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A small non-coding RNA of the invasion gene island (SPI-1) represses outer membrane protein synthesis from the *Salmonella* core genome

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Summary

The *Salmonella* pathogenicity island (SPI-1) encodes ~35 proteins involved in assembly of a type III secretion system (T3SS) which endows *Salmonella* with the ability to invade eukaryotic cells. We have discovered a novel SPI-1 gene, *invR*, which expresses an abundant small non-coding RNA (sRNA). The *invR* gene, which we identified in a global search for new *Salmonella s*RNA genes, is activated by the major SPI-1 transcription factor, HilD, under conditions that favour host cell invasion. The RNA chaperone, Hfq, is essential for the *in vivo* stability of the ~80 nt InvR RNA. Hfq binds InvR with high affinity *in vitro*, and InvR co-immunoprecipitates with FLAG epitope-tagged Hfq in *Salmonella* extracts. Surprisingly, deletion/overexpression of *invR* revealed no phenotype in SPI-1 regulation. In contrast, we find that InvR represses the synthesis of the abundant OmpD porin encoded by the *Salmonella* core genome. As *invR* is conserved in the early branching *Salmonella bongori*, we speculate that porin repression by InvR may have aided successful establishment of the SPI-1 T3SS after horizontal acquisition in the *Salmonella* lineage. This study identifies the first regulatory RNA of an enterobacterial pathogenicity island, and new roles for Hfq and HilD in SPI-1 gene expression.

Introduction

The genomes of many bacterial pathogens contain clusters of genes that encode key virulence determinants, so-called pathogenicity islands (Dobrindt *et al*., 2004). In *Salmonella enterica* serovars, which cause a range of human diseases from gastro-enteritis to typhoid fever, the virulence factors that facilitate invasion of the host's intestinal epithelium are encoded by a ~40 kb region referred to as *Salmonella* pathogenicity island 1 (SPI-1). Originally described as the *inv/spa* genes (Galan and Curtiss, 1989), SPI-1 is an horizontally acquired cluster of ~35 protein-coding genes inserted between the *fhlA* and *mutS* loci, and not found in closely related enterobacterial genomes.

Salmonella pathogenicity island 1 is the best characterized of the five major pathogenicity islands (PAI) presently known in *S. enterica* serovar Typhimurium (*S. typhimurium*) (Hensel, 2004). It encodes a type III secretion system (T3SS) that mediates the translocation of effector proteins from *Salmonella* into mammalian cells (Kimbrough and Miller, 2002; Galan and Wolf-Watz, 2006). Genetic and biochemical analyses have assigned functions to most of the SPI-1-encoded proteins, either as components and secreted effectors of the T3SS apparatus, or as transcription factors that orchestrate SPI-1 expression (Lostroh and Lee, 2001; Galan and Wolf-Watz, 2006).

While traditional genome annotation and analyses have focused on protein-coding regions, genes for small non-coding RNAs (sRNAs) have long been overlooked. Such sRNAs are typically transcribed from intergenic, i.e. non-protein coding, regions of bacterial chromosomes (Vogel and Sharma, 2005). Recent genome-wide screens using diverse methodologies have identified ~80 sRNA genes in non-pathogenic *Escherichia coli* K12, many of which are conserved in *Salmonella* and related pathogenic enterobacterial species (Hershberg *et al*., 2003). More recently, sRNA genes were also identified in bacterial pathogens other than enterobacteria (Pichon and Felden, 2005; Christiansen *et al*., 2006; Livny *et al*., 2006; Mandin *et al*., 2007). Where characterized in detail, the majority of the sRNAs were found to act as antisense RNAs on *trans*-encoded target mRNAs to modulate their translation and/or stability (Storz *et al*., 2004). Pairing to target mRNAs is typically mediated by short, imperfect stretches of complementarity and requires the bacterial Sm-like protein Hfq (Valentin-Hansen *et al*., 2004).

The sRNAs play diverse physiological roles in stress responses, regulation of metabolism, control of bacterial

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envelope composition and bacterial virulence (Lenz *et al*., 2004; Majdalani *et al*., 2005; Storz *et al*., 2005; Romby *et al*., 2006; Vogel and Papenfort, 2006). However, despite the importance of *Salmonella* as a health risk and a model pathogen, little is known about non-coding sRNA functions in the various *Salmonella* species, and no sRNAs have been reported from *Salmonella*-specific virulence regions.

In this work, we have discovered a novel SPI-1 gene that encodes a regulatory sRNA, referred to as InvR (invasion gene-associated RNA). The SPI-1 transcription factor, HilD, and the sRNA chaperone, Hfq, were both identified as being strictly required for InvR expression and stability respectively. Unlike other SPI-1 genes, *invR* does not seem to be involved in effector secretion by the SPI-1 T3SS. Instead, InvR is an Hfq-dependent sRNA that acts outside SPI-1 to regulate outer membrane protein (OmpD) synthesis post-transcriptionally. Collectively, our data describe a new, unsuspected gene within SPI-1, identify a role of Hfq in the expression of a horizontally acquired virulence region, and reveal an unexpected link between SPI-1 and the *Salmonella* core genome.

Results

Identification of a non-coding RNA gene in SPI-1

The *invR* gene was identified using a biocomputational search for novel sRNAs in *S. typhimurium*. Similar to recent sRNA searches in *E. coli* (Argaman *et al*., 2001; Chen *et al*., 2002), we performed a search for 'orphan' pairs of σ^{70}-type promoters and ρ-independent transcription terminators in the intergenic regions (IGRs) of the *Salmonella* LT2 genome (see *Experimental procedures*). This search predicted 46 candidates of *Salmonella* sRNA genes which are not conserved in *E. coli* K12 (Table 1 and Table S1). Many of these sRNA candidate genes appear to be conserved in the early branching *Salmonella* species, *S. bongori*. Comparison of the genomic location of these candidate genes to the co-ordinates of well-established *Salmonella* virulence regions (McClelland *et al*., 2001) revealed an overlap of candidate STnc270 (*invR*) with the invasion gene island, SPI-1 (Fig. 1A and B).

The *invR* gene resides in the 91 bp IGR between *invH* and STM2901, and is encoded on the same strand as these latter two genes (Fig. 1A). *invH* encodes an outer membrane lipoprotein required for a functional outer membrane translocation complex of the SPI-1 T3SS (Altmeyer *et al*., 1993; Crago and Koronakis, 1998; Daefler and Russel, 1998), and it is the last gene at the right border of the island with a defined SPI-1 function. The function of STM2901 is unknown. A σ^{70}-type promoter was predicted ~70 bp downstream of the *invH* stop codon; this promoter is followed closely by a 7 bp inverted repeat and a stretch of T residues, characteristic of ρ-independent transcription terminators (Fig. 1C). These features are conserved in putative *invR* genes of other *Salmonella* species for which genome sequences are available, including the early branching *S. bongori* (Fig. 1C).

Northern blot analysis of the *invR* locus detected an ~80 nt RNA in total RNA samples prepared from various stages of *Salmonella* growth in L-broth (Fig. 2A, lanes 1–6). The size of the InvR RNA was in perfect agreement with the promoter/terminator predictions. Notably, *invR* expression is highly growth rate-dependent and peaks in early stationary phase (OD_{600} of 2), a pattern reminiscent of SPI-1 gene transcription under aerobic growth (Lee and Falkow, 1990; Song *et al*., 2004).

To determine whether *invR* was co-regulated with SPI-1 genes, we probed total RNA isolated from *Salmonella* grown under low oxygen tension and/or high osmolarity (0.3 M NaCl), conditions known to activate SPI-1 gene expression *in vitro* (Lee and Falkow, 1990; Song *et al*., 2004). Consistent with a co-regulation with SPI-1 genes, InvR levels dramatically increased during oxygen limitation compared with growth with aeration, and were further elevated by high osmolarity (Fig. 2B).

The bacterial alarmone, ppGpp (guanosine tetraphosphate), was recently identified as being required for SPI-1 gene expression (Pizarro-Cerdá and Tedin, 2004; Song *et al*., 2004; Thompson *et al*., 2006). Functional deletion of both ppGpp synthetase genes, *relA* and *spoT*, reduced the expression of SPI-1 transcription activators, and consequently invasion gene transcription. To test if *invR* expression is also affected by ppGpp synthesis, we compared InvR levels in wild type and a Δ*relA*/Δ*spoT* mutant strain at various growth points ranging from exponential to stationary phase. Figure 2A (lanes 7–12) shows that *invR* is expressed in the ppGpp mutant at lower levels compared with wild-type *Salmonella*, although the pattern of InvR accumulation during the growth phase is unaffected.

invR *is an independent gene*

The predicted *invR* gene is located downstream of the *invH* gene and in the same orientation. As *invH* does not seem to possess a ρ-independent transcription terminator (Fig. 1C), to determine whether InvR was a primary transcript or a processed species of the upstream encoded *invH* mRNA, the *invR* region was deleted from the *Salmonella* chromosome, and re-introduced by integration into the distant *istR-tisAB* locus (Vogel *et al*., 2004) with a 478 bp DNA fragment containing *invR* and its upstream region (C-terminal portion of *invH*). The complemented strain yielded an InvR-specific Northern blot signal identical to the wild type (Fig. 3A). The same *invR* DNA frag-

Table 1. sRNA candidates.

Name[a]	sRNA[b]	Lgene[c]	Rgene[d]	Orientation[e]	Bongori *E*-value[fd]
STnc10	46114–46050[g]	*STM0038*	*nhaA*	> < >	1.00E-08
STnc20	51926–52260[g]	*STM0042*	*rpsT*	< > <	No hits
STnc30	58792–58923[g]	*lytB*	*STM0050*	> > >	No hits
STnc40	161464–161537	*secA*	*mutT*	> > >	1.00E-18
STnc50	182539–182458	*lpdA*	*STM0155*	> < >	No hits
STnc60	230277–230063[g]	*fhuB*	*stfA*	> < >	No hits
STnc70	670157–670305	*dsbG*	*ahpC*	< > >	7.00E-28
STnc80	967580–967900[g]	*STM0897*	*STM0898*	< > <	No hits
STnc90	974284–974363[g]	*STM0903*	*STM0904*	> > <	No hits
STnc100	975011–975224[g]	*STM0904*	*STM0905*	< > >	5.00E-05
STnc110	976578–976765	*STM0905*	*STM0906*	> > >	No hits
STnc120	1004777–1004432[g]	*STM0929*	*orfB*	< < >	No hits
STnc130	1045232–1045098	*serS*	*dmsA*	> < >	7.00E-43
STnc140	1113681–1113750[g]	*STM1025*	*STM1026*	< > <	4.00E-09
STnc150	1325914–1325649[g]	*icdA*	*STM1239*	> < >	3.00E-19
STnc160	1345782–1345732	*STM1262*	*STM1263*	> < >	No hits
STnc170	1606116–1605784[g]	*STM1528*	*STM1530*	< < >	5.00E-12
STnc180	1807776–1807565[g]	*acnA*	*cysB*	< < <	9.00E-59
STnc190	1937518–1937652	*STM1841*	*kdgR*	> > <	2.00E-09
STnc200	1979598–1979550	*edd*	*zwf*	< < <	9.00E-06
STnc210	2032404–2032580	*yecA*	*STM1939*	< > <	6.00E-35
STnc220	2079068–2078990	*ompS*	*cspB*	> < <	No hits
STnc230	2115370–2115452[g]	*pocR*	*pduF*	< > <	No hits
STnc240	2147409–2147333[g]	*yeeF*	*yeeY*	< < <	9.00E-35
STnc250	2596882–2596789	*acrD*	*yffB*	> < >	2.00E-23
STnc260	2966073–2966247[g]	*STM2816*	*luxS*	< > <	No hits
STnc270	3044923–3045015[g]	*invH*	*STM2901*	> > >	1.00E-31
STnc280	3179540–3179622	*kduI*	*yqeF*	< > <	No hits
STnc290	3194996–3194914	*tnpA_4*	*STM3033*	< < <	No hits
STnc300	3283965–3283807[g]	*STM3123*	*STM3124*	< < >	No hits
STnc310	3393327–3393267	*ygjT*	*ygjU*	> < >	3.00E-09
STnc320	3404895–3404949[g]	*yhaO*	*tdcG*	< > <	2.00E-10
STnc330	3468553–3468497	*greA*	*dacB*	< < >	3.00E-15
STnc340	3635884–3635756[g]	*tnpA_5*	*yhfL*	< < >	3.00E-20
STnc350	3761440–3761373	*uspA*	*yhiP*	> < >	2.00E-13
STnc360	3780254–3780402	*yhjB*	*yhjC*	< > >	1.00E-04
STnc370	3839688–3839758	*STM3654*	*glyS*	< > <	No hits
STnc380	3885736–3885629[g]	*STM3691*	*lldP*	> < >	No hits
STnc390	3902653–3902594	*yibD*	*tdh*	< < <	No hits
STnc400	4051145–4051340[g]	*STM3844*	*STM3845*	> > >	No hits
STnc410	4072507–4072730	*glmU*	*STM3863*	< > <	No hits
STnc420	4251539–4251480	*yiiG*	*STM4041*	> < <	No hits
STnc430	4442059–4441898[g]	*pgi*	*yjbE*	> < >	0.009
STnc440	4559193–4559277[g]	*STM4310*	*tnpA_6*	> > >	1.00E-31
STnc450	4645134–4645079	*ytfL*	*msrA*	< < <	5.00E-23
STnc460	4758332–4758187[g]	*STM4503*	*STM4504*	> < >	3.00E-05

a. sRNA candidate names.
b. Genomic location of sRNA candidate gene according to the *Salmonella typhimurium* LT2 genome.
c, d. Flanking genes of the intergenic region in which the sRNA candidate is located; 'Lgene', 5′ located, upstream gene; 'Rgene' 3′ located, downstream gene.
e. Orientation of sRNA candidate (middle) and flanking genes (> and < denote location of a gene on the clockwise or the counterclockwise strand of the *Salmonella* chromosome).
f. BLAST*E*-value of the sRNA candidate gene for *Salmonella bongori*
g. Multiple candidates for one sRNA gene are predicted due to several promoters predicted for the same sRNA (see Table S1 in *Supplementary material* for details). Given is the candidate with maximal length.

ment born on plasmid (p*invR*) also fully restored InvR expression in the Δ*invR* strain (Fig. S1).

As the above results suggested that InvR is a primary transcript, we carried out a 5′ RACE experiment involving tobacco acid pyrophosphatase (TAP; cleaves 5′-triphosphates) treatment to map the transcription start site of the sRNA gene (Bensing *et al.*, 1996; Vogel *et al.*, 2003). As shown in Fig. 3B, treatment of RNA with TAP prior to 5′ RNA linker ligation resulted in a strongly enhanced 5′ RACE signal for *invR* transcripts (lane T+), indicating that InvR RNA carries a 5′-triphosphate group characteristic of primary transcripts. Cloning of this 5′ RACE fragment positioned the transcription start site at the G residue that is located 7 bp downstream of the *invR*

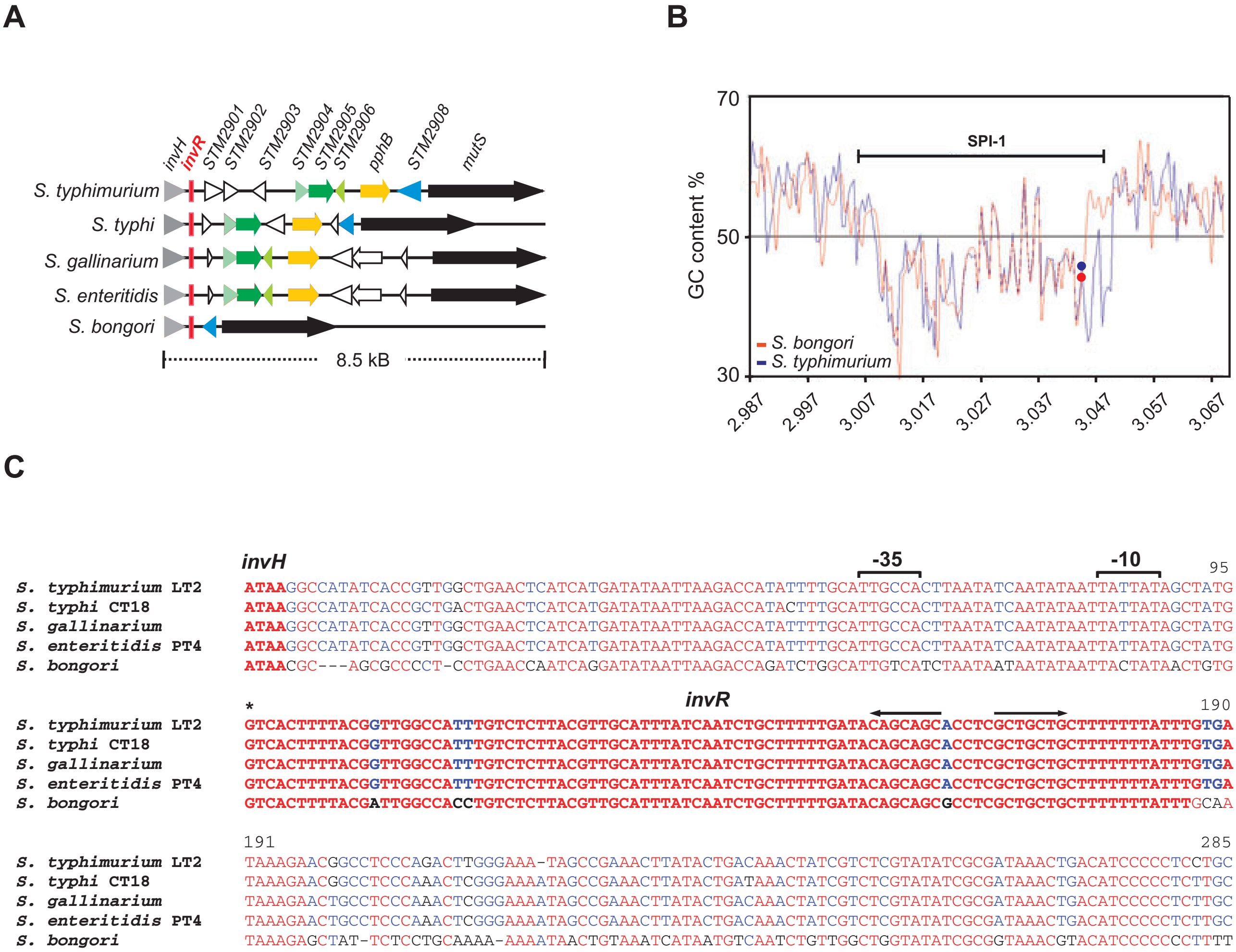
A
invH
invR
STM2901
STM2902
STM2903
STM2904
STM2905
STM2906
pphB
STM2908
mutS
S. typhimurium
S. typhi
S. gallinarium
S. enteritidis
S. bongori
8.5 kB
B
70
50
30
GC content %
SPI-1
S. bongori
S. typhimurium
2.987
2.997
3.007
3.017
3.027
3.037
3.047
3.057
3.067
C
invH
-35
-10
95
S. typhimurium LT2 ATAAGGCCATATCACCGTTGGCTGAACTCATCATGATATAATTAAGACCATATTTTGCATTGCCACTTAATATCAATATAATTATTATAGCTATG
S. typhi CT18 ATAAGGCCATATCACCGCTGACTGAACTCATCATGATATAATTAAGACCATACTTTGCATTGCCACTTAATATCAATATAATTATTATAGCTATG
S. gallinarium ATAAGGCCATATCACCGTTGGCTGAACTCATCATGATATAATTAAGACCATATTTTGCATTGCCACTTAATATCAATATAATTATTATAGCTATG
S. enteritidis PT4 ATAAGGCCATATCACCGTTGGCTGAACTCATCATGATATAATTAAGACCATATTTTGCATTGCCACTTAATATCAATATAATTATTATAGCTATG
S. bongori ATAACGC---AGCGCCCCT-CCTGAACCAATCAGGATATAATTAAGACCAGATCTGGCATTGTCATCTAATAATAATATAATTACTATAACTGTG
*
invR
190
S. typhimurium LT2 GTCACTTTTACGGTTGGCCATTTGTCTCTTACGTTGCATTTATCAATCTGCTTTTTGATACAGCAGCACCTCGCTGCTGCTTTTTTTATTTGTGA
S. typhi CT18 GTCACTTTTACGGTTGGCCATTTGTCTCTTACGTTGCATTTATCAATCTGCTTTTTGATACAGCAGCACCTCGCTGCTGCTTTTTTTATTTGTGA
S. gallinarium GTCACTTTTACGGTTGGCCATTTGTCTCTTACGTTGCATTTATCAATCTGCTTTTTGATACAGCAGCACCTCGCTGCTGCTTTTTTTATTTGTGA
S. enteritidis PT4 GTCACTTTTACGGTTGGCCATTTGTCTCTTACGTTGCATTTATCAATCTGCTTTTTGATACAGCAGCACCTCGCTGCTGCTTTTTTTATTTGTGA
S. bongori GTCACTTTTACGATTGGCCACCTGTCTCTTACGTTGCATTTATCAATCTGCTTTTTGATACAGCAGCGCCTCGCTGCTGCTTTTTTTATTTGCAA
191
285
S. typhimurium LT2 TAAAGAACGGCCTCCCAGACTTGGGAAA-TAGCCGAAACTTATACTGACAAACTATCGTCTCGTATATCGCGATAAACTGACATCCCCCTCCTGC
S. typhi CT18 TAAAGAACGGCCTCCCAAACTCGGGAAAATAGCCGAAACTTATACTGATAAACTATCGTCTCGTATATCGCGATAAACTGACATCCCCCTCTTGC
S. gallinarium TAAAGAACTGCCTCCCAAACTCGGGAAAATAGCCGAAACTTATACTGACAAACTATCGTCTCGTATATCGCGATAAACTGACATCCCCCTCTTGC
S. enteritidis PT4 TAAAGAACTGCCTCCCAAACTCGGGAAAATAGCCGAAACTTATACTGACAAACTATCGTCTCGTATATCGCGATAAACTGACATCCCCCTCTTGC
S. bongori TAAAGAGCTAT-TCTCCTGCAAAA-AAAATAACTGTAAATCATAATGTCAATCTGTTGGCTGGTATATCGCGGTAAACGTACATCCCCCCCTTTT

Fig. 1. Identification of a new sRNA gene in SPI-1.
A. Mosaic structure of the right SPI-1 border (*invH-mutS* region) in diverse *Salmonella* species. Sequences were derived from the whole-genome sequence of *S. typhimurium* LT2 (Accession No.: NC 003197), *S. typhi* CT18 (NC 003198), *S. gallinarium* 287/91 (NCTC 13346), *S. enteritidis* PT4 (NCTC 13349) and *S. bongori* (12419 ATCC 43975). Orthologous genes are indicated by the same colour. The insertion/deletion events at this locus did not affect the *invR* gene.
B. The *invR* gene shares the low GC content of the SPI-1 virulence region. GC content plot of the genomic region from *ygbD* to *pcm* in *S. typhimurium* LT2 (blue) and in *S. bongori* (red). The SPI-1 region from *flhA* to *mutS* is indicated by a horizontal bar. The *invR* loci of *S. typhimurium* and *S. bongori* are indicated by blue and red dots respectively. Genome co-ordinates (2.987–3.067 Mb) refer to the LT2 annotation.
C. Alignment (computed with MultAlin) of the *invH* and *invR* region in various *Salmonella* species [same accession numbers as in (A)] shows the strong conservation of the *invR* gene. The 3′ residues of the *invH* coding region and the InvR RNA sequence are set in bold. The putative –10 and –35 boxes of the *invR* promoter are indicated above the sequence. The transcription start site of *invR* is denoted by an asterisk. The stem structure of the ρ-independent terminator of *invR* is indicated by arrows.

–10 box (Fig. 1C). The results therefore all support the conclusion that InvR is encoded by an independent gene.

InvR RNA is abundant and stable

InvR hybridization yielded a strong signal on Northern blots as compared with other *Salmonella* RNAs, suggesting that the RNA is abundant. Thus, we determined the *in vivo* copy number of the RNA at early stationary phase (OD_{600} of 2), i.e. when InvR expression peaks under standard laboratory growth conditions. Cellular InvR levels were compared in Northern blots relative to known quantities of a slightly longer *in vitro* synthesized InvR transcript (Fig. 3C). Quantification of the hybridization signals put the cellular copy number of InvR at this point of growth at > 100 molecules per cell. We next tested the *in vivo* stability of the RNA in rifampicin-treatment experiments. As shown in Fig. 3D, InvR levels showed little change within 30 min after a total block of transcription, i.e. the RNA decays with a half-life > 30 min. Thus, InvR is both abundant and stable, with an intrinsic half-life exceeding that of many other sRNAs (Vogel *et al*., 2003).

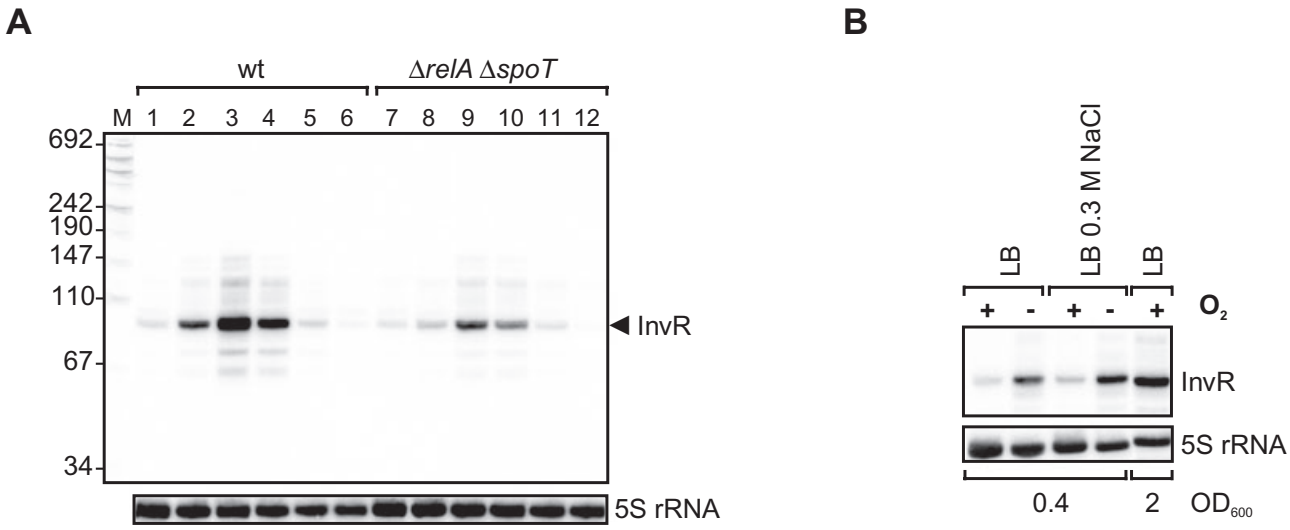

Fig. 2. Expression of the *invR* gene in *S. typhimurium* strain SL1344. Northern blot detection of the ~80 nt InvR RNA with an *invR*-specific probe.
A. RNA was extracted from *Salmonella* wild type and a double deletion of the *relA* and *spoT* genes (Δ*relA*/Δ*spoT*) grown aerobically in L-broth at increasing OD_{600} (wild type lanes 1–6: 0.5, 1, 2, 2.4, 3, 3.2; Δ*relA*/Δ*spoT* lanes 7–12: 0.5, 1, 2, 2.4, 3.3, 3.5). Co-migrating nucleotide size marker bands (M) are shown on the left.
B. InvR levels in cells grown aerobically (+) or under low oxygen (–), and high osmolarity (0.3 M NaCl) as indicated above the lanes. RNA was extracted at exponential and early stationary phase (OD_{600} of 0.4 and 2). Probing for 5S rRNA was used as loading control.

Hfq is essential for InvR accumulation

Many of the sRNAs that are conserved among diverse enterobacteria associate with the bacterial Sm-like protein, Hfq, and require the protein for both intracellular stability and regulatory function. In contrast, the role of Hfq for sRNAs encoded by recently acquired virulence regions or bacterial plasmids is less understood (Huntzinger *et al*., 2005; Will and Frost, 2006; Boisset *et al*., 2007). As Hfq was previously implicated in SPI-1 gene expression (Sittka *et al*., 2007), we tested whether Hfq was also required for InvR expression. Northern blot analysis of a *Salmonella hfq* mutant grown either under standard (Fig. 4A) or SPI-1-inducing conditions (not shown) revealed a complete loss of InvR accumulation in this genetic background. We recently showed that Hfq-dependent sRNAs can accumulate to high levels when expressed from a constitutive $P_{LlacO-1}$ promoter in an *E. coli hfq* mutant strain (Urban and Vogel, 2007). However, this strategy failed to produce abundant InvR RNA in the *Salmonella hfq* mutant strain (Fig. 4B), arguing that Hfq primarily affects InvR stability. To confirm this, we fused the *invR* promoter to a transcriptional reporter gene, and observed that *invR* promoter activity was not affected by the *hfq* mutation (Fig. 5B).

To determine whether Hfq and InvR can directly interact, the RNA was synthesized *in vitro*, and Hfq binding was assayed in gel mobility shift experiments (Fig. 4C). InvR binds Hfq with high affinity and an apparent K_D of ~10 nM. Hfq typically binds to sRNAs in A/U-rich, often single-stranded regions (Valentin-Hansen *et al*., 2004), and an extended A/U-rich region is found immediately upstream of the *invR* terminator stem-loop region (Fig. 1C).

To test whether InvR is associated with Hfq *in vivo*, we sought to co-immunoprecipitate the RNA in bacterial extracts. As preliminary experiments with polyclonal antisera raised against purified *Salmonella* Hfq protein failed to yield sufficient amounts of immunoprecipitated RNA (data not shown), we resorted to a generic method that involves the expression of an epitope-tagged Hfq protein. The chromosomal *hfq* gene was modified such that it expresses Hfq with a C-terminal 3xFLAG epitope tag (Uzzau *et al*., 2001). The resulting strain, hfq^{FLAG}, was indistinguishable from the wild type in terms of growth and *in vitro* virulence assays, e.g. gentamicin protection assay, indicating that the 3xFLAG-tag does not impair Hfq function (Fig. S2). Northern blot analysis of RNA precipitated with Hfq::FLAG protein revealed a strong enrichment of InvR as compared with the control reaction (Fig. 4D). Thus, the sRNA is very likely to be present in a complex with Hfq *in vivo*.

The invR *gene belongs to the SPI-1 regulon and is activated by HilD*

Next, we asked whether *invR* transcription was under the control of SPI-1-encoded transcription factors. The activation of SPI-1 genes is mediated in response to growth rate and environmental conditions by a transcription factor

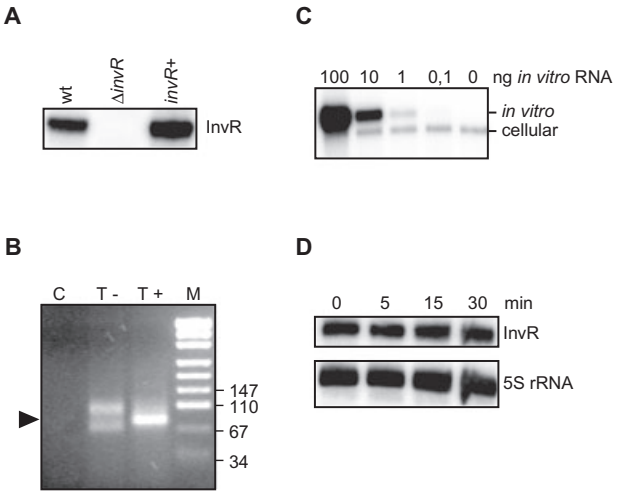

Fig. 3. *invR* is an independent gene and InvR RNA is abundant and stable.
A. InvR levels in the wild-type strain and the *invR* deletion strain (Δ*invR*), the latter strain complemented with an *invR* copy at the distant *istR-tisAB* locus (*invR*+). RNA was prepared from early stationary-phase cells (OD_{600} of 2).
B. 5′ RACE of InvR transcripts was performed with tobacco acid pyrophosphatase-treated (TAP; lane T+) or mock-treated RNA (lane T–) extracted in early stationary phase. DNA served as a control template (lane C). RT-PCR products were separated on a 3% agarose gel. The arrowhead marks the position of the strongly enhanced RT-PCR product upon TAP treatment, which corresponds to the newly initiated InvR transcript. Cloning of this band, followed by sequencing of seven independent clones, identified the G residue marked by an asterisk in Fig. 1C as the uniform 5′ end of InvR RNA. DNA marker sizes (lane M) are given to the right.
C. Abundance of InvR RNA *in vivo*. Increasing amounts (0.1, 1, 10, 100 ng) of *in vitro* synthesized InvR (88 nt) were mixed with total RNA prepared from 2.2×10^8 cells grown to an OD_{600} of 2, and subjected to Northern analysis. Probing with an *invR*-specific oligo detected in parallel the cellular and the *in vitro* synthesized RNA. Approximately 100 InvR molecules per cell correspond to 36 fmol (1 ng) of *in vitro* synthesized RNA.
D. Stability of InvR RNA as determined by rifampicin treatment. RNA from early stationary phase was extracted prior to and 5, 15 and 30 min after treatment, and subjected to Northern analysis. Probing for 5S rRNA served as loading control.

cascade (Ellermeier and Slauch, 2007). On top of this cascade, the transcription factors HilC and HilD, along with RtsA (encoded outside SPI-1), cooperate to transmit environmental signals that lead to de-repression of *hilA* (Bajaj *et al*., 1996; Lucas and Lee, 2001; Schechter and Lee, 2001; Ellermeier *et al*., 2005). HilA is the SPI-1 major transcriptional activator responsible for most of the SPI-1 T3SS and effector gene expression, both directly and indirectly through its activation of InvF (Darwin and Miller, 1999; Eichelberg and Galan, 1999; Lostroh and Lee, 2001). To test whether *invR* was dependent on any of the four SPI1-encoded transcription factors, InvR RNA levels were quantified in strains with disruptions in the individual transcription factor genes. Figure 5A shows that loss of HilD caused a dramatic (50-fold) reduction in InvR levels. Severely reduced activity of the *invR* promoter fusion in this background argues that HilD regulates *invR* at the transcriptional level (Fig. 5B). In contrast, a *hilC* mutation reduced InvR levels less than twofold compared with the wild-type strain, and had only a marginal effect on the *invR* promoter activity. Disruptions in the *hilA* or *invF* genes had no effect on InvR levels (Fig. 5A).

We next tested if ectopically expressed HilD would restore *invR* transcription in the *hilD* mutant. The *hilD* mutant and wild-type strains were transformed with plasmid pBAD-HilD which carries the *hilD* gene under control of an arabinose-inducible P_{BAD} promoter, and InvR levels were determined at early stationary phase in the presence or absence of the inducer (Fig. 5C). Quantification of the Northern blot signals revealed that pBAD-HilD induction resulted in a 75-fold upregulation of *invR* expression in the *hilD* strain, thus fully complementing this mutant. Induction of pBAD-HilD also elevated InvR levels in wild-type cells (threefold), suggesting that the *invR* promoter can be further activated when HilD is highly expressed. In contrast, arabinose did not induce *invR* in the presence of a pBAD control vector. We also tested whether HilD could activate *invR* in the absence of any other SPI1 genes. To this end, the entire SPI-1 region with the exception of *invR* (strain Δ*sitA-invH*) was deleted in the *Salmonella* chromosome. pBAD-HilD fully activated *invR* expression in this mutant background (Fig. 5D). In contrast, a pBAD-HilA plasmid failed to restore *invR* expression, consistent with unchanged InvR levels in the *hilA* mutant (Fig. 5D).

To further validate a direct HilD role in *invR* regulation, we examined binding of the purified transcription factor to a 5′ labelled *invR* promoter double-stranded DNA (dsDNA) fragment in electrophoretic mobility shift assays (EMSAs) *in vitro*. Fragments containing the *hilA* promoter, known to be recognized by HilD (Schechter and Lee, 2001), served as positive control. The promoter of the *E. coli lacZ* gene, which is not recognized by HilD, was included as negative control. The fragments were incubated with increasing concentrations of purified HilD protein, and the resulting complexes were separated on native polyacrylamide gels. Figure 6 shows that HilD formed a singular complex with the *invR* promoter fragment at a concentration (< 5 nM) similar to that required to shift the *hilA* promoter, whereas no complex formation was obtained with the *lacZ* control fragment. Cumulatively, the data indicate that *invR* is a novel member of the SPI1 regulon, and that its promoter is activated directly by HilD.

InvR does not engage in SPI-1 regulation or secretion

The conservation of *invR* as part of SPI-1 and its expression under conditions activating SPI-1-driven host cell invasion suggested an involvement of InvR in SPI-1-encoded functions. One major SPI-1 function is the secre-

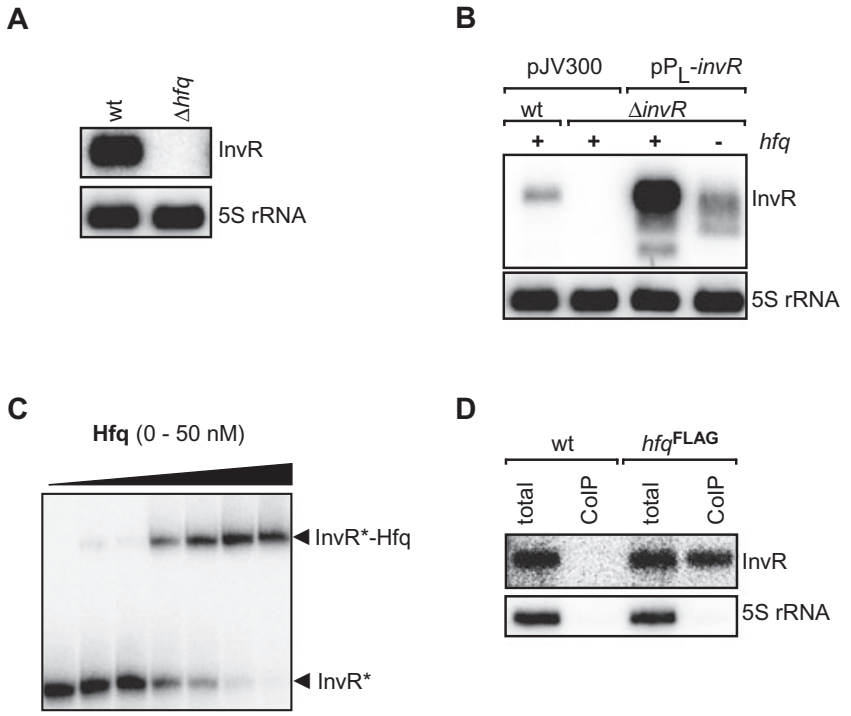

Fig. 4. InvR is an Hfq-dependent sRNA.
A. Northern blot showing that InvR fails to accumulate in an *hfq* deletion strain (JVS-00255) in early stationary phase.
B. InvR expression at early stationary phase in the wild type, Δ*hfq*, Δ*invR*, Δ*invR*/Δ*hfq* (–/+ indicate absence or presence of the *hfq* gene). The strains carried either a control plasmid (pJV300) or a plasmid with *invR* cloned under a constitutive $P_{LlacO-1}$ promoter (pP$_L$-*invR*).
C. Electrophoretic mobility shift assay (EMSA) with *in vitro* synthesized, ^{32}P-labelled InvR RNA (InvR*, 1 nM) in the presence of increasing concentrations of Hfq protein (from left to right: 0, 0.1, 0.5, 1, 10, 25, 50 nM).
D. Co-immunoprecipitation of InvR with C-terminally FLAG-tagged Hfq protein in extracts of an *hfq*^{FLAG} strain. Extracts of the wild-type (wt) strain expressing untagged Hfq served as control. Co-immunoprecipitated RNA (lanes 'CoIP') was probed on Northern blots for InvR or 5S rRNA. Total RNA equivalent to 1/10 of extract used in the immunoprecipitations was run in parallel (lanes 'total').

tion of effector proteins. Analysis of the proteins of culture supernatants from *Salmonella* provides a rough measure of SPI-1 gene induction and the intactness of the SPI-1 secretion apparatus. Surprisingly, neither loss nor ectopically driven overexpression of InvR consistently changed the pattern of secreted effector proteins in early stationary phase (Fig. 7A) and under SPI-1-inducing conditions (Fig. 7B). Next, to measure a possible role of *invR* for SPI-1 secretion more sensitively, translocation assays using a fusion of the SPI-1 effector protein, SipA (also known as SspA), to TEM-1 β-lactamase were performed (Charpentier and Oswald, 2004; Raffatellu *et al*., 2005); SipA translocation had been established by others as a sensitive reporter of an intact SPI-1 secretion apparatus (e.g. Bronstein *et al*., 2000; Lilic *et al*., 2006). The *sipA* fusion is under control of the *sipA* promoter, thus the assay will also be sensitive to any alterations in SPI-1 gene transcription. We found no indication for a role for InvR in SipA-β-lactamase translocation into COS7 cells (Fig. S3). These results strongly suggest that InvR is not involved in SPI-1-dependent secretion, which is supported by preliminary results from an array of *Salmonella* virulence assays (V. Pfeiffer, K. Tedin and J. Vogel, unpubl. results).

InvR represses the abundant OmpD protein

As InvR is Hfq-dependent, we predicted it to act on *trans*-encoded mRNA(s). To identify putative InvR targets, we compared the total protein patterns (Fig. 8A) of wild-type *Salmonella*, the isogenic Δ*invR* strain, and Δ*invR* strains complemented with two multicopy plasmids, p*invR* and pP$_L$-*invR*, which overexpress InvR to different degrees (Fig. 8B). InvR overexpression (plasmid pP$_L$-*invR*) caused the depletion of an abundant 40 kDa protein (Fig. 8A) identified by mass spectrometry analysis of the excised band as the major outer membrane protein, OmpD (also known as NmpC). The concomitant increase in OmpC levels caused by plasmid pP$_L$-*invR* is most likely due to OmpD depletion rather than InvR expression as it is also observed in a Δ*ompD* strain (Fig. 8A).

The InvR-dependent regulation was even more pronounced at the *ompD* mRNA level. Northern blots showed that InvR affects *ompD* mRNA levels in a dose-dependent manner; *ompD* mRNA levels were increased in the Δ*invR* strain but gradually reduced to background levels upon intermediate or strong *invR* overexpression from plasmids p*invR* and pP$_L$-*invR* respectively (Fig. 8B).

To address whether InvR regulates *ompD* transcription or targets the *ompD* mRNA post-transcriptionally, we mapped the *ompD* transcription start site (Fig. S4) and constructed a transcriptional *ompD′–gfp* fusion (Fig. S5). InvR overexpression had no effect on the *ompD′–gfp* fusion expression (Fig. S5), indicating post-transcriptional regulation of *ompD*. This was also supported by the kinetics of sRNA-dependent *ompD* mRNA depletion. Induction of *invR* expression from plasmid pBAD-InvR accelerated *ompD* mRNA decay to a half-life of < 4 min (Fig. 8C). As the normal half-life of *ompD* mRNA under these conditions is ≥ 10 min (Sittka *et al*., 2007), this strongly indicated that InvR directly targets *ompD* mRNA.

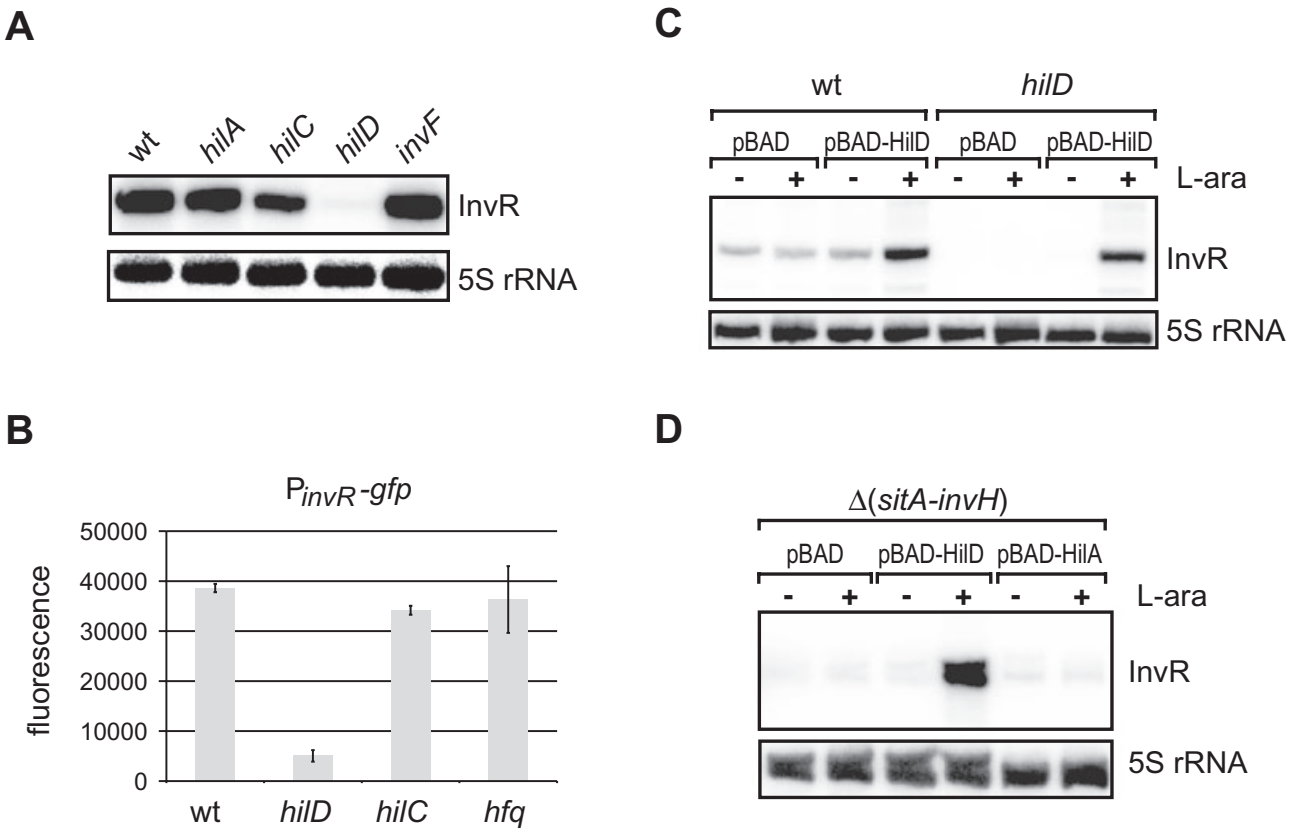

Fig. 5. The *invR* gene belongs to the SPI-1 regulon.
A. Comparison of InvR levels in the wild-type strain and in strains with functional disruptions of the *hilA*, *hilC*, *hilD* or *invF* SPI-1 transcription factor genes, with all strains grown to an OD_{600} of 2.
B. Activity of the *invR* promoter in different genetic backgrounds as determined with a transcriptional P_{invR} –*gfp* reporter plasmid under SPI-1 inducing conditions in the wild type (wt), and in *hilD*, *hilC* or Δ*hfq* mutant strains. Given are mean values (arbitrary units) of GFP fluorescence of triplicate measurements.
C. Ectopic HilD expression restores InvR expression. Shown is a Northern blot of the wild-type or the *hilD* strain, each harbouring a control (pBAD) or an inducible HilD expression plasmid (pBAD-HilD). HilD expression was induced at an OD_{600} of 1.0 with 0.1% L-arabinose (where indicated by +) for 45 min prior to RNA extraction.
D. Induced HilD expression complements *invR* expression to a SPI-1 disruption strain (*spi*-1 is deleted from *sitA* to *invH*). Strains carrying the same plasmids were induced as in (C). A strain carrying a HilA expression plasmid (pBAD-HilA) was included as further control.

Direct negative regulation by Hfq-binding sRNAs is usually dependent on base-pairing with the 5′ UTR of target *omp* mRNAs near the ribosome binding site (Guillier *et al*., 2006; Vogel and Papenfort, 2006). An extended interaction between InvR and the *ompD* mRNA was predicted using the RNAhybrid program (Rehmsmeier *et al*., 2004) (Fig. 8D). Gel mobility shift assays of either labelled InvR RNA with unlabelled 5′ region of *ompD* RNA or *vice versa* showed that the two RNAs form a stable complex *in vitro* (Fig. S6A). Furthermore *in vitro* structure probing with both RNAs using T1 ribonuclease (cleaves at the 3′ end of single stranded G residues) and lead(II) (cleaves RNA in single-stranded regions, loops and bulges) supports the predicted interaction between InvR and *ompD* mRNA *in vitro* (Fig. S6B and C). In summary, these results indicate that post-transcriptional repression of OmpD synthesis by InvR RNA involves specific base-pairing to *ompD* mRNA.

Discussion

Salmonella pathogenicity island 1 is one of the most intensely studied bacterial virulence regions. The ~35 SPI-1 genes encode proteins involved in the assembly of a membrane-spanning supramolecular structure, the T3SS or needle complex, through which effector proteins are secreted into eukaryotic host cells to facilitate *Salmonella* invasion. These genes within SPI-1 encode not only proteins making up the secretion apparatus, but also chaperones, secreted effector proteins and a number of transcription factors involved in the regulation and timing of expression. Here we report a new type of SPI-1 gene, expressing a non-coding RNA not directly involved in SPI-1 regulation or T3SS function, but which affects gene expression of the core *Salmonella* genome.

There is evidence to suggest that *invR* is an ‘ancient’ gene, horizontally acquired along with the SPI-1 virulence

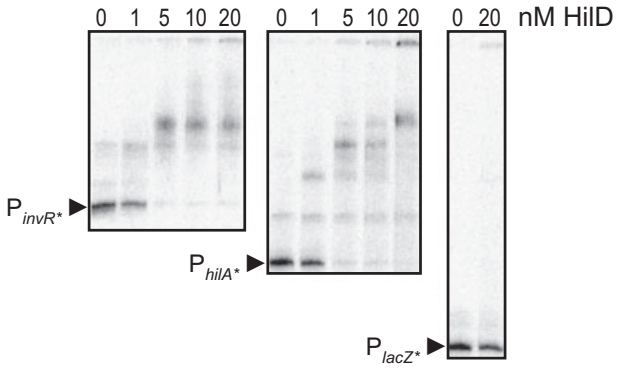

Fig. 6. Purified HilD protein binds to the *invR* promoter region with high affinity *in vitro*. Electrophoretic mobility shift assay (EMSA). Approximately 20 pM of 5′ labelled promoter dsDNA fragments (P_{invR}*, P_{hilA}*, P_{lacZ}*) were incubated with increasing concentrations of purified HilD protein (0, 1, 5, 10, 20 nM). The complexes were separated on a native 5% polyacrylamide gel and visualized by autoradiography. The P_{hilA}* promoter, which is known to be recognized by HilD (Schechter and Lee, 2001), served as positive control. The promoter of the *E. coli lacZ* gene (P_{lacZ}*) not recognized by HilD served as negative control.

region in the first major phase of *Salmonella* evolution. *InvR* is highly conserved in *Salmonella* species (Fig. 1A), including the early branching *S. bongori* that diverged from the *S. enterica* lineage after acquisition of SPI-1 (Reeves *et al*., 1989; Groisman and Ochman, 1994). SPI-1 has a significantly lower average G+C content (47%) than the core *Salmonella* genome (52%), and the *invR* gene (39.6% G+C) shares this low G+C content region (Fig. 1B). *Salmonella* evolution has resulted in a mosaic structure of the right SPI-1 border (*invH-mutS* region). However, these insertions/deletions neither affected *invR* nor its close association with *invH* (Fig. 1A). Finally, *invR* is co-regulated with other SPI-1 genes and strictly requires HilD, the transcription factor that acts at the top of the SPI-1 transcriptional hierarchy (reviewed in Jones, 2005; Ellermeier and Slauch, 2007). HilD is not known to control genes outside SPI-1, except *rtsA* whose gene product is also involved in SPI-1 transcription (Ellermeier *et al*., 2005). Thus, genomic location, conservation and regulation all argue that *invR* was acquired along with SPI-1 very early at (or soon after) the divergence of the *E. coli* and the *Salmonella* lineages. The *invR* sequence appears to be more conserved than its boundaries (Fig. 1C), which argues that *invR* is maintained by selection and that it is a functional gene, i.e. despite the lack of evidence that InvR functions in SPI-1 effector secretion or translocation.

The function of InvR as a repressor of OmpD synthesis reveals a novel coupling between SPI-1 and *Salmonella* core genome expression (summarized in Fig. 9). SPI-1 is regulated by more than a dozen stress–response regulators which respond to environmental stimuli relevant to host cell invasion, and which are collectively encoded by the *Salmonella* core genome (Lostroh and Lee, 2001; Jones, 2005; Ellermeier and Slauch, 2007). Since its horizontal acquisition, SPI-1 has increasingly come under control of common *Salmonella* core genome regulators, namely those involved in responses to conditions found at appropriate anatomic locations in the host. The reciprocal case, i.e. that SPI-1 controls other loci of the *Salmonella* chromosome, was previously limited to SPI-4 and SPI-5, virulence islands activated by the SPI-1 transcription factor, HilA (Ahmer *et al*., 1999). This study shows that the SPI-1 virulence region also encodes a post-transcriptional regulator of *Salmonella* core genome expression, adding a new layer of cross-talk between these regions. Similar to our discovery of the *Salmonella* PAI-encoded InvR, there is evidence that other horizontally acquired elements such as cryptic prophage regions of *E. coli* express sRNAs to interfere with gene expression from the host chromosome, e.g. the DicF and IpeX RNAs (Tetart and Bouche, 1992; Castillo-Keller *et al*., 2006).

We recently demonstrated that the sRNA chaperone, Hfq, is involved in SPI-1 expression and/or functions

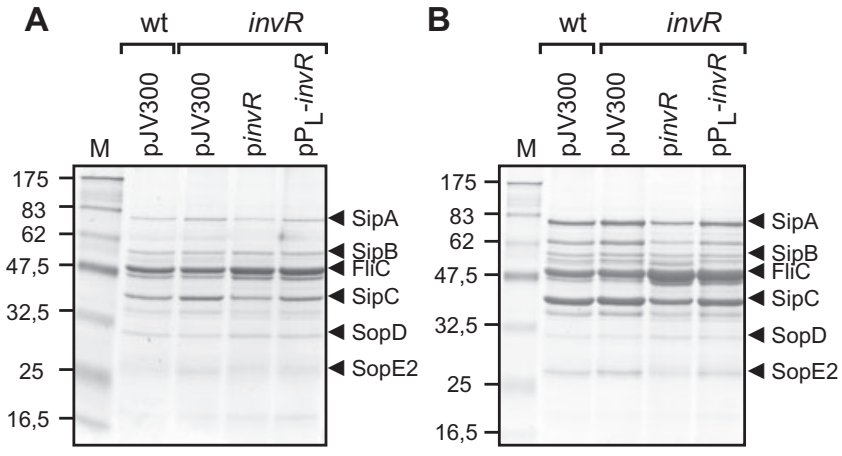

Fig. 7. InvR does not affect the pattern of secreted SPI-1 effector proteins. Secreted proteins were prepared from wild-type (wt) and Δ*invR* strains harbouring a control plasmid (pJV300), a plasmid that expresses *invR* under its own promoter (p*invR*) or under a P_{LlacO} promoter (pP_L-*invR*). Cultures were grown (A) under standard conditions to an OD_{600} of 2.0, or (B) under SPI-1-inducing conditions. Proteins were separated by 12% SDS-PAGE. The gel was stained with Coomassie brilliant blue. The bands of known abundant effector proteins (SipA, SipB, SipC, SopD, SopE2) as well as of flagellin (FliC) are indicated according to Raffatellu *et al*. (2005) and Sittka *et al*. (2007).

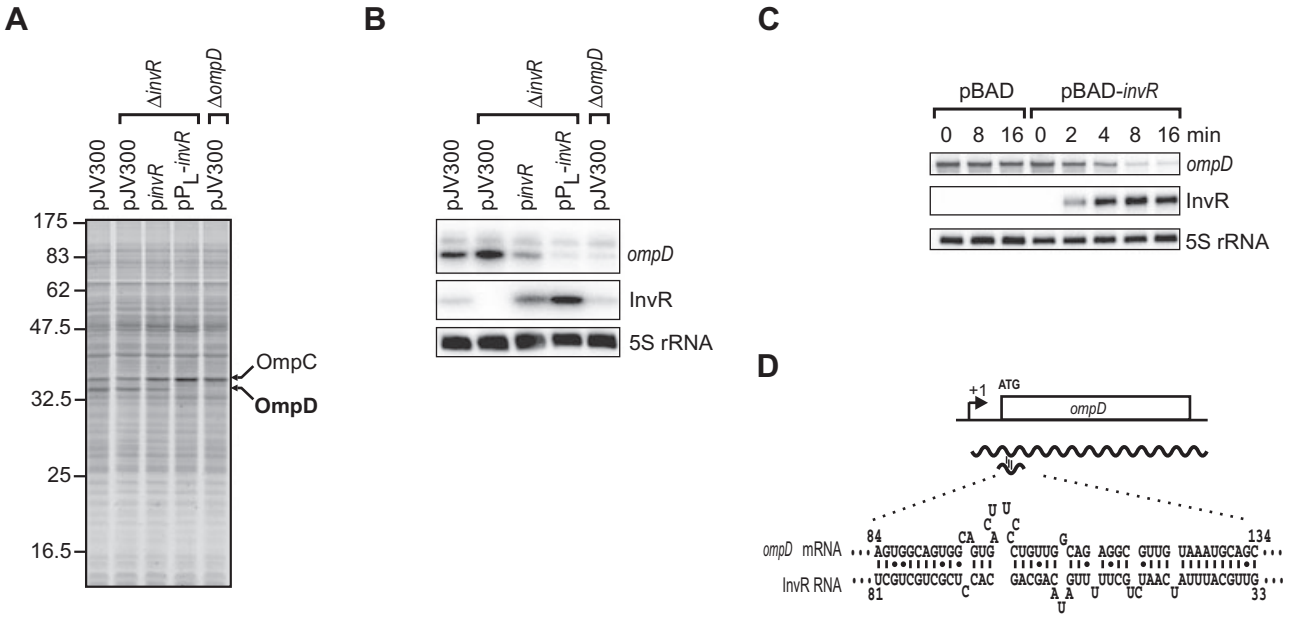

Fig. 8. InvR acts to repress OmpD synthesis.
A. SDS-PAGE of whole-cell protein of wild-type, Δ*invR* and Δ*ompD* strains carrying the control vector (pJV300), or multicopy plasmids with the *invR* locus (p*invR*) or the *invR* gene driven by a constitutive P_{LlacO} promoter (pP_L-*invR*) as indicated above the picture. Samples were taken at early stationary phase. Arrows indicate the OmpD and OmpC proteins.
B. Northern blot of the same strains and growth phase as above to determine steady-state *ompD* mRNA levels in the absence or presence of InvR expression.
C. Northern blot of *Salmonella* Δ*invR* cells carrying a pBAD control vector (pKP8-35) or a pBAD-InvR expression plasmid (pKP7-25) grown to early stationary phase, induced with L-arabinose (0.2%), and RNA was extracted prior to (0 min) or at various time points within 16 min of induction as indicated. As in (B) Northern blots were probed for *ompD* mRNA, InvR and 5S rRNA (loading control). Quantification of the *ompD* signals revealed that InvR expression reduces the half-life of the mRNA to less than 4 min.
D. Predicted antisense interaction of InvR RNA and *ompD* mRNA. Numbers denote the interacting residues in the *ompD* mRNA or InvR RNA (relative to the mapped +1 sites).

(Sittka *et al*., 2007). Moreover, loss of Hfq results in aberrant levels of porins and other envelope proteins (Figueroa-Bossi *et al*., 2006; Sittka *et al*., 2007), the most marked defect being a strong accumulation of OmpD (Sittka *et al*., 2007). As the latter was due to defective translational repression, we hypothesized the existence of Hfq-dependent sRNAs that repress the *ompD* mRNA (Sittka *et al*., 2007). Following the earlier discovery of the RybB sRNA (Papenfort *et al*., 2006), InvR is here revealed as another sRNA to confirm this expectation. Note, however, that RybB and InvR act under different conditions; RybB is strictly controlled by the alternative sigma factor, σ^E, and is induced upon envelope stress (Johansen *et al*., 2006; Papenfort *et al*., 2006; Thompson *et al*., 2007), whereas InvR acts under conditions that favour SPI-1 gene expression.

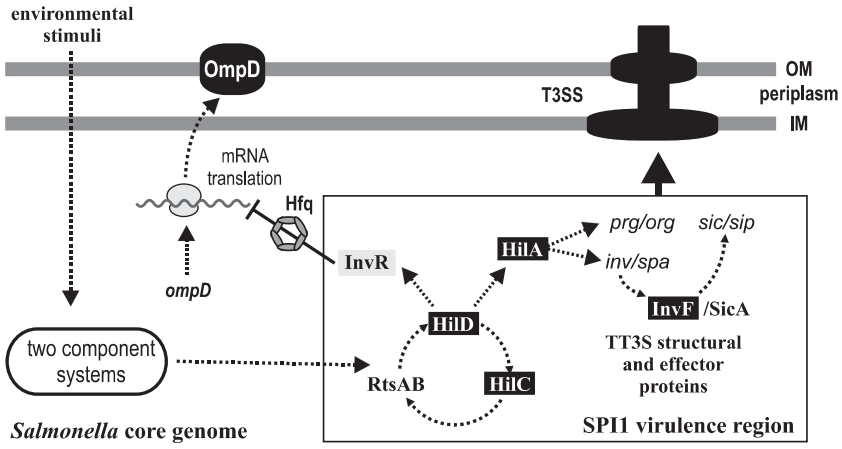

Fig. 9. Proposed model of SPI1-mediated porin repression. Two component systems encoded by the *Salmonella* core genome sense environmental signals that lead to activation of the SPI-1 transcription factor cascade (HilD, HilC, HilA, InvF), and subsequently, to the expression of the SPI-1 T3SS. HilD also activates expression of the SPI-1-encoded, non-coding InvR RNA. Together with the RNA chaperone Hfq, InvR acts to repress synthesis of the major outer membrane protein, OmpD, which is encoded by the *Salmonella* core genome.

OmpD is the most abundant *Salmonella* porin ($> 1 \times 10^5$ molecules per cell; Lee and Schnaitman, 1980). The *ompD* gene is regulated at both the transcriptional and the post-transcriptional level (Santiviago *et al*., 2003), and even a slight increase in OmpD protein levels by ectopic expression can trigger cell lysis, presumably by compromising envelope stability (V. Pfeiffer and J. Vogel, unpubl. data). Intriguingly, several *E. coli* sRNAs that repress abundant outer membrane proteins (OMPs) are encoded adjacent to other *omp* genes, with which they may have been horizontally acquired (Douchin *et al*., 2006; Guillier *et al*., 2006; Vogel and Papenfort, 2006). It has been suggested that as excess OMP synthesis can be deleterious, such sRNAs have evolved to modulate the synthesis of OMPs already present in the recipient strain until newly acquired OMPs become established (Douchin *et al*., 2006). The recent demonstration that *E. coli* IpeX sRNA, which originates from the transcript of a phage-derived porin gene, represses other major porins lends support to this hypothesis (Castillo-Keller *et al*., 2006). In analogy, the InvR-mediated repression of OmpD may have provided an evolutionary advantage for establishing the membrane-anchored T3SS structure upon the arrival of SPI-1 in the *Salmonella* lineage by balancing envelope stability. Subsequently, InvR may have evolved additional functions in *Salmonella* gene expression.

Generally, porins such as OmpD provide surface-exposed epitopes used by the host's immune response after infection (Singh *et al*., 2000), and the immunostimmulatory activity of *Salmonella* OMPs has been well established (Galdiero *et al*., 1990; 1993; Vitiello *et al*., 2004). Preliminary experiments with the *invR* and *ompD* mutant strains failed to detect significant changes in the production of the cytokine IL-8 in HeLa cells upon *Salmonella* infection (V. Pfeiffer and J. Vogel, unpubl. data). However, too little is currently known as to the physiological roles of OmpD (Santiviago *et al*., 2002). Although the protein facilitates *Salmonella* adherence to human macrophages and intestinal epithelial cell lines (Negm and Pistole, 1998; Hara-Kaonga and Pistole, 2004), its overall contribution to pathogenicity has not been clear (Dorman *et al*., 1989; Meyer *et al*., 1998). Thus, future work will focus on the identification of an experimental system in which phenotypes of *invR* or *ompD* mutants can be studied.

Experimental procedures

Bacterial strains, media and growth conditions

Salmonella enterica serovar Typhimurium strain SL1344 was used as the wild-type strain and for mutant construction. The bacterial strains and plasmids used in this study are listed in Tables 2 and 3. Growth under standard and SPI-1-inducing conditions, including antibiotic concentrations, was performed as described in Sittka *et al*. (2007). A culture grown to an OD_{600} of 2 is referred to as early stationary-phase culture throughout the manuscript. For culture grown under low oxygen conditions, 35 ml in Schott flasks was inoculated 1:100 and incubated at 37°C, 50 r.p.m. Samples were taken at an OD of 0.4 or 2. For expression of HilD and HilA from an inducible P_{BAD} promoter, strains carrying plasmids (pCH112, pSA4) were cultured to an OD_{600} of 1. After splitting the culture, one was induced for 45 min by addition of L-arabinose (final concentration of 0.1%). To induce the expression of *invR* from the P_{BAD} promoter, cultures were grown to an OD_{600} of 2 and induced with a final concentration of 0.2% L-arabinose for 2, 4, 8 and 16 min.

Strain construction

Chromosomal mutagenesis of *Salmonella* SL1344 followed the protocol described by Datsenko and Wanner (2000) with few modifications as described in Sittka *et al*. (2007). For construction of the *invR* deletion strain (JVS-00175), the *kan* resistance cassette gene was amplified with oligonucleotides JVO-0299/-0300 (see Table 4 for sequences of deoxyoligonucleotides used as PCR primers and Northern blot probes) from plasmid pKD4. Mutants were verified by colony PCR using the oligonucleotides JVO-0301/-0302. For removal of the *kan* marker the Δ*invR* strain was transformed with the FLP helper plasmid pCP20 (Datsenko and Wanner, 2000). For *invR* complementation at the *istR-tisAB* locus, a *istR-tisAB*::cat deletion strain (JVS-00608) was constructed. The *cat* chloramphenicol resistance cassette gene was amplified from plasmid pKD3 with oligonucleotides JVO-0710/-0711 and transformed into wild-type SL1344 harbouring plasmid pKD46. The deletion was verified by colony PCR using primers JVO-0037/-0038. For complementation of *invR* under control of either a P_L promoter or the native promoter at the *istR-tisAB* locus, DNA fragments were amplified from plasmid pJV872-5 or pVP011, respectively, using primers JVO-0713/-0712. The *cat* gene was amplified using JVO-0710/-0203. The *cat* gene and the *invR* PCR products were used as templates for a fusion PCR protocol to generate P_L-*invR*::cat and *invR*::cat with primer pair JVO-0710/-0712 and were transformed as above. The Δ*istR-tisAB*::cat::P_L-*invR*, the Δ*istR-tisAB*::cat::*invR* locus, as well as Δ*istR-tisAB*::cat, respectively, were transduced using phage P22 into the Δ*invR* strain (Km^R removed; JVS-00487), generating JVS-00610/-00632/-00609. The *sitA-invH* deletion strain was generated by insertion of the *kan* resistance gene of plasmid pKD4 (PCR with primers JVO-0545/-0546) and verified by colony PCR with JVO-0547/-0302. The construction of the chromosomal hfq^{FLAG} strain followed the protocol published by Uzzau *et al*. (2001). Wild-type *Salmonella* SL1344 containing plasmid pKD46 was transformed with a PCR product generated on plasmid pSUB11 and using primers JVO-0935/-0936. Integration yields a strain carrying *hfq*::*FLAG*::*kan*. Verification was carried out using oligos JVO-0076/-0077 in colony PCR. After transduction into fresh wild-type background using phage P22, the kanamycin resistance gene was eliminated using pCP20 (see above), resulting in strain JVS-01338.

Plasmids

To clone the *invR* gene, a PCR product amplified with JVO-0301/-0302 was digested with XbaI/XhoI and ligated into

Table 2. Strains used in this study.

Strain	Name in manuscript	Relevant markers/genotype	Reference/source
S. typhimurium			
SL1344	Wild type	Str^R *hisG rpsL xyl*	Hoiseth and Stocker (1981), source: D. Bumann, MPI-IB Berlin
JVS-00175	Δ*invR*	SL1344 Δ*invR*::Km^R	This study
EE639 (JVS-00212)	*invF*	SL1344 *invF*12-5::Tn*5*::Tet^R (lacZY)	Bajaj *et al*. (1996); functional disruption of *invF*
EE658 (JVS-00214)	*hilA*	SL1344 *hilA*080::Tn*5* (lacZY)	Bajaj *et al*. (1996); functional disruption of *hilA*
EE635 (JVS-00216)	*hilC*	SL1344 *hilC*9::Tn*5*::Tet^R (lacZY)	Schechter *et al*. (1999); functional disruption of *hilC*
RL696 (JVS-00218)	*hilD*	SL1344 *hilD*696::Tn*5* (lacZ)	Lucas and Lee (2001); functional disruption of *hilD*
JVS-00255	Δ*hfq*	SL1344 Δ*hfq*::Cm^R	Sittka *et al*. (2007)
JVS-00289	Δ*invR*/Δ*hfq*	SL1344 Δ*invR*::Km^R/Δ*hfq*::Cm^R	JVS-00175 was transduced with P22 lysate of JVS-00255
JVS-00408	Δ*sitA-invH*	SL1344 Δ*sitA-invH*::Km^R	This study
JVS-00487	Δ*invR*	SL1344 Δ*invR* (Km^R removed)	This study
JVS-00584	*hfq* in Fig. 5B	SL1344 Δ*hfq* (Cm^R removed)	Sittka *et al*. (2007)
JVS-00608	Wild type in Fig. 3A, Fig. S3, Figs 4–6	SL1344 Δ*istR-tisAB*::Cm^R	This study
JVS-00609	Δ*invR* in Fig. 3A, Fig. S3, Figs 4–6	SL1344 Δ*invR*/Δ*istR-tisAB*::cat	This study
JVS-00610	*invR*+ in Fig. S3, Figs 4–6	SL1344 Δ*invR*/Δ*istR-tisAB*::cat::P$_L$ -*invR*	This study
JVS-00632	*invR*+ in Fig. 3A	SL1344 Δ*invR*/Δ*istR-tisAB*::cat::*invR*	This study
JVS-00735	Δ*ompD*	SL1344 Δ*ompD*::Km^R	Sittka *et al*. (2007)
JVS-01338	*hfq*::FLAG	SL1344 *hfq*::FLAG (Km^R removed)	This study
JVS-01505	Δ*relA*/Δ*spoT*	SL1344 Δ*relA*/Δ*spoT*211::Tn*10*	KT4478 (K. Tedin)
SB161	Δ*invG*	SL1344 Δ*invG*	Kaniga *et al*. (1994)
E. coli TOP10		*mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L *end*A1 *nup*G	Invitrogen
E. coli TOP10F′		as TOP10 but additionally carrying F′{*lac*I^q Tn*10* (Tet^R)}	Invitrogen

XbaI/XhoI cut plasmids pZE12-luc, yielding pRT2 (ColE1 plasmid), or pVP003, yielding pVP011 (pSC101* origin). Oligo JVO-0301 binds 256 nt upstream of the +1 site of *invR*, while JVO-0302 binds 131 nt downstream of the *invR* terminator. For generating a plasmid that overexpresses *invR* from a P_{LlacO} promoter (pJV872-5), a PCR fragment amplified with oligos pZE-*Xba*I and JVO-0365 (the latter binds to the +1 site of *invR*) from template pRT2 plasmid was cloned into pZE12-luc as outlined in Vogel *et al*. (2004; cf. construction of P_L-IstR plasmids).

Transcriptional P_{invR}–*gfp* fusion plasmid pVP021 was constructed by cloning an AatII/NheI-digested PCR fragment (primers JVO-1276/1277) into plasmid pAS0046 digested with the same enzymes. The cloned region spans from −386 nt to +16 nt corresponding to the +1 site of *invR*. All plasmids were purified using the Machery-Nagel Plasmid QuickPure Kit. *E. coli* TOP 10 and TOP 10F′ strains were used for cloning.

P22 transduction

P22 lysates were prepared from soft agar plate lysates of donor strains using P22 phage HT/105-1 by standard procedures. Transductions were performed as described (Sternberg and Maurer, 1991) using P22 phage HT/105-1.

Identification of Salmonella*-specific small RNA genes*

Genome sequences and annotations for *Salmonella* LT2 (NC_003197) and *E. coli* K12 (NC_000913) were downloaded from NCBI Genome Database (ftp://ftp.ncbi.nih.gov/genomes/Bacteria/). For identifying putative sRNA encoding genes in this organism, we adopted an approach similar to Chen *et al*. (2002). A database of Inter-Genic (IG, sequence between annotated genes on either strand) sequences, greater than or equal to 100 bp, was created using the GenBank annotation. RNAMotif (Macke *et al*., 2001), which searches for RNA structure motifs, was used for identifying putative ρ-independent terminators in the IG sequences. The 'descriptor' and scoring scheme used were the same as reported in Lesnik *et al*. (2001). We then used *pftools2.3* (Bucher *et al*., 1996; http://www.isrec.isb-sib.ch/ftp-server/pftools/) for searching promoters in intergenic sequences. The profile describing the promoter sequences for σ^{70} of *E. coli*, included in *pftools2.3* package, was used together with a cut-off of 50 (Chen *et al*., 2002). To identify putative sRNA-encoding genes, predicted promoters and terminators (with score better than −4; Lesnik *et al*., 2001) were combined such that both are positioned in the same intergenic sequence and are in the same orientation, and the resulting length (between the end of the predicted promoter and the end of the predicted terminator) is ≥ 50 bp and ≤ 350 bp. To

Table 3. Plasmids used in this study.

Plasmid trivial name	Plasmid stock name	Relevant fragment	Comment	Origin, marker	Reference
	pJV300		ColE1 control plasmid, expresses a ~50 nt nonsense transcript derived from *rrnB* terminator	ColE1, Amp^R	Sittka *et al*. (2007)
	pXG-0		Background control plasmid for GFP reporter fusions (no GFP)	pSC101*, Cm^R	Urban and Vogel (2007)
p*invR*	pRT2	*invR*	ColE1 plasmid for *invR* complementation, based on pZE12-luc, carries a 478 bp *invR* fragment	ColE1, Amp^R	This study
P_L-*invR*	pJV872-5	P_{LlacO}-*invR*	ColE1 plasmid, based on pZE12-luc, expresses *invR* from a P_{LlacO} promoter	ColE1, Amp^R	This study
pBAD	pBAD33		pBAD control plasmid for pBAD-HilA (pCH112) and pBAD-HilD (pSA4)	pACYC184, Cm^R	Guzman *et al*. (1995)
pBAD-HilA	pCH112	P_{BAD}-*hilA-Myc-His*	pHilA; *hilA* ORF in pBAD/*Myc*-His	pBR322, Amp^R	Lostroh *et al*. (2000)
pBAD-HilD	pSA4	P_{BAD}-*hilD*	pHilD; *hilD* in pBAD expression vector	pACYC184, Cm^R	Lucas and Lee (2001)
	pVP003	*luc*	Control plasmid; low-copy-version pZE12-luc	pSC101*, Amp^R	Sittka *et al*. (2007)
	pVP004	Hfq6HIS	Expresses a HIS-tagged Hfq under control of the *hfq* promoter	pSC101*, Amp^R	Sittka *et al*. (2007)
	pVP011	*invR*	pSC101* plasmid for *invR* complementation, based on pVP003, carries a 478 bp *invR* fragment	pSC101*, Amp^R	This study
ompD′–gfp	pVP019	*ompD′–gfp*	*ompD* transcriptional *gfp* fusion plasmid	pSC101*/Cm^R	Sittka *et al*. (2007)
P_{invR}–*gfp*	pVP021	P_{invR}–*gfp*	*invR* transcriptional Gfp fusion plasmid	pSC101*, Cm^R	This study
	pAS0046	*′gfp*	Background control plasmid for transcriptional *gfp* fusion plasmid	pSC101*, Cm^R	Sittka *et al*. (2007)
P_L–*gfp*	pJV859-8/pXG-1	P_{LtetO}–*gfp*	*gfp* control plasmid (constitutive GFP expression)	pSC101*/Cm^R	Urban and Vogel (2007)
	pKD3		Template for Cm^R mutant construction	oriRγ, Amp^R	Datsenko and Wanner (2000)
	pKD4		Template for Km^R mutant construction	oriRγ, Amp^R	Datsenko and Wanner (2000)
	pKD46	P_{araB}-*γ-β-exo*	Temperature-sensitive *lambda red* recombinase expression plasmid	oriR101, Amp^R	Datsenko and Wanner (2000)
	pCP20		Temperature-sensitive FLP recombinase expression plasmid	oriR101, Amp^R and Cm^R	Datsenko and Wanner (2000)
	pSUB11		Template for mutant construction; 3xFLAG-tag sequence linked to a Km^R cassette	R6KoriV, Amp^R	Uzzau *et al*. (2001)
luc	pZE12-luc	*luc*	General expression plasmid	ColE1, Amp^R	Lutz and Bujard (1997)
	pM1644	P_{own}-*sipAM45-β-lactamase*	SipAM45-β-lactamase expression plasmid, controlled by the *sipA* promoter	pBR322, Amp^R	A. Schlumberger *et al*. (2007)

identify sRNA genes present in *Salmonella* but not *E. coli*, predicted sRNA sequences were searched against a local database of *E. coli* genome using BLASTN. Sequences which gave hits with *E*-value better than 1E-05 or normalized score (BLAST score divided by the length of sRNA) better than 0.7 were discarded. The remaining sRNA sequences were further searched against NCBI database using BLASTN and were checked manually. Furthermore, sRNA sequences were searched against a local database of *S. bongori* genome (ftp://ftp.sanger.ac.uk/pub/pathogens/Salmonella/) using BLASTN with an *E*-value cut-off of 0.01.

Stability experiments, RNA isolation and Northern hybridization

The detailed protocol for isolation, Northern blot detection, an stability determination of *Salmonella* RNAs, is described in Sittka *et al*. (2007). InvR was detected with ^{32}P-labelled oligodeoxynucleotide JVO-0222, whereas 5S rRNA signals were detected with labelled oligo JVO-0322. The *ompD* mRNA was detected with a ^{32}P-labelled riboprobe generated by *in vitro* transcription. For stability experiments, rifampicin was added at a final concentration of 500 μg ml^{-1}. Northern blots and gels were visualized on a Phosphorimager (FLA-3000 Series, Fuji), and band intensities quantified with AIDA software (Raytest, Germany).

5′ Race

RNA was extracted from wild-type *Salmonella* grown to an OD_{600} of 2, and treated with DNase I. Six micrograms of DNA-free RNA was treated with TAP (Epicentre; removes the β- and γ-phosphate groups at the capped 5′ end of the RNA) in the presence of SUPERase inhibitor (10 U final in a 50 μl reaction; Ambion) at 37°C for 30 min. Mock treatment of RNA was performed in the same reaction buffer but omitting the enzyme. Subsequently, RNA was ligated to a 5′ RNA-

Table 4. Oligonucleotides used in this study.

Name	Sequence in 5′ to 3′ direction
JVO-0037	GTTTTTCTCGAGCGAGATGGCGCAGTT
JVO-0038	GTTTTTTCTAGACGTCGTTGAGGGTGTCATA
JVO-0076	GAAGTATTACAGGTTGTTGGTG
JVO-0077	GCATCATAACGGTCAAACA
JVO-0203	GGTCCATATGAATATCCTCCTTAG
JVO-0222	GATAAATGCAACGTAAGAGACAAATG
JVO-0273	GTTTTTTGCTAGCAAGGCGATTAAGTTGG
JVO-0299	CTCATCATGATATAATTAAGACCATATTTTGCATTGCCACTTAATATCAAGTGTAGGCTGGAGCTGCTTC
JVO-0300	CAGTATAAGTTTCGGCTATTTCCCAAGTCTGGGAGGCCGTTCTTTATCAGGTCCATATGAATATCCTCCTTAG
JVO-0301	GTTTTTCTCGAGCAGGCGCTCACCTTCTT
JVO-0302	GTTTTTTCTAGAGGATGGGGTATCGTAAGAAT
JVO-0322	CTACGGCGTTTCACTTCTGAGTTC
JVO-0365	GTCACTTTTACGGTTGGC
JVO-0367	ACTGACATGGAGGAGGGA
JVO-0545	CACTATCACTGCATATTGTCGCCATTTCGCAAATAAGAATTATTTTCATTGTGTAGGCTGGAGCTGCTTC
JVO-0546	CTCATCAATACTATTTGCGTTGGCCAGTTGCTCTTTCTGAGCGCCAGGTTGGTCCATATGAATATCCTCCTTAG
JVO-0547	GTTTTTCTCGAGCGTGCTCTCTCCGAACA
JVO-0675	GTTTTTTTAATACGACTCACTATAGGGAGGTCACTTTTACGGTTG
JVO-0676	AAAGCAGCAGCGAGG
JVO-0688	GAGGATGATACTGCTCATAAC
JVO-0689	CAATAGAGATTAGTTTTGTAGCTATC
JVO-0690	GCGCTCAGAAAGAGCAACT
JVO-0691	GCGCAACGCAATTAATGT
JVO-0709	GTTTTTGCTAGCGTGAACTTTACCGTACA
JVO-0710	ATTTCACAATGCCGGAAAACAAAAAACCTCGCCGAAGCGAGGTGTAGGCTGGAGCTGCTTC
JVO-0711	GGCTTGAATCTGAATTACTTAAGGTATTTCAGAACAGCATCAAGGTCCATATGAATATCCTCCTTAG
JVO-0712	GGCTTGAATCTGAATTACTTAAGGTATTTCAGAACAGCATCAACGCGATATACGAGACGA
JVO-0713	CTAAGGAGGATATTCATATGGACCTTTCGTCTTCACCTCGAG
JVO-0817	GTTTTTCTCGAGCCAATAGTCCCCTCCGA
JVO-0818	GTTTTTTCTAGACTGCACGGCATACTCCT
JVO-0935	GCAGGGGTCTACTGCGCAACAGGACAGCGAAGAGACTGAAGACTACAAAGACCATGACGG
JVO-0936	ATCCGACGCCCCCGACATGGATAAACAGCGCGTGAACTTACCATATGAATATCCTCCTTAG
JVO-0943	GTTTTTTTTAATACGACTCACTATAGGTCACTTTTACGGTTGG
JVO-1058	CGTGAACTTTACCGTACA
JVO-1186	TTTTCTCGAGTTAATACGACTCACTATAGGCCATTGACAAACG
JVO-1276	GTTTTGACGTCTCTTCCGTATGTGCC
JVO-1277	GTTTTGCTAGCCGTAAAAGTGACCATAGC
pZE-XbaI	TCGTTTTATTTGATGCCTCTAGA

oligonucleotide adaptor (400 pmol per reaction) using T4 RNA ligase (40 U per 20 μl reaction) at 17°C overnight, purified with a mixture of phenol : chloroform : isopropanol (25:24:1 v/v), and precipitated with 2.5 volumes of an ethanol/sodiumacetate (30:1) solution. Samples were re-suspended in 10 μl of water. Half of the ligated RNA was used for the reverse transcription polymerase chain reaction (RT-PCR) with a random hexamer oligonucleotide primer mix (250 ng per 20 μl reaction) in the presence of SUPERase inhibitor (10 U final) using Superscript III RT (200 U final; Invitrogen) and the following programme: 25°C for 5 min, 50°C for 60 min, 70°C for 15 min. RNase H (1 U) was added, followed by incubation at 37°C for 20 min.

Oligonucleotide JVO-0222 (binds 44 bp downstream of the +1 site of *invR*) and JVO-0367 (antisense to the RNA linker) were used to amplify the 5′ end InvR by PCR with 1.25 U of HotStar Taq polymerase and 1 μl of the cDNA in a 50 μl reaction (incubation as follows: 95°C for 10 min, 95°C for 45 s, 58°C for 45 s, 72°C for 45 s, 35 cycles and 72°C for 7 min). The PCR products were separated on a 3% agarose gel. The TAP-specific band was cut, eluted and sequenced.

EMSA with purified HilD protein

To determine binding of HilD protein to the *invR* promoter region, we performed EMSA following the protocol by Olekhnovich and Kadner (2002) but with slight modifications. The promoter region of the *invR*, the *hilA* or the *lacZ* genes was amplified by PCR with primers JVO-0690/-0222 (bp −437 nt to +45 nt relative to the transcriptional start site), JVO-0688/-0689 (bp −292 nt to +19 nt) or JVO-0273/-0691 (bp −121 nt to +85 nt) respectively. The gel-purified fragments were 5′-end-labelled with [γ-^{32}P]-ATP using polynucleotide kinase, and purified with the NucleoSpin Extract II kit (Machery and Nagel). For the binding assay, 0.2 fmol (20 pM final concentration) labelled DNA fragments were incubated with 1, 5, 10, 20 nM of purified HilD protein (90 μM stock, kindly provided by I.N. Olekhnovich) for 20 min at 37°C in binding buffer (10 mM Tris-HCl pH 8.0, 50 mM KCl, 100 μg ml^{-1} BSA, 10% glycerol, 1 mM DTT, 0.5 mM EDTA) in a 10 μl reaction. Dilutions of HilD were prepared in binding buffer. Three microlitres of loading buffer (50% glycerol, 0.5× TBE, 0.2% bromphenolblue) was added to the reaction. The mixture was separated on a running native 5% polyacrylamide gel cooled

to 4°C at 300 V for 3 h 30 min. The dried gel was analysed using a phosphorimager.

Determination of in vivo *abundance of InvR*

Total RNA from the wild type at an OD_{600} of 2.0 (a total of 3 OD) was isolated using the TRIzol reagent, followed by DNase I treatment (3 OD corresponds to 3.3×10^9 cells). The PCR template for *in vitro* transcription of *invR* with a T7 promoter was amplified with JVO-0675/-0676 (JVO-0675 adds a T7 promoter to the +1 site of *invR*). The DNA was transcribed *in vitro* using the Megascript kit (Ambion), followed by a DNase I treatment and organic extraction. One hundred nanograms (3.6 pmol) of the *in vitro* transcribed InvR RNA (88 nt) corresponds to 2.13×10^{12} molecules. Increasing amounts of the *in vitro* synthesized RNA were mixed with 5 µl of total RNA (corresponding to 2.2×10^8 cells) and separated on a 6% polyacrylamide gel containing 7 M Urea and subjected to Northern hybridization.

In vitro *binding experiments (gel mobility shift assays)*

InvR RNA was synthesized by *in vitro* transcription with T7 RNA polymerase (Megascript, Ambion) from PCR fragments amplified with oligos JVO-0943/-0676. Binding assays, native gel electrophoresis and RNA visualization were performed as described in Sittka *et al*. (2007). RNA and Hfq (hexamer) concentrations are given in the figure legends (see Fig. 4C).

Co-immunoprecipitation

The *hfq* gene was chromosomally tagged with a C-terminal 3xFLAG-tag sequence as described in Uzzau *et al*. (2001), yielding strain hfq^{FLAG}. Cells were cultured to early stationary phase, and collected by centrifugation (40 min, 4000 *g*, 4°C). The pellet was washed once with 2 ml of lysis buffer (20 mM Tris pH 8, 150 mM KCl, 1 mM $MgCl_2$, 1 mM DTT), and snap-frozen in liquid nitrogen. Upon re-suspension in 0.8 ml of lysis buffer, 0.8 ml glass beads (Roth, diameter 0.1 mm) were added, and cells broken by vortexing (30 s burst followed by 30 s chill on ice) for 5 min. Lysis buffer (0.4 ml) was added, followed by centrifugation [30 min at 4°C, 16 000 relative centrifugation force (RCF)]. The cleared lysate (0.1 ml) was removed to prepare total RNA. Twenty-five microlitres of FLAG antibody (Sigma; #F1804) was added to the remaining cleared lysate (0.9 ml), followed by incubation on a rotator at 4°C for 1 h. Fifty microlitres Protein A sepharose beads (Sigma; P-6649-5ML) were added, and incubation continued for 1 h. The suspension was centrifuged (5 min, 4°C, 16 000 RCF), followed by five washes in 1 ml of lysis buffer. Protein/RNA complexes were recovered from the beads by re-suspension in 0.5 ml of water. Phenol : chloroform extracted RNA was concentrated by ethanol precipitation, followed by DNase I treatment. For Northern blot detection, total RNA and co-immunoprecipitated RNA equivalent to 0.25 and 2.5 OD_{600}, respectively, of the original culture volume were used.

GFP reporter assays

Strains carrying the transcriptional *invR*::GFP fusion plasmid (pVP021) or a control plasmid (pAS0046) were inoculated in 5 ml of LB containing 0.3 M NaCl supplemented with 20 µg ml^{-1} chloramphenicol in 15 ml Falcon tubes with a tightly closed lid. The cultures were incubated for 12 h at 37°C. Culture (3×100 µl) was transferred to a 96-well plate, and fluorescence was measured at 37°C using a VICTOR3$_{TM}$ machine (1420 Multilable Counter, Perkin Elmer). All experiments were performed in triplicates. Strains with a non-fluorescent plasmid served as background control (autofluorescence). A detailed protocol of fluorescence measurement is given in Urban and Vogel (2007).

Secreted and whole-cell protein fractions

The protocol for extraction of secreted protein fractions was modified from the protocol described in Kaniga *et al*. (1995). Culture samples were taken from regular LB cultures at an OD_{600} of 2 or from SPI-1-induced cultures, and spun 20 min at 16 100 *g* at 4°C. Proteins from the supernatant were precipitated by adding 25% TCA to a final concentration of 5% followed by 20 min centrifugation at 16 100 *g*, 4°C. The pellet was washed twice in ice-cold acetone and air dried. The pellet was re-suspended in 1× SLB to a final concentration of 1.5 OD per 10 µl. Samples were heated for 5 min at 95°C. For small 12% SDS-PAGE 1 OD, respectively, were loaded per sample. For whole-cell protein fractions 0.1 OD per 10 µl were separated on a 12% SDS-PAGE, and visualized by staining with Sypro Ruby (Bio-Rad).

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