An Integrated Approach for Finding Overlooked Genes in *Shigella*

Junping Peng, Jian Yang, Qi Jin*

State Key Laboratory for Molecular Virolology and Genetic Engineering, Institute of Pathogen Biology, Chinese Academy of Medical Sciences/Peking Union Medical College, Beijing, China

Abstract

**Background:** The completion of numerous genome sequences introduced an era of whole-genome study. However, many genes are missed during genome annotation, including small RNAs (sRNAs) and small open reading frames (sORFs). In order to improve genome annotation, we aimed to identify novel sRNAs and sORFs in *Shigella*, the principal etiologic agents of bacillary dysentery.

**Methodology/Principal Findings:** We identified 64 sRNAs in *Shigella*, which were experimentally validated in other bacteria based on sequence conservation. We employed computer-based and tiling array-based methods to search for sRNAs, followed by RT-PCR and northern blots, to identify nine sRNAs in *Shigella flexneri* strain 301 (Sf301) and 256 regions containing possible sRNA genes. We found 29 candidate sORFs using bioinformatic prediction, array hybridization and RT-PCR verification. We experimentally validated 557 (57.9%) DOOR openon predictions in the chromosomes of Sf301 and 46 (76.7%) in virulence plasmid. We found 40 additional co-expressed gene pairs that were not predicted by DOOR.

**Conclusions/Significance:** We provide an updated and comprehensive annotation of the *Shigella* genome. Our study increased the expected numbers of sORFs and sRNAs, which will impact on future functional genomics and proteomics studies. Our method can be used for large scale reannotation of sRNAs and sORFs in any microbe with a known genome sequence.

Introduction

Genome sequence information has accumulated at a fast pace in recent years. The generation of whole genome sequences creates new opportunities and resources for both basic and applied research. A complete understanding of an organism’s biology depends largely on the accuracy and completeness with which it is annotated. In spite of tremendous advances in gene-finding programs, we are still a long way from thorough and robust annotations for sequenced genomes. A major problem is that many genes have been overlooked, including noncoding RNAs (ncRNAs) and small open reading frames (<100 amino acids; sORFs).

There has been considerable recent interest in ncRNAs, other than ribosomal RNAs (rRNAs) and transfer RNAs (tRNAs), as important regulators in eukaryotes and prokaryotes[1,2,3,4,5]. These RNAs are collectively referred to as small RNAs (sRNAs) in bacteria where they usually regulate gene expression by pairing with other RNAs as part of RNA-protein complexes, or adopt the structures of other nucleic acids [2,6]. sRNAs lack primary sequence common statistical signals that might be exploited by reliable detection algorithms. Thus, the genome-wise annotation of sRNAs has turned out to be a more complex and demanding problem than one expected. In recent years, new bioinformatics and experimental strategies have identified a greater number of novel sRNA candidates in bacteria, including, *Escherichia coli* [7,8,9,10,11,12], *Vibrio cholerae*[13,14,15], *Staphylococcus aureus*[16], *Clostridium perfringens* [17,18], *Chlamydia trachomatis*[19], *Pseudomonas aeruginosa*[20,21], *Bacillus subtilis*[22,23], *Listeria monocytogenes*[24,25], *Salmonella typhimurium*[26,27,28], *Streptococcus pyogenes*[29], *Streptococcus pneumoniae*[30,31], *Myxobacterium tuberculosis*[32], and many others. At present, ~150 bacterial sRNAs have been identified by systematic screens, direct labeling and functional genetic screens[3]. However, the function of the majority of these sRNAs is still unknown. The potential role of sRNA genes in pathogenic bacterial virulence has yet to be clarified.

Bacterial genes average ~1000 nucleotides in sequenced genomes. Annotation of sORFs is difficult, because they are “buried” in an enormous pile of short random open reading frames (ORFs), which, makes them unfavorable targets for random mutagenesis[33]. To maintain a balance between underprediction and overprediction, we usually adopt certain arbitrary cut-offs for gene prediction, such as a 100 codon minimum ORF length. This means that many sORFs are...
not identified, including many with important functions, such as intercellular signals, intracellular toxins, and kinase inhibitors. Systematic analysis of the prevalence of sORFs had been performed in yeast [33,34] and E. coli [35,36] and results show that numerous sORFs were overlooked in initial annotation.

*Shigella* species are Gram negative, non-sporeforming, facultative anaerobes that cause bacillary dysentery, a disease which remains a major worldwide health problem. They are subdivided into four species: *Shigella dysenteriae*, *Shigella flexneri*, *Shigella boydii*, and *Shigella sonnei*. However, multilocus enzyme electrophoresis, multilocus sequence typing, and comparative genomic hybridization suggest that *Shigella* diverged from *E. coli* in several independent events, which means it may not constitute a separate genus [37,38,39,40]. Results from several Shigella genome sequencing projects suggest that many sRNAs and sORFs were overlooked during initial annotation [41,42,43,44,45]. Huang et al. reported that the number of sRNA genes in *S. dysenteriae*, *S. flexneri*, *S. boydii*, and *S. sonnei* were 33, 40, 35, and 38, respectively [46]. However, these results were incomplete. The majority were identified in *E. coli* K12, based on conservation, meaning that sRNAs unique to Shigella were missed. Therefore, we performed a systematic analysis of sRNAs in *Shigella*.

No previous reports exist of global experimental approaches for sRNA and sORF identification in the *Shigella*. Here we present a combined bioinformatic and experimental approach for finding sRNAs and sORFs in *Shigella*. Our search for sRNAs contained four steps. We conducted an initial genomic screen for sRNA candidates in the *Shigella* genome using existing sRNA sequences. We then performed de novo prediction using RNAs, which proved to be an efficient method for detecting sRNAs [47,48]. Our next step was to identify conserved intergenic regions and anti-sense strands of coding sequences. We developed an orthogonal approach to in silico primary sequence analysis that was based on high density oligonucleotide probe arrays, which interrogated both strands of the *S. flexneri* strain 301 (*Sf*301) genome. We interrogated both strands of a genomic sequence using one array, which obtained valuable information on possible antisense gene regulation and provided the basis for a more accurate understanding of gene translation. We concluded the analysis by performing northern blots and RT-PCRs to validate our findings. We also performed bioinformatic prediction, array hybridization and RT-PCR verification for sORFs.

**Results**

**Known sRNAs in Shigella**

Only one sRNA, RnaG, is known from the virulence plasmid (VP) of *Shigella* [49]. We conducted a comparative genomics-based search for sRNAs identified in other bacteria. Based on sequence conservation, we identified 63 other sRNAs in *Shigella* which were experimentally validated (sRNAs were documented by Northern analysis, as shown in Table S1). Sixty were identified in *E. coli* and the remaining three were verified in the pathogens *S. typhimurium* and *P. aeruginosa*. All 63 sRNAs were encoded by chromosomal DNA, where genesize ranged from 50–500 nucleotides. We identified sRNA functional categories, including TPP riboswitch, FMN riboswitch, putative endoribonuclease, bacterial signal recognition particle RNA, tmRNA, 6S RNA, and other functions. Hfq is one of the most abundant RNA-binding proteins in bacteria. Twenty-one *Shigella* sRNAs are known to bind Hfq and are likely to act by base pairing.

**Candidate sRNAs in Shigella**

We used the program RNAz to predict regions encoding conserved RNA secondary structure, on the basis of BLAST sequence alignments between noncoding regions of six *Shigella* genomes. We focused our attention on sequences most likely to encode sRNAs, by excluding regions containing tRNAs, rRNAs, and transposase remnants. We also excluded segments which were conserved directly adjacent to the start of flanking coding genes, i.e., within 40 nt. We identified the corresponding sRNAs in *Sf*301.

Many sRNAs are likely to be transcribed only under specific conditions, so we increased the probability of discovery of these sRNAs with our screening approach. We performed expression profile analysis in five different conditions using a tiling array, in which we excluded repetitive regions and small untranslated regions (UTRs) from our analysis. The sixty four confirmed sRNAs, previously mentioned, were used as controls. We detected 52 sRNAs (81.3%) using RNAz and 41 (64.1%) by array analysis. We identified 55 (54.7%) by both RNAz and array analysis, and 58 (90.6%) by only one method. Earlier studies have reported the presence of rho-independent transcription terminators as evidence for the identification of sRNA[8]. Of the known sRNAs, 49 (76.6%) were predicted by their rho-independent transcription terminators. Giangrossi et al. recently reported RnaG, the first sRNA encoded by the VP of *S. flexneri*, which is transcribed in cis on the complementary strand of icsA and encodes an invasion protein[49]. We detected RnaG by both RNAz and tiling array analysis.

Based on the RNAz predictions and tiling array analyses, 238 and 18 regions were identified respectively as containing possible sRNAs genes (including known sRNAs) in chromosome and VP, as shown in Table S2. According to the sORF prediction, these regions did not appear to encode small peptides. We could not accurately identify the exact transcription start/end sites for candidate sRNA, because our tiling array design had overlapping probes arranged at 25 bp intervals, which does not provide single nucleotide resolution. Thus, the start and end of sRNAs in Table S1 refers to the boundaries of transcriptionally active regions of candidate sRNAs. We verified the sRNAs we detected by tiling array analysis by conducting RT-PCR and detected 165 regions in the chromosome and 18 regions in the VP.

**Identified sRNAs**

We validated our sRNA predictions by northern blot analysis using 18 sequences (12 in the chromosome and 6 in the VP) detected by RNAz prediction, tiling array and rho-independent terminators. We successfully identified transcripts corresponding to sRNAs in nine different intergenic regions. We designated these regions as ‘psrr’ for plasmid-encoded *Shigella* small RNA, and ‘cssr’ for chromosome-encoded *Shigella* small RNA. Table 1 shows novel sRNAs which we predicted to be synthesized from their own transcription initiation sites, which were not predicted to code for proteins using the Glimmer, RBSfinder and GeneMark.hmm ORF prediction algorithms. The sRNA 3’ boundaries are based on rho-independent terminator predictions. Northern blot analysis indicates that the size of the sRNAs ranged from 90–340 nucleotides (Figure 1).

**Candidate sORFs**

We constructed a database of predicted *Sf*301 sORFs using three bioinformatics prediction software programs (data not shown). We excluded ORFs less than 25 amino acids in length and any insertion sequence-related ORFs. We performed tiling array analysis to identify overlooked sORFs in regions previously considered to be intergenic and detected 20 novel sORF candidates located within regions of the *Sf*301 chromosome and 9 in the VP. The size of these sORFs ranged from 28 to 94 codons, including start and stop codons, as shown in Table 2. We
successfully verified all sORFs detected by tiling array analysis using RT-PCR. We performed BLASTX searches for functional annotation against the nonredundant protein database of the NCBI. We found that four newly identified sORFs were not annotated in any genome and four sORFs were only annotated in one E.coli or Shigella strain.

### Table 1. Summary of newly confirmed sRNAs in chromosome and virulence plasmid of *Shigella flexneri* strain 301.

| sRNA genes | Adjacent genes | Strand* | Northern size | 5’ endb | 3’ endb | Methodc |
|------------|----------------|---------|---------------|---------|---------|---------|
| pssrA      | CP0121/ipaJ    | ←→      | ~90           | ~103842 | 103931  | R/M/P   |
| pssrB      | virG/CP0183    | →←      | ~200          | ~152821 | 153020  | R/M/P   |
| cssrA      | map/rpsB       | ←→      | ~110          | ~181629 | 181738  | R/M/P   |
| cssrB      | SF2021/SF2022  | →←      | ~180          | ~2046404| 2046225 | R/M/P   |
| cssrC      | SF2042/SF2043  | ←→      | ~340          | ~2064237| 2063898 | R/M/P   |
| cssrD      | rpsP/ffh       | ←→      | ~200          | ~2745060| 2744861 | R/M/P   |
| cssrE      | yggN/yggL      | ←→      | ~140          | ~3043882| 3043743 | R/M/P   |
| cssrF      | dacB/yhbZ      | ←→      | ~290          | ~3322880| 3322591 | R/M/P   |
| cssrG      | rbsB/rbsK      | ←→      | ~230          | ~3946524| 3946755 | R/M/P   |

*aThe middle arrow represents the sRNA gene, while the flanking arrows indicate the orientation of the adjacent genes, respectively. Genes present on the strand given in the *S. flexneri* strain 301 genome database are indicated by (→), and genes present on the complementary strand are indicated by (←-).

*bThe sRNA 3’ boundaries are from rpo-independent terminator predictions. 5’ boundaries are calculated according to the 3’-ends and northern results.

*cSRNAs were predicted based on different methods. R, RNAz prediction; M, tiling array hybridization; P, RT-PCR verification.

doi:10.1371/journal.pone.0018509.t001

**Figure 1. Detection of small RNAs by Northern blot analyses.** Northern blots were performed with total RNA using strand-specific probes as described in Materials and Methods. The size of RNA markers is indicated on the left. 5s RNA was used as control.

doi:10.1371/journal.pone.0018509.g001
Identification of operon structures

An operon is a series of genes which is co-transcribed in the same transcription unit. Bacterial genes involved in similar functions are often organized into operon structures. DOOR predictions suggested that there were 962 operons in chromosome of Sf301 and 60 in the VP [50]. Table S3 shows that we experimentally validated 557 (57.9%) DOOR operon predictions in the chromosome of Sf301 and 46 (76.7%) in the VP. Table S4 shows 40 additional coexpressed gene-pairs that were not predicted by DOOR. For example, DOOR predicted that operon 75143 in Sf301 was a three gene operon (SF3763–SF3765), but tiling analysis showed that the operon had four genes (SF3762–SF3765) with the inclusion of SF3762. DOOR predictions for a similar operon in E.coli K12 MG1655 matched our result. Table S5 shows predicted operon structures that need to be reanalyzed. Of these, 95 operons contained genes encoding a hypothetical protein. For example, DOOR predicted that operon 74376 in Sf301 was a five gene operon (SF0040-SF0044). However, our results indicated that the operon should be divided into two parts. Thus, our experiment data might assist in increasing the accuracy of operon annotation.

Discussion

We published the first Shigella genome (Sf301) in 2002[44]. In our initial annotation, we identified 449 sORFs in the chromosome and 76 in the VP, with ten sRNAs identified based on conservation. Recently, we characterized four novel sORFs by integrating a shotgun proteomics method with oligonucleotide array analysis [51]. Here we report the first comprehensive screen for sRNAs and sORFs in Shigella, using a combination of bioinformatics and experimental approaches. This is the first genome-wide expression profile of S. flexneri genes, pseudogenes, and noncoding regions, which can be used as a basis for the screening of overlooked genes. Tiling array analysis provided further information on expression patterns in different growth phases.

The first bacterial genome was sequenced in 1995 and approximately 1000 completed microbial genomes are now available [52]. The number of sRNAs and sORFs that have been identified is rapidly increasing. The use of bioinformatics and experimental approaches provides a powerful tool for screening these genes.

Table 2. Summary of candidate sORFs in chromosome and virulence plasmid of Shigella flexneri strain 301.

| ID         | Location       | Length (amino acids) | Strand | Description                                           |
|------------|----------------|----------------------|--------|-------------------------------------------------------|
| BIO00004   | 15610–15401    | 70                   | –      | regulatory protein mokC                                |
| BIO00051   | 259932–259741  | 64                   | −      | hypothetical protein                                   |
| BIO00126   | 511407–511556  | 50                   | +      | putative small toxic membrane polypeptide             |
| BIO00127   | 511910–512059  | 50                   | +      | putative small toxic membrane polypeptide             |
| BIO00144   | 583036–582926  | 37                   | −      | putative outer membrane lipoprotein, cyd operon protein |
| BIO00301*  | 1056382–1056486| 35                   | +      | hypothetical protein                                   |
| BIO00533*  | 1577495–1577376| 28                   | −      | hypothetical protein                                   |
| BIO00534b  | 1577818–1577543| 92                   | −      | hypothetical protein                                   |
| BIO00587b* | 1717264–1717148| 40                   | −      | hypothetical protein                                   |
| BIO00620   | 1809435–1809527| 31                   | +      | hypothetical protein                                   |
| BIO00669   | 1894482–1894333| 51                   | −      | hypothetical protein                                   |
| BIO00670   | 1894620–1894501| 40                   | −      | hypothetical protein                                   |
| BIO00790   | 2213607–2213521| 29                   | −      | hypothetical protein                                   |
| BIO00803   | 2238453–2238557| 35                   | +      | hypothetical protein                                   |
| BIO00855   | 2421445–2421317| 43                   | −      | hypothetical protein                                   |
| BIO00864   | 2469896–2469615| 94                   | −      | hypothetical protein                                   |
| BIO00898   | 2585789–2585685| 35                   | −      | hypothetical protein                                   |
| BIO00936   | 2769587–2769432| 52                   | −      | predicted membrane protein (regulated by cyaR sRNA)    |
| BIO01076   | 3201904–3202023| 40                   | +      | hypothetical protein                                   |
| BIO01336   | 4066446–4066339| 36                   | −      | hypothetical protein                                   |
| BIO01501b  | 9285–9443      | 53                   | +      | hypothetical protein                                   |
| BIO01567*  | 67854–68126    | 91                   | +      | hypothetical protein                                   |
| BIO01585   | 91670–91422    | 83                   | –      | hypothetical protein                                   |
| BIO01587b  | 91991–91860    | 44                   | +      | putative arylsulfatase regulatory protein             |
| BIO01595b  | 105022–105132  | 37                   | +      | hypothetical protein                                   |
| BIO01608   | 135447–135677  | 77                   | +      | hypothetical protein                                   |
| BIO01637   | 153138–153392  | 85                   | +      | adhesion protein, fragment                            |
| BIO01674   | 183288–183455  | 56                   | +      | hypothetical protein                                   |
| BIO01675   | 183646–183792  | 49                   | +      | hypothetical protein                                   |

*aNewly identified sORFs were not annotated in any genome.

*bThese sORFs were only annotated in one E.coli or Shigella strain.

doi:10.1371/journal.pone.0018509.t002
available in the public database (http://www.ncbi.nlm.nih.gov/ genomes/MICROBES/microbial_taxtree.html). Numerous prediction programs have been developed to address the problem of annotation. The main strategies used for genome annotation are mathematical models and algorithm-based computational analysis [52], EST/cDNA sequencing[53] and complete set of protein-encoding ORFs cloning[54]. High throughput next generation sequencing instruments have recently revolutionized genomics and genetics, but genome annotation is not keeping pace with the avalanche of raw sequence data. Many researchers are dedicated to bacterial genome annotation, but serious problems still exist. Many annotated genes found in public database are not protein coding genes, but rather ORFs that occur by chance, whereas many actual genes are missing, including sRNAs and sORFs.

The wealth of genomic sequences now available facilitates comparative sequence analysis, which might potentially identify important sequences that cannot be detected by analysis of individual genomes. Differences in bacteria genomes can reflect processes involved in strain adaptive variation under different natural selection, which can endow them with strain-specific biological traits [55]. Growing evidence suggests that gene acquisition via horizontal gene transfer has played an integral role in the evolution of bacterial genomes, and in the diversification and speciation of enteric bacteria. Shigella species have a lifestyle that is markedly different from that of closely related bacteria. It is widely accepted that the critical step for Shigella speciation was the acquisition of the ancestral form of VP [56]. The functional VP genome is ~220 Kbp in size and it is composed of a mosaic of virulence genes, maintenance genes, IS elements, and hypothetical genes. In addition to VP, several pathogenicity islands are known in the chromosome of Shigella spp.

Transcriptome analysis, using RNA sequencing and high resolution tiling arrays, is beneficial for improving the annotation of sequenced prokaryotic genomes [57]. Tiling array analysis has proved to be a powerful technology, now widely used in eukaryotes and prokaryotes to study transcriptional complexity and identify noncoding transcripts [12,31,58,59,60]. Tjaden et al. assayed the E. coli transcriptome under a range of conditions and identified multiple noncoding transcribed elements, including 5′-UTRs, 3′-UTRs, small RNA molecules, and operons [12]. Kumar et al. identified 50 sRNAs in the intergenic regions of the S. pneumoniae strain TIGR4 using tiling array, of which 36 had no predicted function [31].

A wide range of organisms possess ncRNAs, which have roles in a wide variety of processes, including, chromatin accessibility, activator/repressor binding and function, transcriptional initiation, transcription elongation, RNA processing and modification, messenger RNA stability, and translation [2]. Interest in bacterial sRNAs has been fuelled over the past few decades by the availability of numerous complete bacterial genome sequences, which has led to an explosion in the identification and characterization of sRNAs. However, sRNA identification by comparative genomics analysis is only applicable when sequences of several closely related species are available. Previous systematic screens for sRNAs were mainly conducted with the laboratory strain E. coli K12, which led to the identification of ~80 sRNA genes. We can only find sRNAs shared by pathogenic and nonpathogenic strains by comparisons based on conservation of sRNA sequences and structures. Thus, sRNAs unique to pathogenic strains are excluded.

It is now widely accepted that many sRNAs play central roles in gene expression regulation in response to environmental changes. Previous research shows that some sRNAs directly or indirectly regulate virulence genes, or affect adaptive stress responses that are important for bacterial survival in a host [61]. Several studies indicate that many sRNAs are involved in bacterial pathogenesis, including, RNAIII of S. aureus and CsrBCD of V. cholerae. These sRNAs adapt the expression of virulence genes to stress and metabolic requirements [62]. Padalon-Brauch et al. pointed out that genetic islands (foreign DNA segments) encoding sRNA genes play an important role in networks that regulate bacterial adaptation to environmental changes and stress conditions, thereby controlling virulence [63]. However, very little is known about Shigella sRNAs. Approximately 60 sRNAs are known, but the function of only two sRNAs (RnaG and RyhB) has been studied in Shigella. The S. flexneri virulence gene scd is critical for the intra- and inter-cellular spreading of the pathogen. This gene encodes an invasion protein, which induces host actin polymerization at one pole of the cell [64]. RnaG is transcribed in cis on the complementary strand of scd and regulates at the transcriptional level [49]. S. flexneri requires iron for survival and the genes for iron uptake and homeostasis are regulated by the Fur protein. RyhB expression is repressed by Fur. Oglesby et al. showed that the acid sensitivity defect of the S. flexneri fur mutant is due to RyhB repression of ydkP, which encodes a putative oxidoreductase [65]. Murphy & Payne found that RyhB can repress many virulence genes, including those encoding the type III secretion apparatus, secreted effector proteins, and specific chaperones. This phenomenon occurs via RyhB-dependent repression of the transcriptional activator VirB and iron is implicated as an environmental factor contributing to the complex regulation of Shigella virulence determinants [66].

We have identified and validated nine novel sRNAs in Shigella by combining sRNA identification with tiled microarray probe correlation analysis, transcriptional terminator prediction, and northern blot analysis, but the function of these sRNAs requires further analysis. We also detected 29 novel sORF candidates in S. flexneri and BLASTX indicated that most encoded hypothetical proteins. We performed more detailed analysis to elucidate the functions of these translated products. Several sRNAs were annotated in genomes based on bioinformatics predictions, but for the first time our results provide support at the transcriptional level. Identification of operon structures is critical for understanding coordinated regulation of bacterial transcriptome, which means that successful identification of operon structures can assist in the functional annotation of hypothetical genes, because proteins encoded by genes in the same operon often have related functions, or share biological pathways[50]. We found that identification of co-expression patterns by tiling array experiments was helpful in operon prediction.

Our approach for global identification of sRNAs and sORFs is applicable to any sequenced microbial species and will accelerate and refine genome annotation and gene identification. Methods for finding sRNAs and sORFs, including computational prediction and experimental validation, are available and continue to develop, but they still fail to provide complete annotation. Our mapping and initial characterization of sRNAs throughout the Shigella genome provides significant impetus to the study of these molecules as potential regulators of virulence in Shigella and related pathogens.

Materials and Methods

Genome sequences

Sequence data of six Shigella strains (including VP) were downloaded from GenBank. Accession numbers for the chromosomes are: CP000034, AE005674, AE014073, CP000266, CP000036, and CP000038. Accession numbers for the VPs are: CP000035, AF386526, CP000037, CP000039, AF348706, and AL391753.
**Bioinformatics screening**

Known sRNA sequences were extracted from the sRNAMap and Rfam [46,67] and subjected to BLAST analysis against all sequences mentioned above. We used multiZ to produce a multiple alignment of six chromosomes and VP sequences which were passed on to the RNAz pipeline, according to the manual (cut-off value, P = 0.9). Rho-independent terminators were predicted as previously described in Kingsford et al [68]. Putative sRNA sequences, including a 50 base pair upstream region, were used for promoter prediction with the Neural Network Promoter Prediction program (http://www.fruitfly.org/seq_tools/promoter.html). BLASTN searches were performed against the nonredundant nucleotide database of NCBI to determine newly identified sRNA sequence conservation from other genomes. sORFs (25–100 amino acids) were predicted using Glimmer, RBSfinder, and GeneMark.hmm, using default parameters [69,70,71]. BLASTX searches were performed against the nonredundant protein database of NCBI, for functional annotation.

**Strain and culture conditions**

Shigella flexneri S/301 was cultured overnight at 37°C on Luria-Bertani (LB) agar containing 0.01% Congo red. A single red colony was inoculated into LB medium, without antibiotics, and grown overnight at 37°C and mixed at 250 rpm. An overnight culture of bacteria was prepared for RNA extraction by diluting 1:50 in 100 ml of fresh medium with aeration by rotary shaking (250 rpm). Growth (optical density, OD) was monitored at 600 nm using an Ultraspec 2000 spectrophotometer (Pharmacia Biotech, Sweden). Cells were harvested in different conditions, as follows: at 37°C in LB medium, in three different growth phases, i.e., lag (OD600<0.2), log (0.2<OD600<1.0), and stationary (OD600>1.0); at 37°C in LB medium with 0.01% Congo red in the log and stationary phases.

**RNA isolation, cDNA synthesis, and cDNA labeling**

Total RNA was isolated using a Promega SV total RNA purification kit, according to the manufacturer’s instructions. The concentration and purity of RNA were determined using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, USA). Purity and integrity were confirmed by agarose gel electrophoresis. Contaminating genomic DNA was removed from RNA samples via four 30 min incubations at 37°C with 2 ml of Turbo DNase-free, and DNA removal was verified by PCR. cDNA synthesis and labeling was performed following the direct labelling RNA protocol of the IFR microarray facility (www.ifr.ac.uk/safety/microarrays/protocols). Test samples were fluorescently labeled with Cy5-dCTP (GE Healthcare, USA). Separate labeling reactions were pooled after each respective Cy dye incorporation step and then again divided into aliquots to minimize inconsistencies in probe generation. cDNA was purified with a QIAquick PCR purification kit (Qiagen, Germany), according to the QIAquick spin handbook.

**Chip design, hybridization and data analysis**

We used a custom-made tiling array containing 386144 probes of the Shigella flexneri S/301 genome (NimbleGen Systems, USA) for transcriptomics study. Probes were designed with overlapping probes arranged at 25 bp intervals to represent both DNA strands equally and to be unbiased toward ORFs and/or intergenic regions. Labeled cDNA samples were individually hybridized to the microarray, according to the NimbleGen standard operating procedure. Competitive hybridization was conducted three times for each sample under each test condition. Microarrays were scanned at a 5 μm resolution using a GenePix 4000B scanner (Axon Instruments, CA, USA). Data were extracted using NimbleScan (NimbleGen Systems, USA). Extracted microarray data were analyzed by using NMPP, a user-customized NimbleGen microarray data processing pipeline [72].

We used signals from 280 nonmatching probes, which did not match any region of the genome intentionally placed on our array, to estimate the background level and determine whether a gene was expressed. A gene was considered expressed if its average expression level was greater than five-fold more than the nonmatching probes. All data produced was MIAME compliant and the raw data has been deposited in the Gene Expression Omnibus (GEO) under accession number GSE22800.

**Reverse transcription-PCR (RT-PCR)**

We verified sRNA and sORF candidates using a variation of the reverse transcription-PCR (RT-PCR) procedure. We added a primer complementary to the predicted mRNA and reverse transcriptase. After first-strand cDNA synthesis, the reverse transcriptase was inactivated with heat before we added Taq polymerase, and sRNA-specific primers, and sORF-specific primers. PCR products were analyzed using the Agilent 2100 bioanalyzer (Agilent technologies, USA). We observed PCR products under these conditions only when first strand synthesis was conducted with primers complementary to the predicted mRNA. We used the same RNA in the PCR reaction and a negative control to test for genomic contamination.

**Northern blot hybridization**

We performed northern blot analysis to verify that sRNAs were transcribed. A total of 18 candidate sRNAs were tested by northern blotting. Table S6 shows the probes used in northern blot study. Total RNA (20 μg per lane) was separated by electrophoresis in an 8% polyacrylamide gel, containing 8 M Urea, and transferred to a nylon membrane by electroblotting. RNAs were cross-linked to the membrane by exposure to UV light. The membranes were hybridized with gene-specific 32P end-labeled oligonucleotides, and hybridization signals were visualized using a PhosphorImager (Molecular Dynamics, USA).

**Operons**

Two or more consecutive genes were regarded as part of an operon, if they fulfilled the following criteria: (a) they are expressed and transcribed in same direction, and (b) the intergenic region was identified as a single expressed transcript that overlapped the genes in both directions.

**Supporting Information**

**Table S1** Summary of confirmed sRNAs in Chromosome and virulence plasmid of *Shigella flexneri* (XLS)

**Table S2** List of regions (including known sRNAs) which were identified as containing possible sRNAs genes in chromosome and VP (XLS)

**Table S3** List of confirmed operon predictions (XLS)

**Table S4** List of newly identified co-expressed genes (XLS)
Table S5 List of operon structures need to be re-predicted

Table S6 Probes used in northern blot study

References

1. Huttenhofer A, Schatter P, Poleasek N (2005) Non-coding RNAs: hope or hype? Trends Genet 21: 291–297.
2. Storz G, Altuvia S, Wassarman KM (2005) An abundance of RNA regulators. Annu Rev Biochem 74: 199–217.
3. Livny J, Walder MK (2007) Identification of small RNAs in diverse bacterial species. Curr Opin Microbiol 10: 96–101.
4. Ponting CP, Oliver PL, Reik W (2009) Evolution and functions of long noncoding RNAs. Cell 136: 629–641.
5. Waters LS, Storz G (2009) Regulatory RNAs in bacteria. Cell 136: 615–628.
6. Majdalani N, Vanderpool CK, Gottman S (2005) Bacterial small RNA regulators. Crit Rev Biochem Mol Biol 40: 93–113.
7. Wassarman KM, Repola F, Roseau C, Storz G, Gottman S (2001) Identification of novel small RNAs using comparative genomics and microarrays. Genes Dev 15: 1637–1651.
8. Argaman L, Hershelg R, Vogel J, Bejerano G, Wagner EG, et al. (2001) Novel small RNA-m RNA-regulatory genes in the intergenic regions of Escherichia coli. Curr Biol 11: 941–950.
9. Rivas E, Klein RJ, Jones TA, Eddy SR (2001) Computational identification of noncoding RNAs in E. coli by comparative genomics. Curr Biol 11: 1369–1373.
10. Hall TA, Sampath R, Griffey RH, et al. (2002) A computational approach to identify genes for functional RNAs in genomic sequences. Nucleic Acids Res 29: 3928–3938.
11. Chen S, Lesnik EA, Hall TA, Sampaith R, Grifey RH, et al. (2002) A bioinformatics-based approach to discover small RNA genes in the Escherichia coli genome. Biosystems 65: 157–177.
12. Tjaden B, Saxena RM, Stoylov S, Haynor DR, Kolker E, et al. (2002) Transcriptome analysis of Escherichia coli using high-density oligonucleotide microarrays. Nucleic Acids Res 30: 3732–3739.
13. Livny J, Fogel MA, Davis BM, Waldor MK (2005) sRNAPredict: an integrative computational approach to identify tRNAs in bacterial genomes. Nucleic Acids Res 33: 4106–4109.
14. Liu JM, Livny J, Lawrence MS, Kimball MD, Waldor MK, et al. (2009) Experimental discovery of sRNAs in Vibrio cholerae by direct cloning, 5′-3′tRNA depletion and parallel sequencing. Nucleic Acids Res 37: e46.
15. Song T, Mika F, Lindmark B, Liu Z, Schild S, et al. (2008) A new Vibrio cholerae tRNA-RNA modules colonization and affects release of outer membrane vesicles. Mol Microbiol 70: 100–111.
16. Pichon C, Felden B (2005) Small RNA genes expressed from Staphylococcus aureus genomic and pathogenicity islands with specific expression among pathogenic strains. Proc Natl Acad Sci U S A 102: 14249–14254.
17. Ohtani K, Bhoomik SK, Hayashi H, Shimizu T (2002) Identification of a novel locus that regulates expression of toxin genes in Clostridium perfringens. FEMS Microbiol Lett 209: 113–118.
18. Shimizu T, Yaguchi H, Ohtani K, Imano S, Hayashi H (2002) Clostridial VrR-VrR regulon involves a regulatory RNA molecule for expression of toxins. Mol Microbiol 43: 257–265.
19. Grieshaber NA, Grieshaber SS, Fischer ER, Hackstadt T (2006) A small RNA inhibits translation of the histone-like protein Hc1 in Chlamydia trachomatis. J Bacteriol 188: 352–354.
20. Saito S, Kakeshita H, Nakamura K (2009) Novel small RNA-encoding genes in the invasion gene island (SPI-1) represses outer membrane protein C5. Mol Microbiol 73: 257–265.
21. Perez N, Trevino J, Liu Z, Ho SC, Babitzke P, et al. (2009) A genome-wide analysis of small regulatory RNAs in the human pathogen group A streptococcus. PLoS ONE 4: e7668.
22. Tsai HC, Mukherjee D, Ray VA, Sham LT, Feig AL, et al. (2010) Identification and characterization of noncoding small RNAs in Streptococcus pneumoniae serotype 2 strain D39. J Bacteriol 192: 264–279.
23. Kumar R, Shah P, Swain E, Burgeson RE, Lawrence ML, et al. (2010) Identification of novel noncoding small RNAs from Streptococcus pneumoniae TIGR4 using high-resolution genome tiling arrays. BMC Genomics 11: 350.
24. Arnvig KB, Young DB (2009) Identification of small RNAs in Mycobacterium tuberculosis. Mol Microbiol 73: 397–408.
25. Kastenmayer JP, Ni L, Liu A, Kitchen LE, Au WC, et al. (2006) Functional genomics of genes with small open reading frames (sORFs) in S. cerevisiae. Genome Res 16: 365–373.
26. Kumar A, Harrison FM, Cheung KH, Lan E, Nicholls N, et al. (2002) An integrated approach for finding overlooked genes in yeast. Nat Biotechnol 20: 58–63.
27. Hobbs EG, Astafr JL, Storz G (2010) Small RNAs and small proteins involved in resistance to cell envelope stress and acid shock in Escherichia coli: analysis of a bar-coded mutant collection. J Bacteriol 192: 59–67.
28. Hemm MR, Paul RJ, Miranda-Rios J, Zhang A, Soltanzad N, et al. (2010) Small RNA-encoding genes in the intergenic regions of Escherichia coli genome. Bioinformatics 26: i360–i367.
29. Perez J, Zhang X, Yang J, Wang L, Yang E, et al. (2006) The use of comparative genomic hybridization to characterize genome dynamics and diversity among the serotypes of Shigella. BMC Genomics 7: 218.
30. Yang J, Niu H, Chen L, Zhang X, Yang F, et al. (2007) Revisiting the molecular evolutionary history of Shigella spp. J Mol Evol 64: 71–79.
31. Pupo GM, Lan R, Reeves PR (2000) Multiple independent origins of Shigella clones of Escherichia coli and convergent evolution of many of their characteristics. Proc Natl Acad Sci U S A 97: 10567–10572.
32. Pupo GM, Kaczals K, Lan R, Reeves PR (1997) Evolutionary relationships among pathogenic and nonpathogenic Escherichia coli strains inferred from multilocus enzyme electrophoresis and multisequence studies. Infect Immun 65: 2687–2692.
33. Niu H, Yang F, Zhang Y, Jiang C, Chen L, et al. (2006) Complete genome sequence of Shigella flexneri 5b and comparison with Shigella flexneri 2a. BMC Genomics 7: 173.
34. Yang F, Jiang C, Zhang X, Chen L, Jiang Y, et al. (2005) Genome dynamics and diversity of Shigella species, the etiologic agents of bacillary dysentery. Nucleic Acids Res 33: 6443–6456.
35. Wei J, Goldberg MB, Burtles V, Venkatesan MM, Deng W, et al. (2003) Complete genome sequence and comparative genomics of Shigella flexneri serotype 2a strain 2457T. Infect Immun 71: 2775–2786.
36. Hemm MR, Paul RJ, Miranda-Rios J, Zhang A, Soltanzad N, et al. (2010) Small stress response proteins in Escherichia coli: protein misled by classical prokaryotic studies. J Bacteriol 192: 46–50.
37. Peng J, Zhang X, Yang J, Wang L, Yang E, et al. (2006) The use of comparative genomic hybridization to characterize genome dynamics and diversity among the serotypes of Shigella. BMC Genomics 7: 218.
38. Yang J, Nue H, Chen L, Zhang X, Yang F, et al. (2007) Revisiting the molecular evolutionary history of Shigella spp. J Mol Evol 64: 71–79.
39. Pupo GM, Kaczalski DK, Lan R, Reeves PR (1997) Evolutionary relationships among pathogenic and nonpathogenic Escherichia coli strains inferred from multilocus enzyme electrophoresis and multisequence studies. Infect Immun 65: 2687–2692.
40. Niu H, Yang F, Zhang Y, Jiang C, Chen L, et al. (2006) Complete genome sequence of Shigella flexneri 5b and comparison with Shigella flexneri 2a. BMC Genomics 7: 173.
41. Yang F, Jiang C, Zhang X, Chen L, Jiang Y, et al. (2005) Genome dynamics and diversity of Shigella species, the etiologic agents of bacillary dysentery. Nucleic Acids Res 33: 6443–6456.
42. Wei J, Goldberg MB, Burtles V, Venkatesan MM, Deng W, et al. (2003) Complete genome sequence and comparative genomics of Shigella flexneri serotype 2a strain 2457T. Infect Immun 71: 2775–2786.
43. Hemm MR, Paul RJ, Miranda-Rios J, Zhang A, Soltanzad N, et al. (2010) Small stress response proteins in Escherichia coli: protein misled by classical prokaryotic studies. J Bacteriol 192: 46–50.
44. Peng J, Zhang X, Yang J, Wang L, Yang E, et al. (2006) The use of comparative genomic hybridization to characterize genome dynamics and diversity among the serotypes of Shigella. BMC Genomics 7: 218.
45. Yang J, Nue H, Chen L, Zhang X, Yang F, et al. (2007) Revisiting the molecular evolutionary history of Shigella spp. J Mol Evol 64: 71–79.
53. Wang L, Ma L, Leng W, Liu T, Yu L, et al. (2006) Analysis of the dermatophyte Trichophyton rubrum expressed sequence tags. BMC Genomics 7: 255.

54. Matsuyama A, Arat R, Yasihorda Y, Shiiri A, Kamata A, et al. (2006) ORFeome cloning and global analysis of protein localization in the fusion yeast Schizosaccharomyces pombe. Nat Biotechnol 24: 841–847.

55. Peng J, Yang J, Jin Q (2009) The molecular evolutionary history of Shigella spp. and enteroinvasive Escherichia coli. Infect Genet Evol 9: 147–152.

56. Sansonetti PJ, Kopecko DJ, Formal SB (1982) Involvement of a plasmid in the invasive ability of Shigella flexneri. Infect Immun 35: 852–860.

57. Sorek R, Cossart P (2010) Predatory transcriptomics: a new view on regulation, physiology and pathogenicity. Nat Rev Genet 11: 9–16.

58. He H, Wang J, Liu T, Liu XS, Li T, et al. (2007) Mapping the C. elegans noncoding transcriptome with a whole-genome tiling microarray. Genome Res 17: 1471–1477.

59. David L, Huber W, Granovskiia M, Troedling J, Palm CJ, et al. (2006) A high-resolution map of transcription in the yeast genome. Proc Natl Acad Sci U S A 103: 5326–5325.

60. Akama T, Suzuki K, Tanigawa K, Kawashima A, Wu H, et al. (2009) Whole-genome tiling array analysis of Mycobacterium leprae RNA reveals high expression of pseudogenes and noncoding regions. J Bacteriol 191: 3321–3327.

61. Romby P, Vandenesch F, Wagner EG (2006) The role of RNAs in the regulation of virulence-gene expression. Curr Opin Microbiol 9: 229–236.

62. Toledo-Arana A, Repoila F, Cossart P (2007) Small noncoding RNAs controlling pathogenesis. Curr Opin Microbiol 10: 182–188.

63. Padalon-Brauch G, Herschberg R, Elgrably-Weiss M, Baruch K, Rosenshine I, et al. (2008) Small RNAs encoded within genetic islands of Salmonella typhimurium show host-induced expression and role in virulence. Nucleic Acids Res 36: 1913–1927.

64. Bernardini ML, Mounier J, d’Hauteville H, Coquis-Rondon M, Sansonetti PJ (1989) Identification of icsA, a plasmid locus of Shigella flexneri that governs bacterial intra- and intercellular spread through interaction with F-actin. Proc Natl Acad Sci U S A 86: 3867–3871.

65. Oglesby AG, Murphy ER, Iyer VR, Payne SM (2005) Fur regulates acid resistance in Shigella flexneri via RyhB and ydeP. Mol Microbiol 56: 1354–1367.

66. Murphy ER, Payne SM (2007) RyhB, an iron-responsive small RNA molecule, regulates Shigella dysenteriae virulence. Infect Immun 75: 3470–3477.

67. Gardner PP, Daub J, Tate JG, Nasrrooki EP, Kolhe DL, et al. (2009) Rfam: updates to the RNA families database. Nucleic Acids Res 37: D136–140.

68. Kingford CL, Ayanbule K, Salzberg SL (2007) Rapid, accurate, computational discovery of Rho-independent transcription terminators illuminates their relationship to DNA uptake. Genome Biol 8: R22.

69. Delcher AL, Hamou D, Kasif S, White O, Salzberg SL (1999) Improved microbial gene identification with GLIMMER. Nucleic Acids Res 27: 4636–4641.

70. Suzek BE, Ermakleva MD, Schreiber M, Salzberg SL (2001) A probabilistic method for identifying start codons in bacterial genomes. Bioinformatics 17: 1123–1130.

71. Lukashin AV, Borodovsky M (1998) GeneMark.hmm: new solutions for gene finding. Nucleic Acids Res 26: 1107–1113.

72. Wang X, He H, Li I, Chen R, Deng XW, et al. (2006) NMPP: a user-customized NimbleGen microarray data processing pipeline. Bioinformatics 22: 2955–2957.