2015

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Genome Rearrangements Can Make and Break Small RNA Genes

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Accepted: July 12, 2015
Data deposition: This project has been deposited at NCBI Sequence Read Archive (SRA) under the accession SRP044074 and SRP009089.

Abstract
Small RNAs (sRNAs) are short, transcribed regulatory elements that are typically encoded in the intergenic regions (IGRs) of bacterial genomes. Several sRNAs, first recognized in Escherichia coli, are conserved among enteric bacteria, but because of the regulatory roles of sRNAs, differences in sRNA repertoires might be responsible for features that differentiate closely related species. We scanned the E. coli MG1655 and Salmonella enterica Typhimurium genomes for nonsyntenic IGRs as a potential source of uncharacterized, species-specific sRNAs and found that genome rearrangements have reconfigured several IGRs causing the disruption and formation of sRNAs. Within an IGR that is present in E. coli but was disrupted in Salmonella by a translocation event is an sRNA that is associated with the FNR/CRP global regulators and influences E. coli biofilm formation. A Salmonella-specific sRNA evolved de novo through point mutations that generated a σ70 promoter sequence in an IGR that arose through genome rearrangement events. The differences in the sRNA pools among bacterial species have previously been ascribed to duplication, deletion, or horizontal acquisition. Here, we show that genomic rearrangements also contribute to this process by either disrupting sRNA-containing IGRs or creating IGRs in which novel sRNAs may evolve.

Key words: sRNA, E. coli, Salmonella, intergenic regions, gene origination.

Introduction
RNAs that do not code for proteins are critical to gene regulation in all domains of life. In bacteria, small RNAs (sRNAs) are typically 50–200 nucleotides (nt) in length and are usually encoded in genomic regions between protein-coding genes (intergenic regions or IGRs). They can control gene expression by modulating transcription, translation, or mRNA stability (Storz et al. 2011). The application of technologies that interrogate entire transcriptomes has revealed unexpectedly large numbers of sRNAs in bacterial genomes (Raghavan, Groisman, et al. 2011; Kroger et al. 2012). But unlike protein-coding genes, the mechanisms by which new sRNA genes arise and the forces that shape the sRNAs contents of genomes are not well understood (Gottesman and Storz 2011). Some sRNAs, such as 6S RNA (Wassarman and Storz 2000), are broadly conserved among bacteria, whereas several others are species- or even strain-specific (Gottesman and Storz 2011; Skippington and Ragan 2012). The sRNA transcriptomes of the enterics Escherichia coli and Salmonella enterica show substantial overlap; however, some of the orthologous IGRs display different patterns of expression and several sRNAs are present in only one of the species (Raghavan et al. 2012).

Differences in sRNA gene contents among bacteria can arise from lineage-specific loss or from the emergence of new sRNAs through duplication (Lenz et al. 2004; Wilderman et al. 2004) or horizontal acquisition (Pichon and Felden 2005; Sittka et al. 2008). An examination of the distribution of sRNAs within the E. coli/Shigella complex showed that the variation in the presence of known sRNAs was dominated by gene loss through deletions (Skippington and Ragan 2012). However, because this study focused only on those sRNAs that were originally characterized in a single strain of E. coli, it was biased toward the recognition of deletion events as it could not detect unique sRNAs in the genomes of other strains. Applying a broader phylogenetic perspective, homologs of a dual-function sRNA, SgrS, have been detected in distantly related Gammaproteobacteria (Horler and
Vanderpool 2009), and an exhaustive survey of sRNAs revealed that most E. coli sRNAs originated after Enterobacteriales split from other Gammaproteobacteria (Peer and Margalit 2014). This lineage-specific sRNA accumulation seems to be related to the evolution of the RNA-binding protein Hfq; however, the mechanisms by which new bacterial sRNAs emerge or are lost remain largely unknown.

In eukaryotes, there are cases where novel regulatory RNAs have evolved through gene duplication, by de novo origination from noncoding sequences, and from the degradation of protein-coding genes (Kaessmann 2010); but in bacteria, the mechanisms by which new regulatory RNAs arise are much less clear. Because new genes can form through the chimeric assembly of fragments from various sources—one well-known example of this is the jingwei gene of Drosophila (Long and Langley 1993)—we first adopted a structural genomics and RNA sequencing (RNA-seq)-based approach to identify new sRNA genes and then tested for sRNA functions. This combination of comparative and experimental analyses identified several previously unrecognized sRNAs and uncovered the sources of these differences in sRNA repertoires. We find that genome rearrangements have disrupted and formed IGRs containing functional sRNAs, thereby causing disparity in the sRNA contents of related bacterial species.

**Materials and Methods**

**RNA Sequencing**

For sRNA discovery, E. coli K-12 MG1655 (GenBank NC_000913.2) and S. enterica subsp. enterica serovar Typhimurium str. 14028S (GenBank NC_016856.1) were grown in lysogeny broth (LB) to OD600 ≈ 0.5 and then harvested by centrifugation. Total RNA was extracted from bacterial pellets using TRI reagent (Life Technologies) and cleaned with TRI-reagent (Life Technologies). Strand-specific RNA-seq libraries were synthesized (Raghavan et al. 2012), and each library was sequenced on the Illumina GA II platform (35 cycles) at the Yale Center for Genome Analysis.

**Mapping Sequencing Reads**

To characterize regions that are differentially expressed, sequencing reads were mapped onto E. coli (NC_000913.2) using the CLC Genomics Workbench. Genes with at least one read per sample and at least 20 total reads across all samples were chosen based on raw gene read counts from CLC mapping. Differential expression analysis of genes was performed using the DESeq R package (Anders and Huber 2010). Genes were chosen for downstream analysis based on significance (P < 0.05, FDR-corrected). Gene Ontology (GO) terms were found using Database for Annotation, Visualization and Integrated Discovery (DAVID) and the GO FAT filter (Huang et al. 2009). GO-term enrichment tests were also performed with DAVID. GO-terms overrepresented among differentially expressed genes were chosen based on the level of statistical significance (P < 0.05, Benjamini-corrected).

**sRNA Target Identification**

To identify sRNA-regulated genes, EcsR1 was cloned into the NheI and HindIII sites behind the arabinose-inducible promoter on plasmid pBAD using the polymerase chain reaction (PCR) primers 5’-CCG CTA GGC TTT TAG CCG CAT AAA GTG TAA C-3’ and 5’-CTA AGC TTT CCT GCC CGC TGT TAT GCC G-3’. *Escherichia coli* or *Salmonella*, transformed with either the empty pBAD vector (control) or pBAD+ EcsR1 (test), were grown in LB to OD600 ≈ 0.5 and induced with 0.2% arabinose for 15 min, as previously described (Durand and Storz 2010). RNA was extracted and processed for Illumina sequencing as above. Four Illumina mRNA-seq libraries (two control samples and two test samples) were prepared for each bacterium and multiplexed into a single lane of an Illumina HiSeq 2000 (101 cycles) at the Genomic Sequencing and Analysis Facility at University of Texas at Austin.

**Measuring Hfq Stabilization of sRNAs**

An Hfq-deleted strain of E. coli (JW4130-1) and its isogenic parent strain (BW25113) (Baba et al. 2006) were obtained from Yale Coli Genetic Stock Center and grown to mid-log phase (OD600 ≈ 0.5) in LB. Total RNA was DNase-treated, and 1 μg used as template for preparing cDNA. The abundances of EcsR1 in the wild-type and Hfq-deleted strains were determined by quantitative PCR (primers: 5’-TTT TTG TGT AAT GCG GGC TTT TTC TGC TTA-3’, and 5’-GCC GGC TTT TTC TGC TTA TT-3’), and calculated from Ct (threshold cycle) values.

**Identification of Unique IGRs**

Orthologous genes common to *E. coli* and *Salmonella* were identified using a reciprocal BLAST best-hit approach (Raghavan et al. 2012). Gene order of each orthologous gene-pair was determined with GeneOrder 4.0 (Mahadevan and Seto 2010), and in cases where the genomic locations were not syntenic in the two species, we searched for gene-pairs with adjacent novel IGRs using Artemis.
Identification of -10 Promoter Elements and sRNA Homologs

Transcriptional start sites (TSSs) for novel sRNAs were identified from RNA-seq data as described previously (Raghavan, Sage, et al. 2011; Raghavan et al. 2012). TSSs for flanking genes were identified as above and were confirmed using published data (Kroger et al. 2012; Keseler et al. 2013). The σ70 -10 motif has a 6-bp consensus sequence TATAAT; however, promoters often have imperfect matches to the consensus and can be located anywhere in a window ranging from approximately 4 to 18 bp upstream of the TSS (Huerta and Collado-Vides et al. 2003). To identify potential -10 elements associated with sRNAs, we searched this 15-bp window for any hexamers that matched at least 4 of the 6 bp in the consensus sequence including the two most highly conserved positions, A2 and T6 (Huerta et al. 2006). Bacterial genomes were queried for homologs of sRNAs identified in this study by analyzing a combination of sequence identity, secondary structure conservation, and genomic location as described previously (Raghavan, Groisman, et al. 2011).

Detection of sRNA 3'-Ends

A modified Rapid Amplification of cDNA Ends (RACE) procedure (Raghavan, Groisman, et al. 2011) was used to determine the 3'-ends of sRNAs as follows: Total RNA, depleted of 16S and 23S rRNA using a MICROBExpress kit (Life Technologies), was dephosphorylated with alkaline phosphatase (NEB), and a short oligonucleotide adapter (5'-P-UCG UAU GCC GUC UUC UGC UUG UidT-3') was ligated to 3'-ends using T4 RNA ligase (NEB). The 3' -adapter-ligated RNA was reverse-transcribed using a primer complementary to the adapter (5'-CAA GCA GAA GAC GCC ATA CGA-3'), and the resulting cDNA was used as template in PCR reactions using primers specific to sRNAs (EcsR1: 5'-AGA TGA CAC TTT TTT TGT AAT GAC G-3'; EcsR2: 5'-TAT CGC GCT ACT TCA GGA TGA TGT A-3') along with the adapter-complementary primer. Amplicons were resolved on 3% low-range ultra agarose (Bio-Rad) gels to determine their lengths, and their nucleotide sequences were determined by Sanger sequencing.

Biofilm Assay

EcsR1-deletion strain of E. coli was constructed using λ Red-mediated recombination (Datsenko and Wanner 2000). Escherichia coli or Salmonella strains grown overnight at 37°C in LB (or LB with 100 μg/ml ampicillin) were diluted 1:100 in fresh media and grown in 96-well microtiter plates for 48 h at 28°C without shaking. Planktonic growth (OD600) of E. coli and Salmonella strains measured on a Victor X5microplate reader (Perkin Elmer) did not significantly differ from each other. Supernatants containing nonadhered cells were discarded, and samples were washed twice with distilled water and the attached biofilm in each well was stained with 0.1% crystal violet for 30 min. Unbound stain was removed by washing with distilled water. To quantify biofilm production, the crystal violet associated with biofilms was dissolved in 100% ethanol and absorbance (A600) was measured, and normalized to the OD600 value of each strain, as described previously (Gualdi et al. 2008). Average intensity of biofilm formation for each strain was generated from at least four replicate experiments.

Results

Genome Rearrangements Form Unique IGRs

To identify IGRs that are unique to either E. coli or Salmonella, we compared the genomic locations of all orthologous genes in the two genomes. Because E. coli and Salmonella genomes are largely syntenic, the majority of IGRs situated between orthologous gene-pairs in the two genomes are also syntenic. However, there are several instances where orthologous protein-coding genes are situated at different relative locations in each genome (apparent as data points that do not lie along the diagonal in supplementary fig. S1, Supplementary Material online). After examining each of these cases, we identified chimeric IGRs present in either E. coli or Salmonella that were generated through the rearrangement of 68 genes (supplementary table S1, Supplementary Material online).

Unique IGRs Contain Novel sRNAs

We performed a directional RNA-seq analysis on E. coli and Salmonella grown under identical conditions to determine whether any of the species-specific IGRs contained highly transcribed regions. After mapping sequencing reads onto the respective genomes, we detected “transcriptional peaks,” which usually indicate the presence of sRNAs, in four of the species-specific IGRs, two in E. coli and two in Salmonella (fig. 1). Transcripts mapping to the corresponding locations in the E. coli and Salmonella genomes have been observed in previous studies (Tjaden et al. 2002; Dornenburg et al. 2010; Kroger et al. 2013) further verifying their transcriptional status, and there were no potential open reading frames (ORFs) of substantial length within these transcripts indicating that they represent sRNAs.

TSSs and 3'-ends of the transcribed sequences detected in these IGRs were identified from RNA-seq data (Raghavan et al. 2012), and a modified 3'-RACE procedure (Raghavan, Groisman, et al. 2011) was used to confirm the sRNAs in E. coli (supplementary fig. S2, Supplementary Material online), yielding the following results: 1) The sRNA (EcsR1) within the IGR between uspF and ampN genes in E. coli is 126 nt (genomic location 1433654–1433779), 2) the sRNA (EcsR2) within the IGR between yagU and ykgJ genes in E. coli is 166 nt (genomic location 302905–303070), 3) the sRNA (SesR1) within the IGR between STM14_1512 and
STM14_1513 genes in *Salmonella* is 105 nt (genomic location 1347963–1348067), and 4) the sRNA (SesR2) within the IGR between STM14_1869 and STM14_1870 genes in *Salmonella* is 111 nt (genomic location 1636380–1636490).

Homologs of EcsR1 and EcsR2 are present in all 66 *E. coli* genomes available in the RefSeq database (supplementary table S2, Supplementary Material online). SesR1 homologs were detected in all 44 *S. enterica* and *S. bongori* genomes (supplementary table S3, Supplementary Material online), and a recent study reported an sRNA (STnc1990) at the homologous position in *S. enterica* Typhimurium SL1344 (Kroger et al. 2013). SesR2 is conserved in 20 *S. enterica* genomes and in the two sequenced *S. bongori* strains (supplementary table S3, Supplementary Material online). However, the STM14_1869–STM14_1870 IGR is not maintained in *S. bongori* due to the loss of the STM14_1870 ortholog.

Because SesR2 is absent from a few *S. enterica* serovars but present in the *S. bongori* outgroup, this sRNA is ancestral to *Salmonella* and was subsequently lost in some *S. enterica* lineages.

**A *Salmonella*-specific IGR Formed Through HGT-mediated Genome Rearrangement**

The STM14_1869–STM14_1870 IGR is present in *Salmonella* but not in *E. coli*. *Escherichia coli* possesses a gene, yjgH, that is orthologous to STM14_1869, but contained no ortholog for STM14_1870. Further analysis uncovered that STM14_1870 and its neighboring gene STM14_1871 constitute the toxin and antitoxin, respectively, of the StbED toxin–antitoxin (TA) system (Unterholzner et al. 2013) (fig. 2).

Orthologs of stbED TA genes are present on several enterobacterial plasmids and prophages, and are horizontally transferred between bacteria (Anantharaman and Aravind 2003; Unterholzner et al. 2013). Additionally, the succeeding gene in the *Salmonella* genome, STM14_1872, is also homologous to a gene of bacteriophage origin (fig. 2), further indicating that the IGR between STM14_1869 and STM14_18670 was created by the introduction of genes through horizontal gene transfer (HGT)-mediated events.

**Evolution of a New sRNA in a *Salmonella* IGR**

To determine whether SesR2 was introduced along with its horizontally acquired neighboring genes into *Salmonella*, we searched the IGRs downstream of the stbE gene in several enterobacterial genomes, but could not detect homologous sRNAs (fig. 2). Because a promoter is required for the new sRNA to be transcribed, we compared the region that contains the sRNA’s TSS (5’-end of STM14_1869) with its homologous sequence in *E. coli* (5’-end of yjgH) and in other enterics. As shown in figure 3 and supplementary figure S3, Supplementary Material online, a putative σ70 promoter (CA TAAT, located -6 to -11 bp from sRNA’s TSS) is uniquely present in *Salmonella*, indicating that this sRNA originated de novo in the *Salmonella*-specific IGR. Additionally, the promoter and sRNA sequences are conserved in *S. bongori* and in those *S. enterica* serovars that maintain an intact STM14_1869–STM14_1870 IGR.

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**FIG. 1.**—Expression profiles within nonsyntenic IGRs. Putative sRNAs were detected by RNA-seq analysis of transcript levels within IGRs in *E. coli* (A, B) and *Salmonella* (C, D). For uniformity, the number of sequencing reads mapped to IGRs is limited to 2,000 (dashed line). Arrows showing the orientation of ORFs and putative sRNAs are not drawn to scale.
**Salmonella Typhimurium str. 14028S chromosome**

STM14_1869 sRNA stbE stbD STM14_1872

**Erwinia pyrifoliae Ep1/96 plasmid pEP36**

**Klebsiella pneumoniae str. SB3432 chromosome**

**Citrobacter rodentium ICC168 plasmid pCROD2**

**Shigella sonnei Ss046 plasmid pSS_046**

[Fig. 2]—Salmonella IGR formed through an HGT-mediated genome rearrangement. Most homologs of STM14_1870 (stbE, blue arrow) and STM14_1871 (stbD, green arrow) are situated on bacterial plasmids, and STM14_1872 (purple arrow) is a prophage gene. Both the STM14_1869–STM14_1870 IGR and the sRNA (SesR2) are present only in Salmonella. In Shigella, an insertion sequence (IS1294) flanks the stbE gene.

[Fig. 3]—Evolution of a new sRNA promoter. Sequences immediately upstream of STM14_1869 (4471230–4471245) and its ortholog yjjG in E. coli (1636369–1636384) are aligned. Numbers of RNA-seq reads mapping to this region are shown (black, Salmonella; blue, E. coli). The new Salmonella σ70 promoter and sRNA (SesR2) transcription start site are boxed. Asterisks indicate point mutations that differentiate the Salmonella sequence from the corresponding region in E. coli.

Rearrangement-Induced Loss of a Salmonella IGR

In E. coli, the ompN and uspF genes are adjacent, separated by a 140-bp IGR that contains EcsR1; whereas in Salmonella, the ompN gene is in an alternate location, situated between the STM14_1775 and rstA genes. To determine the ancestry of these gene arrangements—specifically whether the uspF–ompN IGR was gained by E. coli or lost by Salmonella—we analyzed the organization of the orthologous regions in the genomes of other enteric bacteria. The uspF–ompN IGR is present and intact in other enteric species (Klebsiella pneumoniae, Enterobacter aerogenes, and Citrobacter kosenii) establishing that this IGR predates the split between E. coli and Salmonella and was lost in Salmonella due to the relocation of ompN gene (figs. 4 and 5). As a consequence of this genome rearrangement in Salmonella, EcsR1 was split into two fragments located ≈200 kb apart, neither of which is transcribed (supplementary fig. S4, Supplementary Material online).

EcsR1 Is Associated with Global Regulators in E. coli

The uspF–ompN IGR and EcsR1 are present in all strains of E. coli, which suggests that it maintains a regulatory function. To identify genes that are potentially under the control of EcsR1, we examined the effect of its overexpression on E. coli genes genome-wide, an approach that has been used previously to characterize the regulatory targets of sRNAs (Durand and Storz 2010; Beisel and Storz 2011). When analyzed by RNA-seq, the expression levels of 43 genes were significantly different (P < 0.05) in the EcsR1-overexpressing strain when compared with wild-type E. coli (supplementary table S4, Supplementary Material online). A GO analysis uncovered bacterial membrane (GO:0031090), carbohydrate catabolic process (GO:0016052), and nitrate metabolic process (GO:0042126) as processes that were significantly enriched (P < 0.05) in our data set. Eleven downregulated genes were associated with these GO terms, of which nine were regulated by CRP and/or FNR (supplementary table S5, Supplementary Material online) (Constantinidou et al. 2006; Keseler et al. 2013). In concert with these observations, a 22-nt palindromic sequence with features resembling the consensus CRP-binding site and a putative 15-nt FNR-binding sequence (fig. 5) were identified upstream of EcsR1, indicative of the sRNA being part of the CRP and FNR regulons. Expression of another E. coli RNA, FnrS, is known to be affected by both FNR and CRP (Durand and Storz 2010), showing that CRP and FNR regulons overlap and may control multiple sRNAs. It has been shown previously that the transcriptional regulator CRP can control the expression of both an sRNA and the sRNA’s target genes, and this “feed-forward loop” is thought to aid in the efficient modulation of gene expression in E. coli (Beisel and Storz 2011).

Because many sRNAs in E. coli associate with and are stabilized by the RNA-binding protein Hfq (De Lay et al. 2013), we examined whether Hfq stabilizes EcsR1. We measured the abundance of EcsR1 in wild-type and Hfq-deficient strains of E. coli, and found it to be significantly more abundant in the...
EcsR1 Impacts Biofilm Formation

Because CRP and FNR control carbohydrate and nitrate metabolism during biofilm formation (Van Alst et al. 2007; Karatan and Watnick 2009), we constructed EcsR1-deletion and EcsR1-overexpression E. coli strains and measured the impact of this sRNA on biofilm formation. As shown in figure 6, biofilm production increased significantly ($P < 0.0001$) in the EcsR1-deleted strain when compared with wild-type E. coli. Reintroduction of a plasmid-borne copy of EcsR1 into the deletion strain reduced biofilm formation.

FIG. 4.—Distribution of the uspF–ompN IGR among enteric species. (A) Alignment of genomic regions containing the uspF–ompN IGR in E. coli and three other enteric species. Note that in both Citrobacter koseri and Klebsiella pneumoniae, small ORFs (gray arrows situated between uspF [purple] and ompN [blue]) have been predicted to occur in this IGR. (B) Phylogenetic tree (modified from Petty et al. 2010) showing the presence or absence of the uspF–ompN IGR among species.

wild-type strain (supplementary fig. S5, Supplementary Material online), reinforcing its identity as an sRNA.
formation to the same level as that of the wild-type strain (fig. 6), indicating that biofilm-inhibition is an sRNA-specific phenotype.

**Expression of E. coli EcsR1 in Salmonella Activates Invasion-Associated Genes**

Biofilm production is important to virulence of enteric pathogens, so we tested the effects of EcsR1 on biofilm production in *Salmonella* by reintroducing the sRNA in an expression vector. There was no significant difference in biofilm production between the wild-type and EcsR1-overexpression strains; the overexpression of EcsR1 in *Salmonella* alters the expression of 128 genes genome-wide (supplementary table S6, Supplementary Material online). GO analysis revealed nine processes (representing 27 genes) that were significantly enriched within this gene set, with “pathogenesis” (GO:0009405) being the most highly significant (supplementary table S7, Supplementary Material online). Among genes regulated by this sRNA, 22 are known to promote *Salmonella* invasion of host cells, most of which are situated within SPI-1 pathogenicity island (Fàbrega and Vila 2013).

**Discussion**

Our search for species-specific sRNAs was directed toward IGRs that were unique to either *E. coli* or *Salmonella* because most bacterial regulatory sRNAs are contained within these noncoding regions, although 3'-untranslated regions (UTRs) of mRNAs and promoters within mRNAs can also give rise to sRNAs (Chao et al. 2012; Guo et al. 2014). We found that genome rearrangements have altered IGRs and, in doing so, caused disparity in sRNA contents of these two species. A newly discovered sRNA (EcsR1), situated within the IGR between the *uspF–ompN* genes in *E. coli*, is absent from *Salmonella* due to the translocation of a genomic segment containing the *ompN* gene. This sRNA is associated with the FNR and CRP regulons, and its expression impacts *E. coli* biofilm formation. Additionally, we identified an sRNA (SesR2) unique to *Salmonella* that evolved de novo in an IGR that
was formed through a phage-mediated genome rearrangement. Although disparities in genome architectures are common among related species, these are the first known cases where rearrangements have caused the generation and destruction of sRNAs.

The main source of rearrangement events in bacterial genomes is homologous recombination across identical sequences. *Escherichia coli* and *Salmonella* contain numerous classes of repeat elements that can serve as templates for exchange (Rocha 2004). In addition, recombination between bacteriophage sequences in a genome can result in altered genome architectures in related bacteria (Brunner et al. 2004); large proportions of both *E. coli* and *Salmonella* genomes consist of prophage genes (Bobay et al. 2013). Notwithstanding the large number of targets for homologous exchange, the gene order of *E. coli* and *Salmonella* has been well conserved despite an estimated 100-Myr divergence (Ochman et al. 1999). The major difference in their repertoires of DNA recombination and repair enzymes (Tamas et al. 2002) but also to selection on gene order and position (Suyama and Bork 2001; Ballouz et al. 2010; Treangen and Rocha 2011). Our analyses show that some fraction of the rearrangements that shuffle IGRs may affect organismal fitness by disrupting or generating regulatory elements.

Although the IGR between *yagU* and *ompN* was disrupted and split in the *Salmonella* genome, some portions of it—approximately 70 nt of the 5'-end of *EcsR1* and 20 nt of its 3'-end—are still recognizable adjacent to the *uspF* and *ompN* genes in *Salmonella* (fig. 5). It is likely that these sRNA segments are not transcribed and are not functional in *Salmonella* (supplementary fig. S4, Supplementary Material online, and Kroger et al. 2013) because nucleotide substitutions in the putative CRP- and FNR-binding regions (supplementary fig. S6, Supplementary Material online) have rendered them inactive. The reintroduction of *EcsR1* into *Salmonella* did not affect biofilm production but instead triggered the increased expression of several virulence genes, particularly those within the SPI-1 pathogenicity island (supplementary table S7, Supplementary Material online). Multiple factors, including the biofilm machinery, are known to regulate the expression of these invasion-associated genes (Fabrega and Vila 2013), suggestive of links between the different phenotypes produced by this sRNA in *E. coli* and *Salmonella*. Additional experiments are necessary to understand how *EcsR1* induces diverse phenotypes in the two species; nevertheless, our findings demonstrate the potential of sRNAs to influence bacterial adaptation and evolution.

In addition to losing the biofilm-reducing sRNA (*EcsR1*), *Salmonella* has gained, again by a rearrangement event, an IGR that contains an sRNA (SesR2) that is not present in other enteric species. Because none of the corresponding regions in *E. coli* displays any appreciable transcript production, the evolution of this new sRNA in *Salmonella* also required the de novo formation of a new promoter sequence (fig. 3 and supplementary fig. S3, Supplementary Material online). Multiple factors, including the biofilm machinery, are known to regulate the expression of these invasion-associated genes (Fabrega and Vila 2013), suggestive of links between the different phenotypes produced by this sRNA in *E. coli* and *Salmonella*. Additional experiments are necessary to understand how *EcsR1* induces diverse phenotypes in the two species; nevertheless, our findings demonstrate the potential of sRNAs to influence bacterial adaptation and evolution.
complementary to the 5′-UTR of STM14_1869 (TSS of STM14_1869 is located 63 bp upstream of coding region), it could be functioning as an antisense RNA to regulate STM14_1869 expression, as shown previously for other genes in Salmonella (Lee and Grosman 2010).

In bacteria, differences in protein-coding gene contents between closely related species are either due to new genes that arose by gene duplication or HGT (Lerat et al. 2005; Blount et al. 2012; Nasvall et al. 2012), or due to gene loss through pseudogenization and deletion (Mira et al. 2001; Kuo and Ochman 2010). Although the mechanisms that shape bacterial sRNA gene repertoires are not well understood, duplication, deletion, and HGT have also been attributed to this process (Gottesman and Storz 2011). In this report, we show that genome rearrangements that create and disrupt IGRs can result in the gain or loss of sRNA genes in bacteria. Because sRNAs regulate myriad metabolic processes, this disparity in sRNA repertoires between closely related bacteria might also contribute to niche adaptation and speciation events.

**Supplementary Material**

Supplementary figures S1–S6 and tables S1–S7 are available at Genome Biology and Evolution online (http://www.gbe.oxfordjournals.org/).

**Acknowledgments**

The authors thank Cindy Barlan, Daniel Phillips, Todd Smith and Abraham Moses for technical help, and Kim Hammond for assistance with figures. This work was supported by Portland State University and by grants from the National Institute of Health to H.O.

**Literature Cited**


Associate editor: Ruth Hershberg