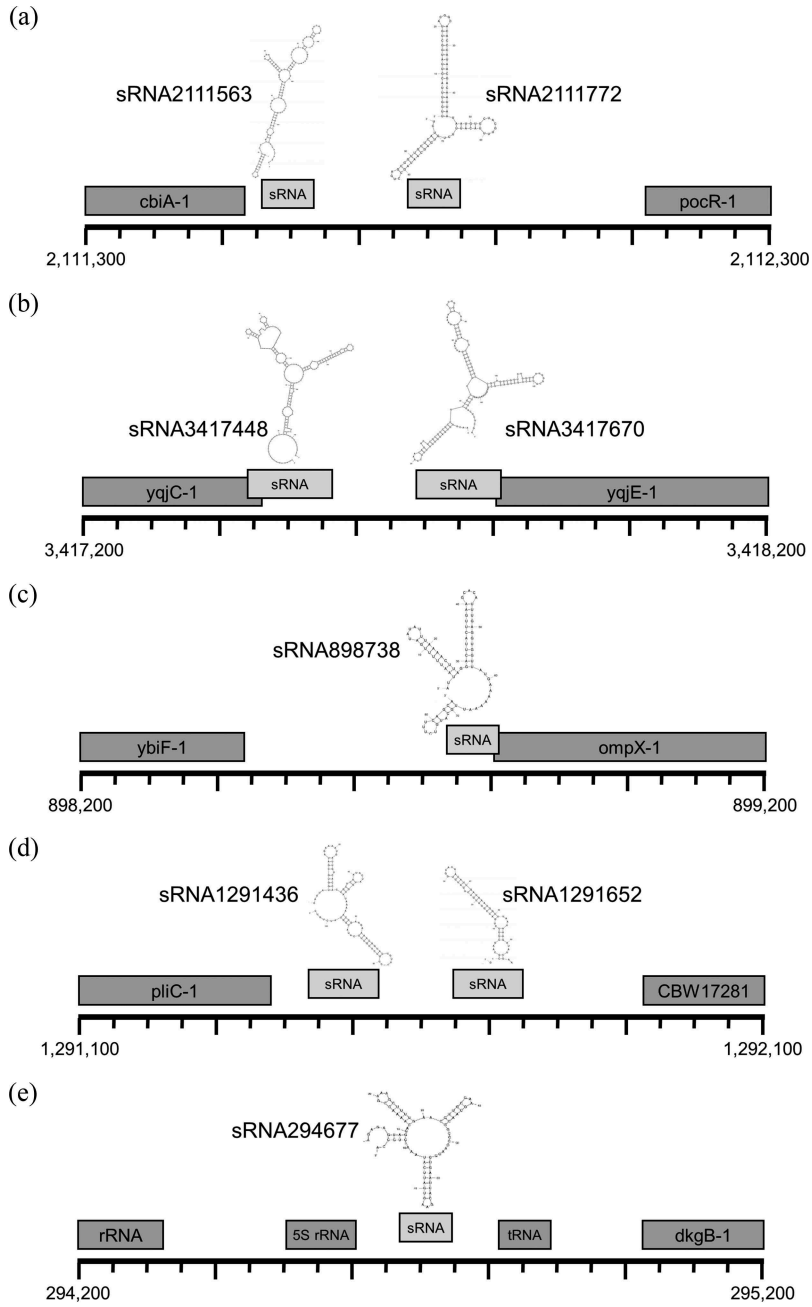

Figure 3. Select sRNA loci. Cartoons depicting 1kb genomic regions roughly centred on 1 or 2 sRNA sequences are illustrated. The most thermodynamically stable secondary structures of individual sRNAs (as predicted by Mfold [31]) are included above respective sRNA sequences (light grey). Names and positions of neighbouring genes occurring within the 1kb region as defined in the current Ensembl build [44] are also indicated (dark grey). SL1344 chromosomal positions are shown (bottom left and right). (a) sRNA2111563 and sRNA2111772 locus. (b) sRNA3417448 and sRNA3417670 locus. (c) sRNA898738 locus. (d) sRNA1291436 and sRNA1291652 locus. (e) sRNA294677 locus.

a combinational strategy taking both proximity and hybridization kinetics into consideration in order to identify the most likely targets of desiccation-induced sRNAs. Of note, predicted primary targets of all differentially expressed sRNAs were clustered according to their gene ontology (GO) terms, focusing on their molecular function. Interestingly, we find the targets of sRNAs differentially expressed in desiccation appear to present mainly catalytic, binding and transcription regulator activities (Supplementary File 6), and the enzymes predicted to be the most affected mostly correspond to transferases, oxidoreductases and lyases. That said, one sRNA differentially expressed in 24 and 72-h desiccated cells, sRNA2111563, was predicted to interact with and potentially repress *proP*, which encodes an osmoprotectant proline/betaine transporter crucial for osmoprotection and survival of desiccated *Salmonella enterica* Serovar Typhimurium [41]. We find this novel sRNA significantly repressed after 72 h of desiccation, suggesting a higher reciprocal expression of *proP*. In fact, *proP* is known to be overexpressed in low-moisture environments, and mutant Δ*proP* shows significant reduction in viability when compared to wild type strain in the same conditions [41]. In addition, we find another of our putative sRNAs (sRNA1448803) is predicted to regulate *ssaL-1* a type III secretion system apparatus protein previously shown to be necessary for surviving desiccation [42]. Although experimental assays will be required to confirm the

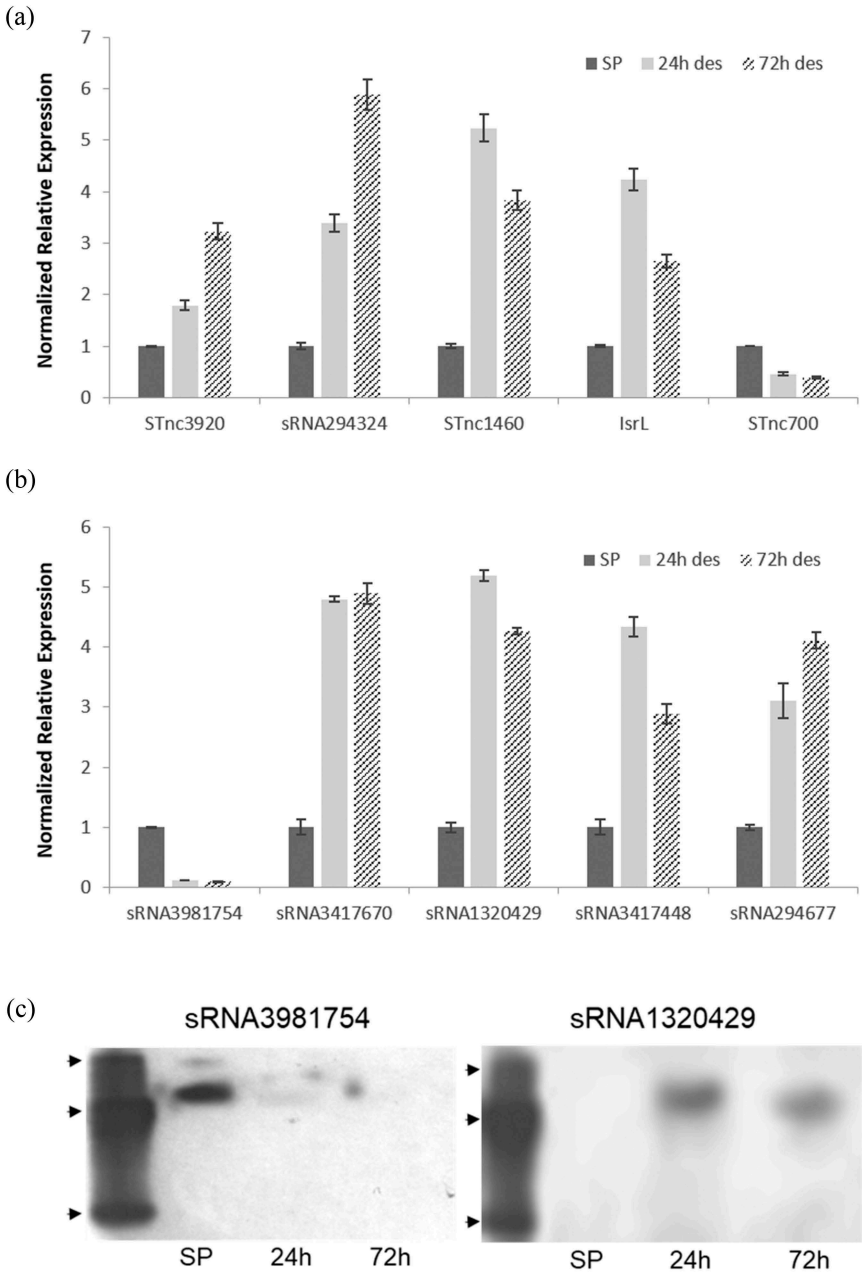

Figure 4. qRT-PCR and small transcript northern validation of sRNA differential expressions. (a) Annotated sRNA qPCR expressions. (b) Putative sRNA qPCR expressions. The specificity of each amplification was verified via melting curves, and a control without reverse transcriptase was included in parallel. Gene expressions were calculated via the Delta-Delta cycle threshold method [56]. SP, stationary phase; 24 h des, 24 h desiccated; 72 h des, 72 h desiccated. Expressions were normalized to SP. Error bars in A and B indicate SD (n = 3). (c) sRNA3981754 (left) and sRNA1320429 (right) small transcript northern blot. Arrowheads indicate 25, 50 and 200 nt oligonucleotide bands in custom ssDNA 5′ biotinylated ladder. SP, stationary phase. 24 h, 24 h desiccated. 72 h, 72 h desiccated.

role of these sRNAs (and several of our other putative sRNAs) in regulating the desiccation stress response, we find it exciting that several of our predicted targets are associated with characterized desiccation responses in *Salmonella* (Table 2). As such, we now suggest that these logical target associations, taken with our demonstration that a loss of specific sRNAs identified in this study can significantly impair the ability of *Salmonella* to survive desiccation, strongly indicate that many of the sRNAs described in this report represent uncharacterized, critical regulators of desiccation [25,43].

Of note, the 71 novel sRNAs described here bring the total number of *Salmonella enterica* serovar Typhimurium sRNAs to over 450 [15,24,25,41,44–48]. Excitingly, mounting evidence suggests there are many additional *Salmonella* sRNAs yet to be described. Although our work only considered intergenic sRNAs, recent reports suggest that functional ncRNAs can also be derived from gene untranslated regions (UTRs), and that some sRNAs are entirely embedded within protein-coding regions [49]. As such, it is tempting to speculate that sRNAs may actually outnumber protein-coding genes in this and perhaps many additional prokaryotic genomes. That said, the functional dissection of the specific roles for each of these novel genetic regulators represents the next major hurdle to realizing a more thorough understanding of the genetic networks driving bacterial biology.

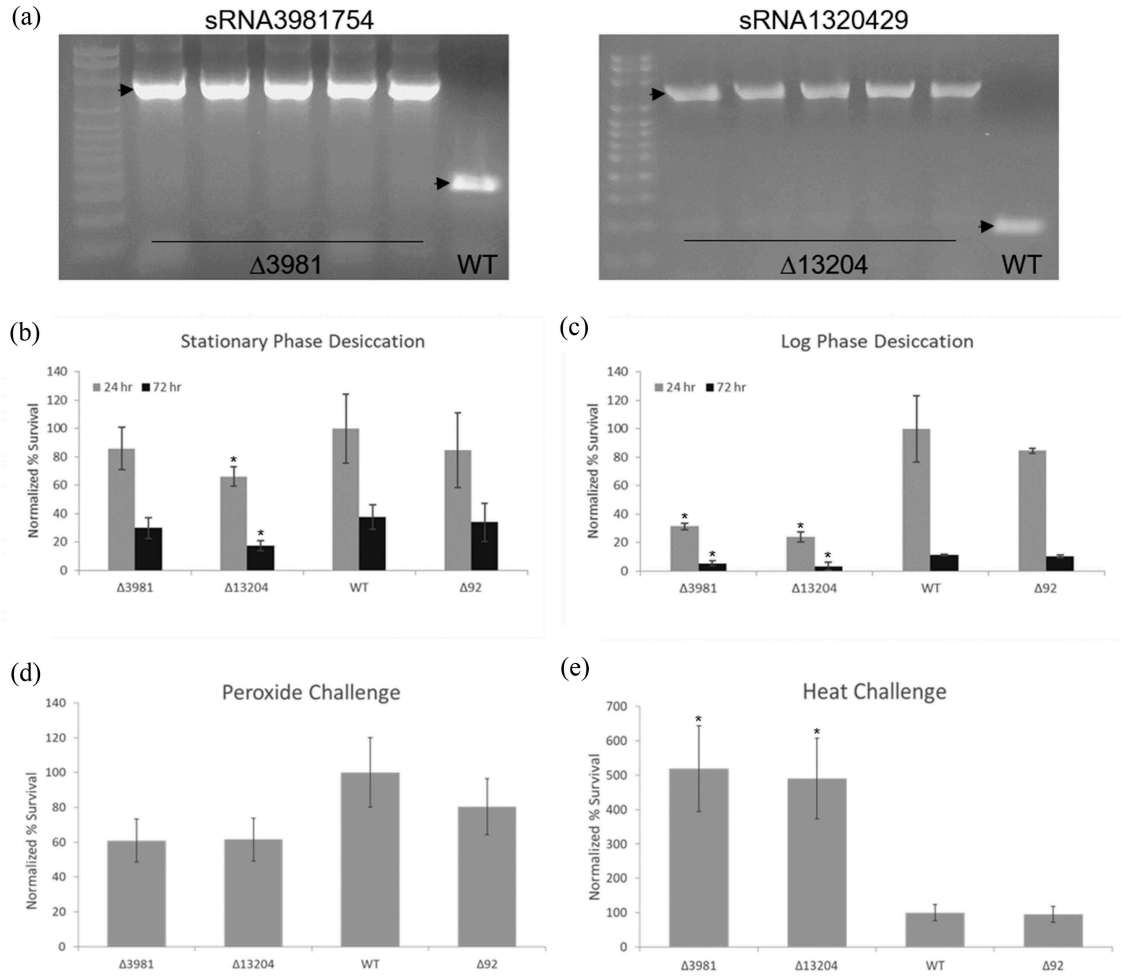

Figure 5. Effects of silencing sRNA1320429 and sRNA3981754. (a) PCR confirmation of sRNA genomic replacement with chloramphenicol resistance cassette. Arrowheads indicate amplicons generated utilizing primers flanking indicated sRNA loci. Lane 1, l00 bp ladder. Lanes 2-6, mutant colonies (harbouring ~1,000 bp insertions at indicated loci). Lane 7, wild type sRNA amplicons (<200 bp). (b) Desiccation of stationary phase deletion mutants and wild type *Salmonella*. Survival determined after desiccation for 24 and 72 h by comparison to wild type samples maintained under the same conditions. (c) Survival of log phase deletion mutants and wild type *Salmonella* subjected to desiccation for 24 and 72 h. (d) Survival of peroxide-challenged mutants and wild type *Salmonella*. (e) Survival of mutant and wild type *Salmonella* after heat exposure (55°C) for 45 min. Error bars in B-E indicate SD (n = 5). Survivals were normalized to wild type (WT). * indicates $p \leq 0.05$; p-values determined by unpaired two-tailed t-test. Δ13204, sRNA1320429 deletion mutant; Δ3981, sRNA3981754 deletion mutant; Δ92, sRNA deletion mutant previously generated using the same methodology [15].

Materials and methods

Strain and growth conditions

Salmonella enterica serovar Typhimurium strain SL1344 was grown and maintained on LB agar (1% w/v tryptone/1% w/v NaCl/0.5% w/v yeast extract/1.5% w/v agar). To prepare the cell suspension, a fresh overnight-grown culture was streaked onto 2 LB Agar plates and grown overnight at 37°C. On the next day, a sterile cell scraper was used to harvest the cells. The scraped cells were washed 3 times with sterile distilled water for 5 min at 5,000 g. At the end of the last wash, water was added up to reach OD_{600} = 1.0 (~ 8×10^8 cells/ml).

Salmonella desiccation and small RNA sequencing

Cells were desiccated following a protocol used in a previous work examining the *Salmonella* transcriptome under dehydration [50], with minor modifications. Petri dishes containing 15 mL of the cell suspension were left open inside of a laminar flow hood (relative humidity of 40%) for up to 72 h at room temperature. Control samples were maintained at the same conditions but in water suspension in a 50 mL conical tube. 24 and 72-h cells were harvested from Petri dishes by adding 10 mL RNA storage solution (25 mM sodium citrate, 10 mM EDTA, 70% w/v ammonium sulphate, pH 5.2). Cells were pelleted (4,500 rpm for 10 min at room temperature) and washed two times with a sterile PBS solution. The final pellet was resuspended in 1 mL PBS solution, then samples frozen in liquid nitrogen and stored at −80°C. Final frozen samples were shipped to the Genomic Services Lab at HudsonAlpha (HudsonAlpha Institute for Biotechnology, Huntsville, AL, USA) where RNA isolation and small RNA-seq using an Illumina HiSeq v4 genome sequencer were performed (paired ends, 100 bp, 25M reads). HudsonAlpha performed RNA isolations following standard Trizol (Invitrogen) protocols then employed the NEBNext Multiplex Small RNA Library Prep Set for Illumina (NEB) coupled with automated agarose gel size selection (30 to 200 nt) using the Pipin Prep Instrument (Sage Science) for small RNA library prep. Adapter sequences were removed with cutadapt [51], and

reads were mapped against *S.* Typhimurium SL1344 genome using BWA-MEM [52].

Identification and analysis of known small RNAs (sRNAs)

Reads from the RNA-seq library were mapped against the intergenic regions of SL1344 reference genome and known sRNAs from *Salmonella* using the CLC Genomics Workbench (CLC bio – v9.0; Finlandsgade, Dk) with the following parameters: mapping settings (minimum length fraction = 0.9, minimum similarity fraction = 0.8, and maximum number of hits for a read = 15) and paired settings (minimum distance = 180 and maximum distance = 250, including the broken pairs counting scheme). Expression values were reported in reads per kilobase of exon model per million mapped reads (RPKM), and the normalized value for each sample was calculated in transcripts per million (TPM). The expression data were log2 transformed and normalized to identify sRNAs exhibiting a fold change ≥2.0 during desiccation.

Identification of sRNA consensus sequences

To define consensus sRNA sequences with inconclusive mapping from RNA-seq data, Integrative Genomics Viewer (IGV) was employed to refine the sRNA locations within the larger intergenic regions of the SL1344 chromosome [53,54]. Indexed BAM files for each culture sample (SP, 24 h desiccated, and 72 h desiccated) and a BED file containing chromosomal positions for the intergenic regions were uploaded to IGV to determine expression levels within these regions along the SL1344 chromosome. Chromosomal position of peak expression within each intergenic region was determined and chromosomal positions (upstream and downstream) where peaks fell below 75% of the maximum were identified in each sample. Start and stop positions in each of the three samples where a putative sRNA was expressed were averaged resulting in the final sRNA consensus sequence calls.

Computational analyses of novel sRNAs

After definition of sRNA, consensus sequences through peak calling in IGV, the newly identified sRNA sequences were aligned to the original raw RNA-seq read data for each sample (SP, 24 h desiccated, and 72 h desiccated) using BLAST+ (2.2.27) with best hit parameters and an e-value of $1e^{-1}$ [55]. Alignments were also required to have percent identity ≥95% and length ≥28 bp (-evalue 1e-1 -best_hit_score_edge 0.05 -best_hit_overhang 0.25 -perc_identity 95 -max_target_seqs 1). Resulting alignments were then counted for each unique sequence read (sRNA) in each of the three samples, and counts were normalized using the total reads from the raw RNA-seq data for each sample to allow comparison of expression across all three sample conditions. Candidate sRNA sequences were aligned to Rfam, sRNATarBase, and SalCOM databases to determine similarity to known sRNAs [24,25,29,30]. NCBI BLAST megablast tool (word size: 16) was used to search for homologous sequences in all available bacterial genomes (excluding *Salmonella*). SRNAs were considered conserved if genomic sequences with ≥80% identity to the full length of a novel sRNA were identified in at least one other bacterial genera [56]. All RNA and protein-coding genes located within 300 bp upstream or downstream of the sRNA in Ensembl were also identified to analyse the expressions of flanking and overlapping genes [44], and putative sRNA expressions required to differ from overlapping genes by ≥200%. The computational tool 'IntaRNA' (http://rna.informatik.uni-freiburg.de) was used to predict potential, primary sRNA targets, and target genes were further analysed using Blast2GO and UniProtKB (https://www.uniprot.org/) [39,55,57]. Predicted secondary structures for each sRNA were generated using Mfold [31].

Real-time quantitative qRT-PCR analysis

Total RNA was isolated from control (24-h water-suspended) and desiccated samples (24 and 72 h) using the RNeasy PowerMicrobiome Kit (Qiagen, Cat No.:26000–50). qPCR was performed using the iTaq™ Universal SYBR® Green One-Step Kit (Bio-Rad, Cat No.: 172–5150) according to the manufacturer's instructions. Five annotated genes were selected to validate the small RNA-Seq data: *STnc3920, STnc1460, STnc700, IsrL* and *sRNA294324*. Five previously unannotated genes were also selected for validation: sRNA3981754, sRNA3417670, sRNA1320429, sRNA3417448, and sRNA294677. The gene *rpoD* was used as a control to normalize the values as it has shown not to be differentially expressed under these conditions. The reactions were performed in duplicate in a 384-well plate containing 5 µL of iTaq universal SYBR® Green reaction mix, 0.125 µL of the iScript reverse transcriptase, 300 nM of each forward and reverse primers, 45 ng of RNA and nuclease-free water to a total reaction mix volume of 10 µL. Primers are listed in Supplementary File 8. qRT-PCR was conducted in the CFX384 Touch Real-Time PCR Detection System (Bio-Rad). The real-time PCR program was as follows: initial reverse transcription reaction at 50°C for 10 min, polymerase activation and DNA denaturation at 95°C for 1 min, followed by 40 cycles of amplification at 95°C for 10 sec and 60°C for 15 sec. All the PCR amplifications were performed in triplicates. DNase-treated RNA was used in all sample extractions, and a control without reverse transcriptase was included. Specificity of amplifications was verified using melting curves. Gene expression was calculated via the Delta-Delta cycle threshold method [58].

Deletion and over expression of select sRNAs

Select sRNAs were deleted from the SL1344 genome to analyse the phenotypic effects of their removal under various conditions. The sRNAs chosen for deletion were sRNA1320429 and sRNA3981754, and deletion mutants were generated employing the previously described Lambda-Red recombinase method [17,34]. Briefly, lambda-red recombinase genes from the pKD46 plasmid were induced with arabinose in wild type SL1344 cells. The pKD3 plasmid was used to amplify the chloramphenicol resistance cassette flanked by sequences corresponding to the sRNAs selected for knockout. Resulting SL1344 mutant strains with chloramphenicol resistance in place of either sRNA3981754

or sRNA1320429 were confirmed by colony PCR before use in further analysis. Δ92, an sRNA deletion mutant we previously generated using the same methodology [15] was utilized as an sRNA deletion control as it had previously been identified by both our group (as sRNA924744) and independently (as STnc1200) [24].

Following Lambda Red protocol, colonies having incorporated the antibiotic resistance cassette (selected by growth on chloramphenicol plates) were re-suspended in 10 µL of 1x PBS and boiled for 15 min at 95°C. Samples were then pulse centrifuged to remove cell debris and 2 µL of resulting supernatant used for colony PCR amplifications. Prior to visual confirmation by being run on a 1.5% agarose gel and imaged with EtBr, colony PCR amplifications were performed in 25 µL reactions at standard concentrations (2.5 µL of 10x NH_4 PCR buffer, 1.25 µL of 50mM $MgCl_2$, 1 µL of 10mM dNTP, 0.2 U Taq (Bioline USA, Inc., Randolph, MA), 0.5 µM each primer) and using standard cycling parameters (94°C – 2 min (94°C – 45 s, 56°C – 30 s, 72°C – 45 s) × 30 cycles, 72°C – 2 min), using the following primers:

sRNA13204F – CGCAGGATAGCGAGCAATAACC
sRNA13204R – CGCAGTGCTTATGCCAACGC
sRNA3981F – GCAGAATGGGCGGATGTATATAC
sRNA3981R – CTAACGCGTTTTGGCGAGCAC.

PCR amplifications of expression construct inserts were performed in 40 µl reactions at standard concentrations (1.5 mM MgCl2, 0.2 mM dNTP, 1x Biolase PCR buffer, 0.5 U Taq (Bioline), 0.5 uM each primer) and using standard cycling parameters (94°C – 3 min (94°C – 30 s, 55°C – 30 s, 72°C – 60 s) x 30 cycles, 72°C – 3 min). sRNA expression vector sRNA insert primers 5′ to 3′ were: s132f – CAGCGAGCGATGATTTG; s132r – GCGAAAAAACGAAAGGATGG; s398f – GTACCAATGGATACCG; s398r – GAGCGTACACGTAGTACG. Resulting amplicons were separated on a 1% agarose gel and a band excised from the appropriate lane at ~350 bp. Gel extractions were then cloned into Topo TA PCR 2.1 and sequenced. Resultant amplicons were cloned into Topo PCR 2.1 and sequenced. RT-PCR reactions were performed to confirm expression using the Tetro cDNA synthesis kit (Bioline) and random hexamer primers per standard manufacturer protocols followed by PCR amplifications (as above) with the following primers:

S132rtF53 – GTCGGGAAAGTCGGG with
S132rtR53 – GGCAGAGCGGCTATTAATG and
S398rtF53 – ATCAGCTACTGATTGAAAGTTATACC with
S398rtR53 – TTATTTATGCGCGTTGAGAATCC.

For sRNA add-back expression experiments, deletion mutants and controls were grown to mid-log phase and subsequently washed 2x with sterile ddH2O and 2x with sterile 10% (v/v) glycerol solution to allow for electro-competency. Using Bio-Rad E. coli Pulser (1.8kV, 0.1 cm E cuvette), 60uL aliquot of each EC mutant or control was immediately electroporated with 850 ng of TOPO vector sRNA expression construct or empty TOPO control and plated on Ampicillin-selective agar plates.

Desiccation survival

To assess the effects of removing and/or over expressing selected sRNAs from the SL1344 genome, mutant and wild type strains were desiccated under the same conditions as described previously [42]. After 24 and 72 h, desiccated cells were harvested from Petri dishes using a sterile cell scraper and resuspended in 10 mL sterile distilled water. Desiccated resuspensions and control samples were serially diluted (1:5) in sterile distilled water, spread onto LB agar plates, and incubated overnight at 37°C. Colony-forming units (CFUs) were determined for countable dilutions, and CFUs between 30 and 300 were used to calculate desiccation survival for each sample. The same procedure was also repeated using *Salmonella* cells in log-phase growth.

Heat and peroxide challenge

To assess tolerance to heat and hydrogen peroxide challenges, deletion mutants and wild type *Salmonella* were exposed to these conditions under the following parameters. Cultures were grown overnight in LB broth with shaking at 37°C. The next morning, overnight cultures were inoculated 1:100 into fresh LB broth and grown to an OD_{600} of approximately 0.3–0.4 to generate log-phase cells. Aliquots of log-phase culture were serially diluted (1:5) in sterile distilled water and plated on LB agar to serve as control. For challenge with hydrogen peroxide, 10 µL of H_2O_2 (1M) and 10 µL of log-phase culture were added to 980 µL of fresh LB broth and incubated at 37°C with shaking for 45 min. For heat tolerance assessment, log-phase cultures were incubated at 55°C with shaking for 30 min. Following each treatment, aliquots from each sample were serially diluted and plated in the same manner as control, and all plated samples were incubated at 37°C overnight. CFUs were then determined and used to calculate survival under each condition.

Small transcript northern blots

Total RNA from SL1344 cultures was isolated with Trizol® (Life Sciences) per standard manufacture protocol. A 15% acrylamide/bis-acrylamide (29:1) gel containing 8 M urea (48% (w/v)) and 1X TBE was prerun for 30 min at 100 V in a vertical mini-PROTEAN tank (Bio-Rad). Gels were flushed and loaded with 10 µg of total RNA in 2X TBE/Urea sample buffer (Bio-Rad), then run at 200 V until the bromophenol blue dye front reached the gel bottom. As a size reference, 1 µl of pooled, commercially synthesized biotin 5′ end–labelled DNA oligonucleotides (25, 50 and 200 bp each at 1 µM) was also loaded in 2X TBE/Urea sample buffer. After removal from the electrophoresis plates, gels were gently rinsed with water then washed in 0.5X TBE for 5 min on an orbital shaker. After electrophoresis, RNA was electro-transferred (Mini Trans-Blot Electrophoretic Transfer Cell apparatus, Bio-Rad) to Biodyne B Pre-cut Modified Nylon Membranes 0.45 µm (Thermo Scientific) for 2 h at 20 V in 0.5X TBE. After removal from the transfer stack, membranes were gently washed in 1X TBE for 15 min on an orbital shaker, then UV cross-linked at 1200 mJ for 2 min (Stratalinker, Stratagene). Prehybridization was performed in North2South® Hybridization Buffer (Thermo Scientific) at 42°C for 30 min, after which 30 ng (per millilitre of hybridization buffer) of each appropriate biotin 5′ end–labelled oligonucleotide was added directly to the hybridization buffer as probe.

Probe1320429 5pBio- CAGTGTCGACCATATTAGGCTCGCCGATAG

Probe3981754 5pBio- AGCTACTGATTGAAAGTTATACCAAAGCGC

Probe1320429ctl 5pBio- CTATCGGCGAGCCTAATATGGTCGACACTG

Probe3981754ctl 5pBio- GCGCTTTGGTATAACTTTCAATCAGTAGCT

Blots were hybridized overnight with gentle rotation at 23°C. Hybridization buffer was removed the following day, and membranes washed and developed using the Thermo Scientific™ North2South® Chemiluminescent Hybridization and Detection Kit per manufacturer instructions then imaged on a LI-COR C-DiGit Chemiluminescent Blot Scanner.

Acknowledgments

This work was facilitated by the University of South Alabama (USA) College of Arts & Sciences Department of Biology, the USA College of Allied Health Professions Department of Biomedical Sciences, and the USA College of Medicine Department of Pharmacology. This study was also financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

Funding was provided in part by NSF CAREER grant 1350064 (GMB) awarded by Division of Molecular and Cellular Biosciences (with co-funding provided by the NSF EPSCoR program). Graduate funding was also provided in part by Alabama Commission on Higher Education ALEPSCoR grants 150380 (JTR),160330 (VMK), and 180435 (DH). Postdoctoral funding was provided by São Paulo Research Foundation (FAPESP) Grants # 2014/17387-8 and # 2015/19400-4 (AC). A.S. Sant'Ana acknowledges the support of 'Conselho Nacional de Desenvolvimento Cientifico e Tecnológico' (CNPq) (Grants #302763/2014-7; #305804/2017-0); Alabama Commission on Higher Education [180435]; Alabama Commission on Higher Education [160330]; Alabama Commission on Higher Education [150380]; Conselho Nacional de Desenvolvimento Científico e Tecnológico [305804/2017-0]; Fundo de Apoio ao Ensino, à Pesquisa e Extensão, Universidade Estadual de Campinas [2015/19400-4]; Fundo de Apoio ao Ensino, à Pesquisa e Extensão, Universidade Estadual de Campinas (BR) [2014/17387-8]; Conselho Nacional de Desenvolvimento Científico e Tecnológico (BR) [302763/2014-7].

ORCID

Shivam V. Amin http://orcid.org/0000-0002-0899-106X
Justin T. Roberts http://orcid.org/0000-0003-4433-1234
Jaire A. Ferreira-Filho http://orcid.org/0000-0001-9690-7704
Anderson S. Sant'Ana http://orcid.org/0000-0001-5052-2315
Michael P. Spector http://orcid.org/0000-0003-2876-6027
Glen M. Borchert http://orcid.org/0000-0002-6182-7154

References

[1] Hombach S, Kretz M. Non-coding RNAs: classification, biology and functioning. Adv Exp Med Biol. 2016;937:3–17.
[2] Cech TR, Steitz JA. The noncoding RNA revolution-trashing old rules to forge new ones. Cell. 2014 Mar;157(1):77–94.
[3] Fischer JW, Leung AKL. CircRNAs: a regulator of cellular stress. Crit Rev Biochem Mol Biol. 2017 Apr;52(2):220–233.
[4] Sun X, Zheng H, Sui N. Regulation mechanism of long non-coding RNA in plant response to stress. Biochem Biophys Res Commun. 2018 Sep;503(2):402–407.
[5] Tehrani SS, Karimian A, Parsian H, et al. Multiple functions of long non-coding RNAs in oxidative stress, DNA damage response and cancer progression. J Cell Biochem. 2018 Jan;119(1):223–236.
[6] Hollins SL, Cairns MJ. MicroRNA: Small RNA mediators of the brains genomic response to environmental stress. Prog Neurobiol. 2016 Aug;143:61–81.
[7] Olejniczak M, Kotowska-Zimmer A, Krzyzosiak W. Stress-induced changes in miRNA biogenesis and functioning. Cell Mol Life Sci. 2018 Jan;75(2):177–191.
[8] Amaral PP, Dinger ME, Mattick JS. Non-coding RNAs in homeostasis, disease and stress responses: an evolutionary perspective. Brief Funct Genomics. 2013 May;12(3):254–278.
[9] Cavanagh AT, Wassarman KM. 6S RNA, a global regulator of transcription in Escherichia coli, Bacillus subtilis, and beyond. Annu Rev Microbiol. 2014 Sep;68(1):45–60.
[10] Holmqvist E, Wagner EGH. Impact of bacterial sRNAs in stress responses. Biochem Soc Trans. 2017;45:1203–1212.
[11] Perry RN. Desiccation survival of parasitic nematodes. Parasitology. 1999;119 Suppl:S19–S30.
[12] Giarola V, Hou Q, Bartels D. Angiosperm plant desiccation tolerance: hints from transcriptomics and genome sequencing. Trends Plant Sci. 2017 Aug;22(8):705–717.
[13] Esbelin J, Santos T, Hébraud M. Desiccation: An environmental and food industry stress that bacteria commonly face. Food Microbiol. 2018 Feb;69:82–88.
[14] Sharma CM, Vogel J. Experimental approaches for the discovery and characterization of regulatory small RNA. Curr Opin Microbiol. 2009 Oct;12(5):536–546.
[15] Amin SV, Roberts JT, Patterson DG, et al. Novel small RNA (sRNA) landscape of the starvation-stress response transcriptome of Salmonella enterica serovar typhimurium. RNA Biol. 2016 Mar;13(3):331–342.
[16] Deng X, Li Z, Zhang W. Transcriptome sequencing of Salmonella enterica serovar Enteritidis under desiccation and starvation stress in peanut oil. Food Microbiol. 2012 May;30(1):311–315.
[17] Spector MP, Kenyon WJ. Resistance and survival strategies of Salmonella enterica to environmental stresses. Food Res Int. 2012 Mar;45(2):455–481.
[18] Smirnov A, Förstner KU, Holmqvist E, et al. Grad-seq guides the discovery of ProQ as a major small RNA-binding protein. Proc Natl Acad Sci U S A. 2016 Oct;113(41):11591–11596.
[19] Vogel J, Sharma CM. How to find small non-coding RNAs in bacteria. Biol Chem. 2005 Jan;386(12):1219–1238.
[20] Hoe CH, Raabe CA, Rozhdestvensky TS, et al. Bacterial sRNAs: regulation in stress. Int J Med Microbiol. 2013;303(5):217–229.
[21] Oliva G, Sahr T, Buchrieser C. Small RNAs, 5′ UTR elements and RNA-binding proteins in intracellular bacteria: impact on metabolism and virulence. FEMS Microbiol Rev. 2015 May;39(3):331–349.
[22] Mandin P, Guillier M. Expanding control in bacteria: interplay between small RNAs and transcriptional regulators to control gene expression. Curr Opin Microbiol. 2013 Apr;16(2):125–132.
[23] Altuvia S, Weinstein-Fischer D, Zhang A, et al. A small, stable RNA induced by oxidative stress: role as a pleiotropic regulator and antimutator. Cell. 1997 Jul;90(1):43–53.
[24] Kröger C, Colgan A, Srikumar S, et al. An infection-relevant transcriptomic compendium for Salmonella enterica serovar Typhimurium. Cell Host Microbe. 2013 Dec;14(6):683–695.
[25] Kroger C, Dillon SC, Cameron ADS, et al. The transcriptional landscape and small RNAs of Salmonella enterica serovar Typhimurium. Proc Natl Acad Sci. 2012 May;109(20):E1277–E1286.
[26] Calderón IL, Morales EH, Collao B, et al. Role of Salmonella Typhimurium small RNAs RyhB-1 and RyhB-2 in the oxidative stress response. Res Microbiol. 2014 Jan;165(1):30–40.

[27] Kim JN, Kwon YM. Genetic and phenotypic characterization of the RyhB regulon in Salmonella Typhimurium. Microbiol Res. 2013 Jan;168(1):41–49.
[28] Babicki S, Arndt D, Marcu A, et al. Heatmapper: web-enabled heat mapping for all. Nucleic Acids Res. 2016 Jul;44(W1): W147–W153.
[29] Kalvari I, Argasinska J, Quinones-Olvera N, et al. Rfam 13.0: shifting to a genome-centric resource for non-coding RNA families. Nucleic Acids Res. 2018 Jan;46(D1):D335–D342.
[30] Wang J, Liu T, Zhao B, et al. sRNATarBase 3.0: an updated database for sRNA-target interactions in bacteria. Nucleic Acids Res. 2016 Jan;44(D1):D248–D253.
[31] Zuker M. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res. 2003 Jul;31 (13):3406–3415.
[32] Wassarman KM, Repoila F, Rosenow C, et al. Identification of novel small RNAs using comparative genomics and microarrays. Genes Dev. 2001 Jul;15(13):1637–1651.
[33] Schlüter J-P, Reinkensmeier J, Daschkey S, et al. A genome-wide survey of sRNAs in the symbiotic nitrogen-fixing alpha-proteobacterium Sinorhizobium meliloti. BMC Genomics. 2010 Apr;11(1):245.
[34] Murphy KC. Use of bacteriophage lambda recombination functions to promote gene replacement in Escherichia coli. J Bacteriol. 1998 Apr;180(8):2063–2071.
[35] Evans DJ, Brown MR, Allison DG, et al. Susceptibility of bacterial biofilms to tobramycin: role of specific growth rate and phase in the division cycle. J Antimicrob Chemother. 1990 Apr;25 (4):585–591.
[36] Mayhall CG, Apollo E. Effect of storage and changes in bacterial growth phase and antibiotic concentrations on antimicrobial tolerance in Staphylococcus aureus. Antimicrob Agents Chemother. 1980 Nov;18(5):784–788.
[37] Purdy MA, Tenovuo J, Pruitt KM, et al. Effect of growth phase and cell envelope structure on susceptibility of Salmonella typhimurium to the lactoperoxidase-thiocyanate-hydrogen peroxide system. Infect Immun. 1983 Mar;39(3):1187–1195.
[38] Vogel J, Wagner EGH. Target identification of small noncoding RNAs in bacteria. Curr Opin Microbiol. 2007 Jun;10(3):262–270.
[39] Busch A, Richter AS, Backofen R. IntaRNA: efficient prediction of bacterial sRNA targets incorporating target site accessibility and seed regions. Bioinformatics. 2008 Dec;24(24):2849–2856.
[40] Podolak R, Enache E, Stone W, et al. Sources and risk factors for contamination, survival, persistence, and heat resistance of salmonella in low-moisture foods. J Food Prot. 2010 Oct;73 (10):1919–1936.
[41] Finn S, Handler K, Condell O, et al. ProP is required for the survival of desiccated salmonella enterica serovar typhimurium cells on a stainless steel surface. Appl Environ Microbiol. 2013 Jul;79(14):4376–4384.
[42] Mandal RK, Kwon YM. Global screening of salmonella enterica serovar typhimurium genes for desiccation survival. Front Microbiol. 2017 Sep;8:1723.
[43] Hébrard M, Kröger C, Srikumar S, et al. sRNAs and the virulence of Salmonella enterica serovar Typhimurium. RNA Biol. 2012 Apr;9(4):437–445.
[44] Zerbino DR, Achuthan P, Akanni W, et al. Ensembl 2018. Nucleic Acids Res. 2018 Jan;46(D1):D754–D761.
[45] Ryan D, Mukherjee M, Nayak R, et al. Biological and regulatory roles of acid-induced small RNA RyeC in Salmonella Typhimurium. Biochimie. 2018 Jul;150:48–56.
[46] Srikumar S, Kröger C, Hébrard M, et al. RNA-seq brings new insights to the intra-macrophage transcriptome of Salmonella Typhimurium. PLoS Pathog. 2015 Nov;11(11):e1005262.
[47] Lévi-Meyrueis C, Monteil V, Sismeiro O, et al. Expanding the RpoS/σS-network by RNA sequencing and identification of σS-controlled small RNAs in Salmonella. PLoS One. 2014 May;9 (5):e96918.
[48] Colgan AM, Kröger C, Diard M, et al. The impact of 18 ancestral and horizontally-acquired regulatory proteins upon the transcriptome and sRNA landscape of Salmonella enterica serovar Typhimurium. PLoS Genet. 2016 Aug;12(8):e1006258.
[49] Dar D, Sorek R. Bacterial noncoding RNAs excised from within protein-coding transcripts. MBio. 2018 Sep;9(5).
[50] Gruzdev N, McClelland M, Porwollik S, et al. Global transcriptional analysis of dehydrated Salmonella enterica Serovar Typhimurium. Appl Environ Microbiol. 2012 Nov;78 (22):7866–7875.
[51] Chen C, Khaleel SS, Huang H, et al. Software for pre-processing Illumina next-generation sequencing short read sequences. Source Code Biol Med. 2014;9:8.
[52] Li H, Durbin R. Fast and accurate long-read alignment with Burrows–Wheeler transform. Bioinformatics. 2010 Mar;26 (5):589–595.
[53] Robinson JT, Thorvaldsdóttir H, Winckler W, et al. Integrative genomics viewer. Nat Biotechnol. 2011 Jan;29(1):24–26.
[54] Thorvaldsdóttir H, Robinson JT, Mesirov JP. Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. Brief Bioinform. 2013 Mar;14 (2):178–192.
[55] Camacho C, Coulouris G, Avagyan V, et al. BLAST+: architecture and applications. BMC Bioinformatics. 2009 Dec;10(1):421.
[56] Thompson JD, Gibson TJ, Higgins DG. Multiple sequence alignment using ClustalW and ClustalX. Curr Protocals Bioinf. 2002 Aug;Chapter 2:Unit 2.3.
[57] Götz S, Garcia-Gomez JM, Terol J, et al. High-throughput functional annotation and data mining with the Blast2GO suite. Nucleic Acids Res. 2008 Jun;36(10):3420–3435.
[58] Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc. 2008;3(6):1101–1108.