

# Small RNA-based feedforward loop with AND-gate logic regulates extrachromosomal DNA transfer in *Salmonella*

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**Horizontal gene transfer via plasmid conjugation is a major driving force in microbial evolution but constitutes a complex process that requires synchronization with the physiological state of the host bacteria. Although several host transcription factors are known to regulate plasmid-borne transfer genes, RNA-based regulatory circuits for host–plasmid communication remain unknown. We describe a posttranscriptional mechanism whereby the Hfq-dependent small RNA, RprA, inhibits transfer of pSLT, the virulence plasmid of *Salmonella enterica*. RprA employs two separate seed-pairing domains to activate the mRNAs of both the sigma-factor  $\sigma^5$  and the Ricl protein, a previously uncharacterized membrane protein here shown to inhibit conjugation. Transcription of *ricI* requires  $\sigma^5$  and, together, RprA and  $\sigma^5$  orchestrate a coherent feedforward loop with AND-gate logic to tightly control the activation of Ricl synthesis. Ricl interacts with the conjugation apparatus protein TraV and limits plasmid transfer under membrane-damaging conditions. To our knowledge, this study reports the first small RNA-controlled feedforward loop relying on posttranscriptional activation of two independent targets and an unexpected role of the conserved RprA small RNA in controlling extrachromosomal DNA transfer.**

RprA | sRNA | feedforward control | plasmid conjugation | Hfq

Intercellular transmission of plasmid DNA is key to bacterial evolution and diversity (1). In the widespread family of F-like conjugative plasmids, environmental cues and mating partner availability affect conjugation frequency, and unregulated conjugation comes with significant fitness costs for the host (2). It is well understood how conjugation is regulated by plasmid-borne factors. For example, conjugation of the self-transmissible F-like plasmid pSLT [which encodes several virulence genes and is required for systemic disease (3)] of *Salmonella* species depends on TraJ, the transcriptional activator of the transfer (*tra*) operon (4). Synthesis of TraJ itself is precisely controlled by a *cis*-antisense RNA, FinP, which in concert with the dedicated RNA chaperone, FinO, inhibits translation of the *traJ* mRNA (5). As a result, most cells reside in a conjugational OFF-state under regular physiological conditions (6).

Besides plasmid-encoded factors, core genome-encoded proteins such as the leucine-responsive regulatory protein (Lrp), the ArcAB two-component system, and Dam methylation affect pSLT conjugation (7, 8). These factors usually respond to specific ecological cues; for example, ArcAB responds to microaerophilic conditions, the environment that bacteria will encounter in the intestine of infected hosts (9). In addition, host-produced compounds such as bile salts repress pSLT conjugation, but the underlying molecular mechanisms are unknown (10).

The regulatory networks of the host that restrict plasmid conjugation to permissive conditions must integrate various physiological signals. This may involve small RNAs (sRNAs) that can cross-connect gene expression at the posttranscriptional level through their ability to repress or activate multiple target mRNAs (11, 12). Intriguingly, the RNA chaperone Hfq, which helps many

sRNAs to regulate their targets (13, 14), has been reported to affect the transfer of F-like plasmids (15), suggesting that host–plasmid communication does involve regulatory activities of noncoding RNA molecules. However, Hfq-dependent sRNAs controlling plasmid conjugation were hitherto unknown.

In this work, we have studied the role of RpoS regulator RNA A (RprA) in *Salmonella enterica*. RprA is one of three sRNAs (the others being DsrA and ArcZ) that activate translation of *rpoS* mRNA encoding the alternative sigma-factor  $\sigma^S$  (16–18). All three sRNAs act by an anti-antisense mechanism whereby their base pairing with the *rpoS* mRNA opens an inhibitory structure in the 5′-untranslated region (5′-UTR) to promote translation initiation (19). In *Escherichia coli*, expression of RprA is induced during stationary-phase growth (20) through either of two signal transduction pathways, Rcs (21) or CpxAR (22). The fact that both Rcs and CpxAR respond to insults to the bacterial cell envelope (23, 24) suggests a role for RprA in the extracytoplasmic stress responses and membrane homeostasis.

Here, we describe that in *Salmonella* RprA controls a large set of mRNAs in addition to *rpoS*, including *ricI* (STM4242) mRNA. Similar to its known *rpoS* target (21), RprA activates the *ricI* mRNA through opening of an inhibitory structure in the 5′-UTR. However, unlike *rpoS* regulation, which is regulated through the 5′ end of RprA, it is the conserved 3′ end of RprA that recog-

## Significance

**Horizontal gene transfer is a major force in bacterial evolution, and a widespread mechanism involves conjugative plasmids. Albeit potentially beneficial at the population level, plasmid transfer is a burden for individual cells. Therefore, assembly of the conjugation machinery is strictly controlled, especially under stress. Here, we describe an RNA-based regulatory circuit in host–plasmid communication where a regulatory RNA (RprA) inhibits plasmid transfer through posttranscriptional activation of two genes. Because one of the activated factors ( $\sigma^5$ ) is necessary for transcription of the other (Ricl), RprA forms the centerpiece of a posttranscriptional feedforward loop with AND-gate logic for gene activation. We also show that the synthesis of Ricl, a membrane protein, inhibits plasmid transfer, presumably by interference with pilus assembly.**

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nizes the *ricI* mRNA, revealing RprA as the first (to our knowledge) regulatory RNA with two activating seed-pairing domains.

From a physiological point of view, RicI is shown to inhibit pSLT conjugation through interaction with anchor protein TraV of the type IV secretion apparatus to restrict the number of conjugation pili. RprA activates the synthesis of RicI in the presence of bile salts, and components of the Rcs phosphorelay as well as  $\sigma^S$  are required for this process. Thus, RprA and  $\sigma^S$  act in concert to activate RicI synthesis via a feedforward loop (FFL) with AND-gate decision logic to control plasmid transfer. Donor cells lacking one component of this regulatory mechanism, i.e., RprA,  $\sigma^S$ , or RicI, display increased conjugation rates, a phenotype that is exacerbated under conditions of envelope stress.

## Results

**Two Isoforms of RprA Regulate Target mRNAs.** The function of RprA as a posttranscriptional activator of  $\sigma^S$  synthesis has been well established in *E. coli* (20, 21, 25, 26). By contrast, this “core” sRNA, which is conserved in many enterobacterial species, was reported to have little if any role in the closely related pathogen, *Salmonella* Typhimurium (27), although both RprA and its target region in the *rpoS* mRNA are fully conserved (28, 29). To address this discrepancy, we monitored RprA expression in *Salmonella* on a Northern blot using a probe complementary to the conserved 3′ end of the sRNA. Expression of RprA peaked during stationary phase, and we detected two forms of RprA: a full-length transcript of ~107 nt and a shorter processed 3′-end fragment of ~50 nt (Fig. 1*A* and *B*). This is in agreement with previous studies in *E. coli* (30) and RNA-seq experiments in *Salmonella* (28, 31, 32).

To identify mRNA targets of both RprA forms, we pulse-expressed (10 min) either the full-length or the processed sRNA from a pBAD promoter and scored global transcriptome changes on microarrays, comparing to an empty vector control (33). Induction of the full-length RprA altered the expression of 64 genes (Fig. 1*C*), one of which was *rpoS* (+2.5-fold), rectifying that  $\sigma^S$  activation by RprA is functionally conserved in *Salmonella*. As expected from the previously mapped RprA-*rpoS* RNA interaction (21, 34), processed RprA did not activate *rpoS* expression. Twelve genes were regulated by both the full-length and processed RprA, 11 of which were repressed by RprA (Fig. 1*C*). We did not observe the previously reported RprA-mediated repression of *csgD* (35, 36), perhaps because the *csgD* promoter was silent under the experimental conditions used here (37). This notwithstanding, our pulse expression data suggested that the two forms of RprA recognize several targets by different seed regions and predicted processed RprA to be a regulator in its own right.

One gene, *ricI* (also known as STM4242), was up-regulated by both forms of RprA (Fig. 1*C*). To test the contribution of each isoform to RicI synthesis, we added a 3×FLAG epitope to the chromosomal *ricI* gene and monitored RicI protein levels upon induction of full-length or processed RprA. Indeed, both forms of RprA equally activated RicI expression (Fig. 1*D*), whereas only full-length RprA induced  $\sigma^S$  production.

**A Second Seed Region in RprA Activates RicI Synthesis.** Next, we sought to understand how RprA activates RicI production. As our pulse expression approach suggested posttranscriptional control, we looked for evidence of activation by the aforementioned “anti-antisense” mechanism (12), whereby the sRNA opens a self-inhibitory structure in the 5′-UTR of its target mRNA (21). Indeed, in silico analysis of the secondary structure of the *ricI* mRNA (from the transcriptional start site to the fifth codon; see below) using the Mfold algorithm (38) predicted a discontinued RNA duplex formed between nucleotides 38–62 and 95–119 (relative to the transcriptional start site; see below) of the *ricI* mRNA (Fig. 2*A*). This structure would sequester the Shine-

Dalgarno sequence (SD) and the GUG translation start codon of *ricI*.

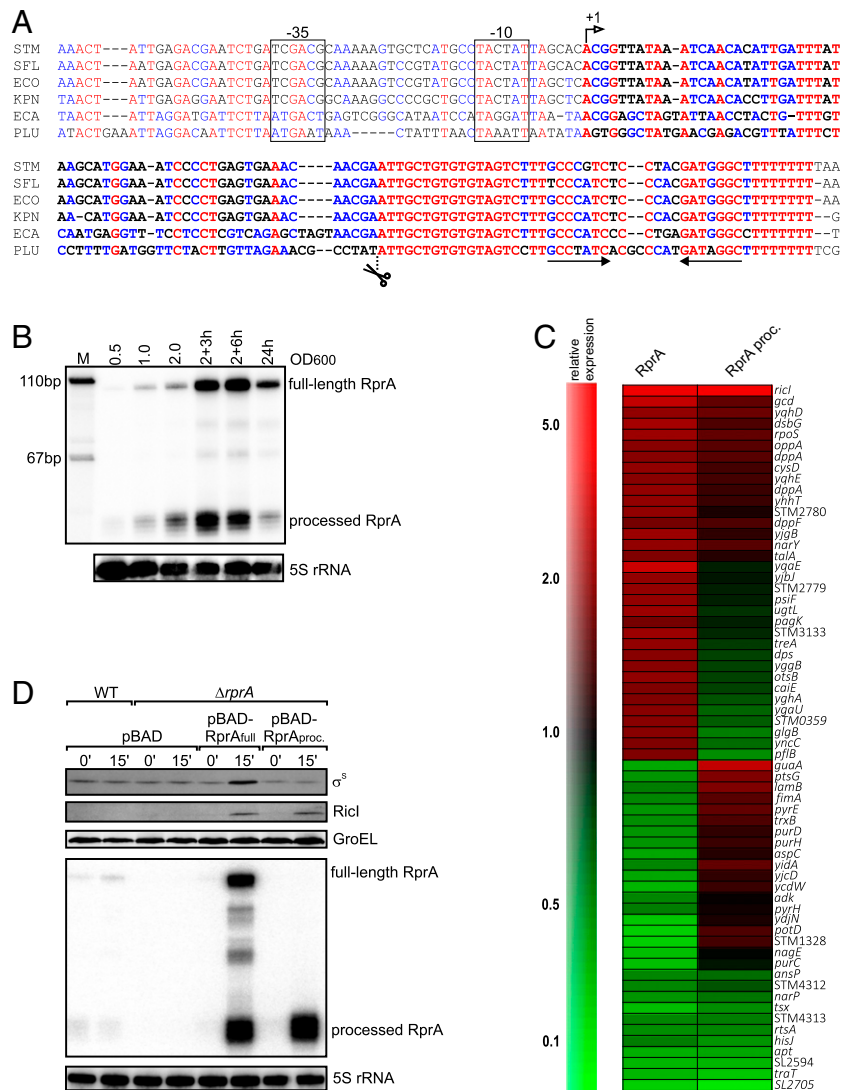
To validate this predicted hairpin and its potential function in translation control, we cloned the sequence of the *ricI* mRNA—from its transcriptional start site to the 10th codon—into a *gfp*-based reporter plasmid designed to report posttranscriptional regulation (39). This construct gave only modest GFP expression. However, when we truncated the *ricI* mRNA at its 5′ end (transcription start at A48; Fig. S1*A*), essentially deleting the sequence predicted to sequester the translational start site, a >50-fold increase in the level of RicI::GFP was observed (Fig. S1*B*). Moreover, a single C→G change opposite the first nucleotide of the start codon (42 nt downstream of the transcriptional start site; see below) increased the expression of the full-length reporter ~13-fold (Fig. S1*B*). In further support of our model that a 5′ hairpin sequesters the *ricI* start codon, two other mutations, G44→C or C113→G (Fig. 2*A* and *B*, compare lane 1 vs. 2 and 3), also increased RicI::GFP synthesis. As expected, however, the combination of these two latter mutations restored wild-type expression levels of RicI::GFP, most likely by restoring mRNA hairpin formation. Together, this mutational analysis suggests that RicI synthesis is intrinsically repressed by intramolecular base pairing in the 5′ region of its mRNA.

Next, we used the *ricI::gfp* reporter to establish that RprA activates translation of this target by preventing self-sequestration of the *ricI* mRNA. Indeed, coexpression of RprA from a compatible plasmid increased RicI::GFP levels by approximately threefold (Fig. 2*C*, lane 1 vs. 2), whereas it had no effect on GFP levels expressed from the pXG-1 control plasmid (Fig. S1*C*) (39). As expected if RprA acted by suppressing hairpin formation, the sRNA had no effect on the truncated (Fig. S1*D*) or “open” (C42→G) variants of *ricI::gfp* (Fig. S1*E*).

Because the processed RprA form sufficed for activation (Fig. 1*C* and *D*), we used its sequence to search for an RprA binding site in the 5′-UTR of *ricI*. The *RNA-hybrid* algorithm (40) predicted a consecutive stretch of nine Watson-Crick base pairs formed between the proximal end of the processed RprA and the internal antisense element of the *ricI* mRNA (Fig. 2*A*). To test this prediction, we constructed an RprA variant with a point mutation in the seed region (G63→C, Fig. 2*A*); as expected, RprAC63 was unable to activate the *ricI::gfp* reporter (Fig. 2*C*, lane 3). Conversely, mutating the corresponding position 45 in the *ricI* 5′-UTR (*ricIG45::gfp* reporter, Fig. 2*A*) abrogated reporter activation by wild-type RprA (Fig. 2*C*; lane 5). Note that this nucleotide is not paired in the intrinsic hairpin and hence will not alter RicI::GFP expression (Fig. 2*C*; lane 1 vs. 4). By contrast, combining both mutants (RprAC63 and *ricIG45::gfp*) fully restored target activation (Fig. 2*C*; lane 6). Thus, RprA uses a similar mechanism but different seed sequences to activate the synthesis of  $\sigma^S$  and RicI.

## Membrane Stress and the Rcs Phosphorelay Activate RicI Production.

The *ricI* gene (also known as STM4242) is conserved in all sequenced *Salmonella* species, including the ancestral *Salmonella bongori* and the human-specific serovar *Salmonella typhi*, but absent in other enterobacterial relatives such as *E. coli*, *Klebsiella pneumoniae*, and *Shigella flexneri* (Fig. S2). Although its biological role has not been investigated, RicI has been reported as a bile salt-induced protein (41). To address this, we monitored production of 3×FLAG-tagged RicI protein in both wild-type and  $\Delta$ rprA cells upon exposure to bile. In *Salmonella* wild-type cells, RicI levels increased approximately fourfold within 15 min after treatment, with a further increase to approximately eightfold after 120 min (Fig. 3*A*, lanes 1–5). By contrast, the  $\Delta$ rprA mutant failed to increase RicI production (Fig. 3*A*, lanes 6–10). These results confirm bile as a potent activator of RicI production but also implicate RprA as an essential factor in this process.

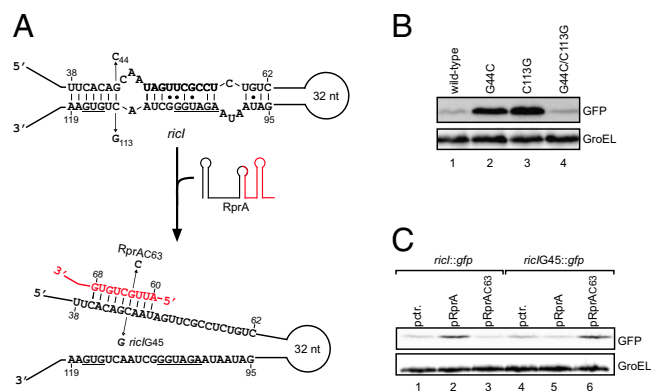


**Fig. 1.** Multiple target regulation by RprA in *Salmonella*. (A) Alignment of the *rprA* gene from selected enterobacterial species (ECA, *Erwinia carotovora*; ECO, *Escherichia coli* K12; KPN, *Klebsiella pneumoniae*; PLU, *Photobacterium luminescens*; SFL, *Shigella flexneri*; STM, *Salmonella enterica* sv. Typhimurium). Transcription control regions  $-10$  and  $-35$  are boxed, the transcription initiation site is marked by an arrow. Scissors indicate the RprA processing site, and inverted arrows refer to the rho-independent terminator. (B) Northern blot analysis of RprA in *Salmonella*. Samples were collected at several stages of growth (OD<sub>600</sub> of 0.5, 1.0, and 2.0, at 3 and 6 h after cells had reached OD<sub>600</sub> of 2.0, and after 24 h of cultivation). 5S rRNA served as loading control. (C) Microarray analysis of RprA full-length and RprA processed pulse expression. Expression profiles of pulse-induced full-length and processed RprA were compared with samples carrying control plasmids. A heat map of genes regulated by full-length RprA (more than twofold) is shown and compared with regulation by processed RprA. (D) Western and Northern blot analyses of  $\sigma^S$ , RicI::3 $\times$ FLAG, and RprA production after pulse expression of full-length and processed RprA. Wild-type and  $\Delta rprA$  carrying the indicated plasmids were grown to early stationary phase (OD<sub>600</sub> of 1.5) and induced for pBAD expression. 5S rRNA (Northern blot) and GroEL (Western blot) served as loading controls.

Bile is a detergent-like substance that can disrupt bacterial membranes (42) and thereby activate the Rcs stress response. Because the Rcs system strongly induces the *rprA* promoter in *E. coli* (21), and other cell envelope-damaging conditions trigger RprA synthesis through RcsB (21, 43–45), we hypothesized that the bile-induced increase in RicI synthesis is indirect, resulting from the Rcs-mediated activation of RprA. To test this, we constructed single-gene deletion strains of various components of the Rcs signaling cascade and evaluated bile-induced changes in the levels of RprA and RicI levels in these mutants. As expected, wild-type cells activated both RprA and RicI expression in the presence of bile (Fig. 3B, lane 1 vs. 2), whereas cells lacking *rprA* (lanes 3 and 4), *rscF* (lanes 5 and 6), or *rscB* (lanes 9 and 10) failed to activate RicI. This corresponded well with a loss of bile-induced activation of RprA in the  $\Delta rcsB$  and  $\Delta rcsF$  mutants,

with the  $\Delta rcsC$  mutant showing intermediate RprA induction that seemed insufficient for RicI activation (lanes 7 and 8). Given the known relationship of RprA and  $\sigma^S$ , we also tested a  $\Delta rpoS$  strain. Surprisingly, bile did not increase RicI levels in the  $\Delta rpoS$  strain, although RprA was fully activated (Fig. 3B, lanes 11 and 12). This suggested that also  $\sigma^S$  was essential for RicI activation but it would act downstream of RprA.

To better understand the role of  $\sigma^S$  in RicI activation, we sought to override Rcs signal transduction by constitutive expression of RprA (from plasmid pKP-112) in the *rprA*, *rscB*, and *rpoS* mutant strains. Midexponential cultures (low endogenous RprA expression; Fig. 1B) were probed for RicI production (Fig. 3C). In support of our previous results, plasmid-borne overexpression of RprA strongly induced RicI expression in wild-type cells and complemented the *rprA* and *rscB* mutant strains (Fig.



**Fig. 2.** Anti-antisense activation of *ricI*. (A) Graphical presentation of the *ricI* 5'-UTR alone (Top) or in complex with RprA (Bottom). Numbering of *ricI* and RprA is relative to their transcription start site. *ricI* base pairs with the 5' terminal end of the processed RprA form. Arrows denote mutations introduced in *ricI::gfp* and RprA, respectively. (B) Western blot analysis of *ricI::gfp* variants (as indicated in A, Top) expressed in *Salmonella*  $\Delta rprA$  cells. (C) Western blot analysis of  $\Delta rprA$  *Salmonella* harboring plasmid pRprA or mutant plasmid, pRprAC63, in combination with either wild-type *ricI::gfp* or mutant *ricIG45::gfp* fusion plasmids. GroEL served as loading control.

3C, lanes 1–6). However, RicI levels remained low in the  $\Delta rpoS$  mutant, suggesting that the function of  $\sigma^S$  was independent of RprA-mediated posttranscriptional activation of *ricI* mRNA.

**$\sigma^S$  Is Required for Transcription of *ricI*.** To further address the requirement of  $\sigma^S$  for RicI synthesis, we tested whether RprA activated the *ricI::gfp* reporter (Fig. 2B, lane 1) in the  $\Delta rpoS$  mutant strain. The *ricI::gfp* reporter gene is transcribed from a constitutive P<sub>LetO</sub> promoter that is insensitive to absence of  $\sigma^S$ . There was no difference to the previously observed approximately threefold activation in wild-type *Salmonella* (Fig. S3A), suggesting that  $\sigma^S$  influences RicI expression at an earlier step, i.e., transcription.

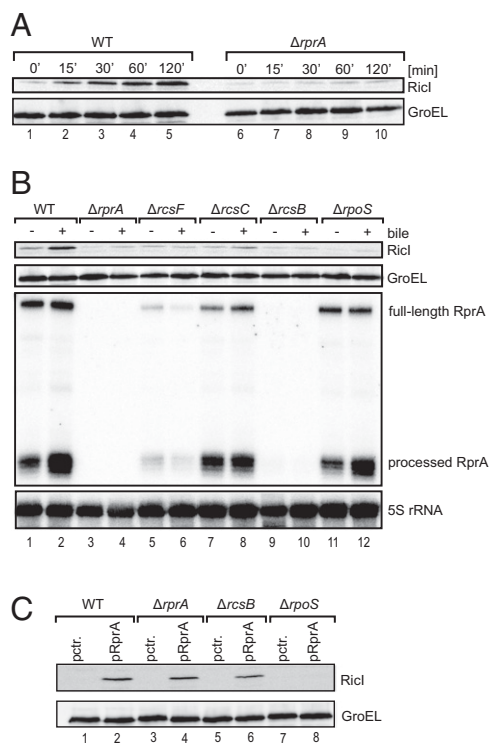
The transcriptional start site of *ricI*, mapped here by 5'-RACE (Fig. S3B) and previously by global dRNA-seq analysis of the *Salmonella* transcriptome (46), is a guanine that lies 114 nt upstream of the start codon (Fig. 4A). Intriguingly, the associated promoter contains a highly conserved cytosine at position –13, which is a hallmark of  $\sigma^S$ -dependent promoters; this nucleotide contacts amino acid E458 in  $\sigma^S$  and counterselects for binding of the housekeeping  $\sigma^{70}$  (47). To test a potential  $\sigma^S$  dependence of the *ricI* promoter, we inserted a *lacZ* reporter gene downstream of it in the *Salmonella* chromosome (48). Promoter activity assays in the wild-type and in a  $\Delta rprA$  mutant revealed comparable transcriptional activity of the two strains with peaking activity under stationary-phase growth conditions (Fig. 4B). In contrast, *Salmonella* lacking the *rpoS* gene failed to activate the *ricI* promoter under all conditions tested, indicating that  $\sigma^S$  controls *ricI* transcription.

This was further confirmed by mutating C-13, which eliminated the  $\sigma^S$  dependency of *ricI* transcription as expected; i.e., a *gfp* reporter gene fused to a C-13→G variant of the *ricI* promoter was insensitive to the presence or absence of an intact *rpoS* gene (Fig. 4C). Collectively, these results suggest that both transcriptional activation by  $\sigma^S$  and posttranscriptional activation by RprA are essential for *ricI* expression.

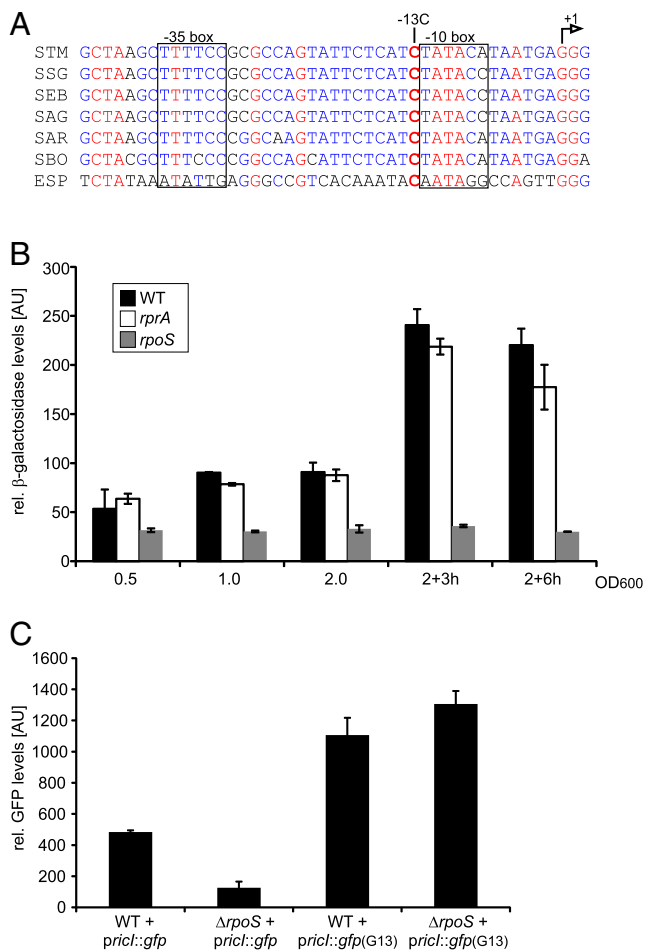
**An RNA-Controlled FFL with AND-Gate Logic Regulates RicI Production.** The dual requirement of RprA and  $\sigma^S$  in the activation of RicI resembles a coherent type 1 FFL (49). However, whereas such FFLs are typically controlled by transcription factors, the type 1 FFL activating RicI depends on dual base-pairing interactions of a

regulatory RNA, which we predicted to work through an AND-gate logic: the up-regulation of RicI synthesis requires both  $\sigma^S$  and RprA (Fig. 5A).

To examine the effectivity of this regulatory scheme, we mutated the *rprA* gene at two positions (Fig. 5B): we changed adenine 37 to cytosine (RprAC37) abolishing activation of the *rpoS* mRNA (21), and guanine 63 to cytosine (RprAC63) to abrogate activation of the *ricI* mRNA (Fig. 2A and C). These mutant *rprA* alleles were expressed from an inducible pBAD promoter to test their ability to up-regulate a chromosomal *ricI::lacZ* translational reporter under the control of the endogenous *ricI* promoter. Induction of wild-type RprA resulted in a >50-fold increase in reporter activity over the course of 60 min (Fig. 5C). By contrast, neither the RprAC63 nor the RprAC37/C63 double mutant could activate the reporter (Fig. 5C). Likewise, the RprAC37 single mutant, which fully activates the posttranscriptional *ricI::gfp* reporter (Fig. S4A), failed to activate the translational *ricI::lacZ* reporter (Fig. 5C). To further test this scheme, we treated  $\Delta rprA$  cells carrying either the *rprA*, *rprAC37*, or *rprAC63* allele (on a low-copy plasmid) with 3,4-dichlorobenzyl carbamimidothioate (A22) and followed the kinetics of RprA,  $\sigma^S$ , and RicI production. A22 inhibits the actin-like MreB protein and provides superior activation of the Rcs phosphorelay compared with bile salts (Fig. S4B) (45, 50). As expected from our previous results (Fig. 5C), only wild-type RprA provided full induction of RicI and  $\sigma^S$ , whereas RprAC63 failed to activate RicI and RprAC37 displayed only reduced  $\sigma^S$



**Fig. 3.** Bile-induced expression of RicI. (A) Western blot analysis of RicI::3xFLAG expression. Wild-type and  $\Delta rprA$  cells were grown to late exponential phase (OD<sub>600</sub> of 1.0) and treated with bile salts (3% final concentration). Whole-protein samples were collected at the indicated time points and probed for RicI::3xFLAG production. (B) Wild-type and the indicated *Salmonella* mutants ( $\Delta rprA$ ,  $\Delta rcsF$ ,  $\Delta rcsC$ ,  $\Delta rcsB$ , and  $\Delta rpoS$ ) were cultivated in LB media (with or without 3% bile salts) to OD<sub>600</sub> of 1.0 and probed for bile-mediated activation of RicI::3xFLAG (Western blot) and RprA (Northern blot). (C) RicI::3xFLAG production in the context of RprA overexpression. Wild-type,  $\Delta rprA$ ,  $\Delta rcsB$ , and  $\Delta rpoS$  cells transformed with a control plasmid (pctr.) or the RprA overexpression plasmid (pRprA) were tested for RicI::3xFLAG expression on Western blot.



**Fig. 4.**  $\sigma^S$  activates *ricI* transcription. (A) Alignment of the *ricI* promoter sequence from *Salmonellae* and *Enterobacter* species (ESP, *Enterobacter* sp. 638; SAG, *S. enterica* sv. Agona; SAR, *S. enterica* sv. *arizonae*; SBO, *Salmonella bongori*; SEB, *S. enterica* sv. *Bovismorbificans*; SSG, *S. enterica* sv. *Schwarzengrund*; STM, *S. enterica* sv. *Typhimurium*). Transcription control regions  $-10$  and  $-35$  are boxed, and the transcription initiation (+1) site is marked by an arrow. Residue C-13 is shown in bold. (B) Wild-type,  $\Delta rprA$ , and  $\Delta rpoS$  cells carrying the transcriptional *ricI*::*lacZ* reporter were monitored for  $\beta$ -galactosidase production at the indicated stages of growth. (C) Wild-type and  $\Delta rpoS$  cells transformed with either a wild-type (*pricl*::*gfp*) or the mutant [*pricl*::*gfp* (G13)] reporter were cultivated to early stationary phase (OD<sub>600</sub> of 2.0) and assayed for GFP production.

induction and did not up-regulate RicI (Fig. S4C). Together, these data indicate that RprA acts in a sequential order: activation of *rpoS* precedes activation of *ricI* because  $\sigma^S$  must activate *ricI* transcription first.

To test this circuit under more physiological conditions, i.e., without RprA overexpression, we monitored expression of a *ricI*::*lacZ* fusion at various stages of *Salmonella* growth. In wild-type cells, reporter activity peaked in stationary phase after increasing  $\sim 12$ -fold from exponential phase (Fig. 5D). As expected, introduction of either a  $\Delta rpoS$  or a  $\Delta rprA$  allele abrogated this increase in RicI::LacZ levels. To uncouple transcriptional activity of  $\sigma^S$  from posttranscriptional regulation by RprA, we introduced the mutation G44 $\rightarrow$ C (Fig. 2A) in the *ricI* 5'-UTR (*ricI*<sup>\*</sup>) on the *Salmonella* chromosome. This "ON" mutation interferes with stem-loop formation of the *ricI* untranslated leader, resulting in high RicI::GFP production in the absence of RprA (Fig. 2B). Similarly, this mutation induced RicI::LacZ expression by  $\sim 16$ -fold at early stages of growth (Fig. 5D) and rendered reporter activity insensitive to a secondary deletion of the *rprA*

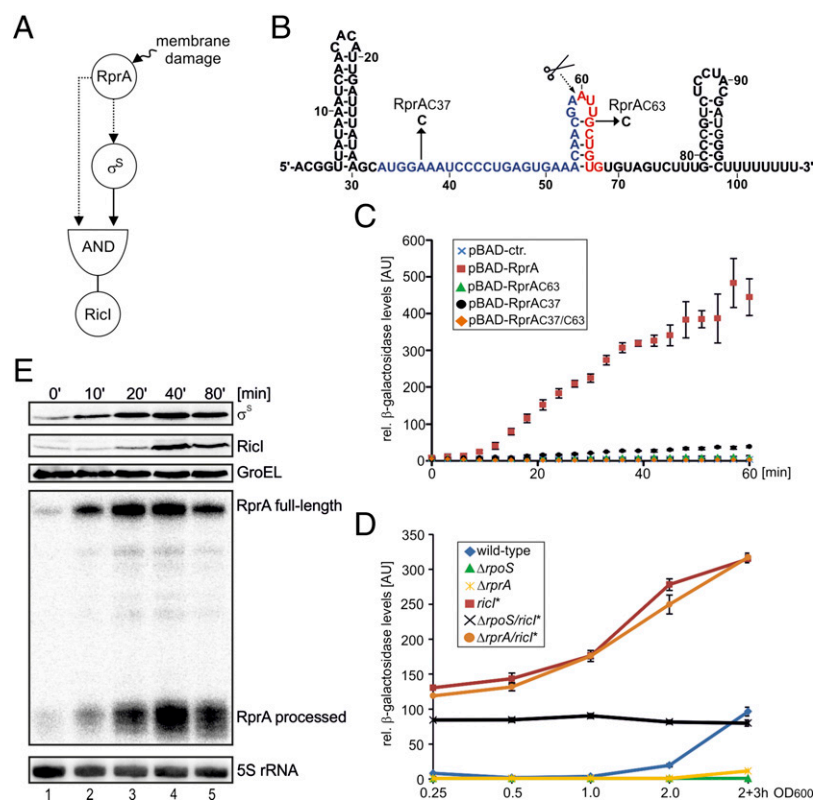
gene, because translation is already derepressed. Introduction of the *ricI*<sup>\*</sup> allele into a *rpoS* mutant also increased RicI::LacZ production at early stages of growth but failed to increase expression at higher cell densities (Fig. 5D). These results support the combinatorial activity (AND-function) of  $\sigma^S$  and RprA in activation of RicI.

A key characteristic of type 1 FFLs is their delay function upon signal perception (51). This is easy to understand in the context of RprA,  $\sigma^S$ , and RicI: RprA has to activate *rpoS* until sufficient  $\sigma^S$  is produced to generate the *ricI* mRNA serving as a second target for RprA. To investigate whether production of  $\sigma^S$  precedes immediate posttranscriptional activation of the *rpoS* mRNA. By contrast, a cross-comparison of  $\sigma^S$  and RicI levels showed that RicI production was significantly delayed. RicI levels increased  $\sim 2.5$ -fold after 20 min of treatment and increased afterward to  $\sim 15$ -fold (Fig. 5E, lanes 1, 3, and 5). Using a similar approach, we also monitored expression of RprA, RicI, and  $\sigma^S$  following deactivation of the circuit. Specifically, we treated wild-type cells with A22 for 30 min and collected and washed the cells followed by reinoculation into fresh media. We discovered that shutoff of  $\sigma^S$  production is almost immediate, whereas reduction of RicI expression to "prestress" levels required  $\sim 60$  min (Fig. S4D). These differences in protein levels might depend on specific proteolytic factors targeting the  $\sigma^S$  protein (50). Interestingly, we found that  $\sigma^S$  degradation also preceded inhibition of RprA expression: expression levels of full-length RprA and processed RprA reached the prestress status  $\sim 15$  min after cells were reinoculated in fresh media. Together, our data provide evidence for a previously unidentified variant of the type 1 FFL network that functions through the regulatory activity of two base-pairing domains of a single sRNA. Deactivation of the circuits depends on the individual stabilities of the three components, with RicI being most stable and  $\sigma^S$  showing almost immediate degradation once the stress is removed.

**RicI Inhibits *Salmonella* Virulence Plasmid Transfer.** The RprA-mediated up-regulation of RicI and the evident connection with the  $\sigma^S$  stress response network prompted us to investigate the biological role of these three factors more closely. Although the biological function of RicI was unknown, BLAST-P searches suggested similarity of RicI to a variety of proteins from different bacterial genera (Fig. S5), most of which with candidate functions in plasmid conjugation.

To investigate a potential role of RprA-mediated RicI activation in conjugation of the pSLT virulence plasmid in *Salmonella*, we compared the plasmid transfer rates of  $\Delta rprA$ ,  $\Delta ricI$ , and  $\Delta rpoS$  donors with the transfer rate of the wild type. Deletions of  $\Delta rprA$ ,  $\Delta ricI$ , or  $\Delta rpoS$  increased plasmid conjugation  $\sim 8$ - to 11-fold (Fig. 6A, bars 1–4), suggesting an inhibitory function for RicI in pSLT transfer. Double mutants  $\Delta rprA \Delta ricI$  and  $\Delta rprA \Delta rpoS$  yielded conjugation rates similar to those of the single-mutant variants (Fig. 6A), suggesting that RprA, RicI, and  $\sigma^S$  act in the same biological pathway to inhibit pSLT transfer.

Bile salts are an important factor for *Salmonella* pathogenicity (52) and have been reported to decrease pSLT transfer (9, 10). Because RprA expression is induced by bile (Fig. 3B), we wondered whether bile salts could affect conjugation frequency through RprA,  $\sigma^S$ , and RicI. To test this hypothesis, we compared conjugation rates of wild type,  $\Delta rprA$ ,  $\Delta ricI$ , and  $\Delta rpoS$  mutants in the presence of 4% bile. As expected, bile strongly decreased conjugal transfer from wild-type donors (compare



**Fig. 5.** An FFL with AND-gate logic controls RicI production. (A) Schematic display of the FFL regulating RicI production. Both RprA and  $\sigma^S$  are required for RicI expression. Dashed lines indicated posttranscriptional regulation, and solid lines denote control at the transcriptional level. (B) Secondary structure of RprA. Mutations tested in C are indicated by arrows. Scissors mark the RprA processing site. (C) *Salmonella* carrying the translational *ricI::lacZ* reporter were transformed with the indicated plasmids and tested for  $\beta$ -galactosidase production upon induction of pBAD expression. (D) The indicated strains (wild type,  $\Delta rpoS$ ,  $\Delta rprA$ ,  $\Delta ricI$ \*,  $\Delta rpoS/\Delta ricI$ \*, and  $\Delta rprA/\Delta ricI$ \*) carrying the translational *ricI::lacZ* reporter were assayed for  $\beta$ -galactosidase production at the indicated time points of growth. (E) Analyses of  $\sigma^S$ , RicI::3 $\times$ FLAG, and RprA expression after A22-mediated induction of the Rcs pathways. Samples were collected at the indicated time points and probed for  $\sigma^S$  and RicI::3 $\times$ FLAG (Western blot) as well as RprA (Northern blot) production. GroEL and 5S rRNA served as loading controls.

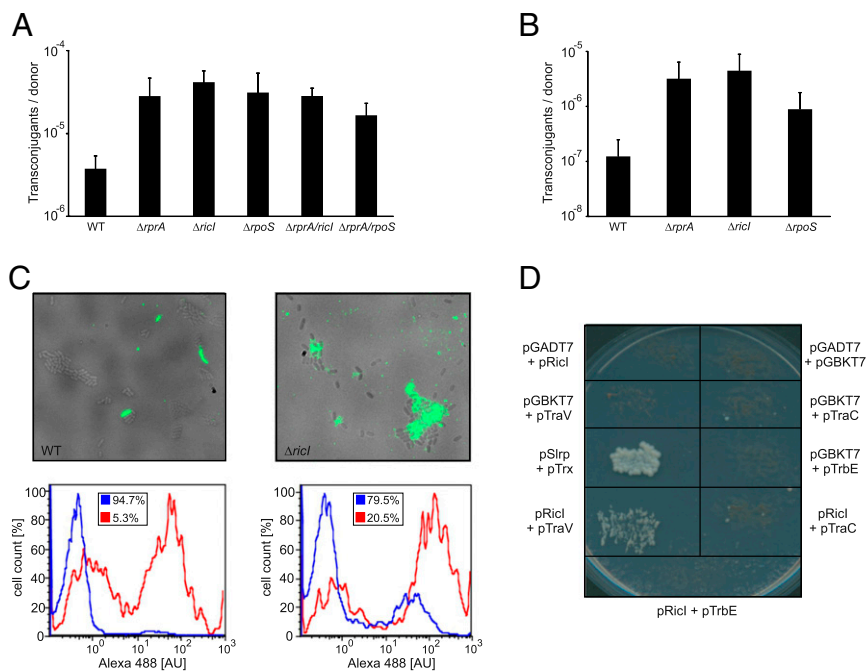
Fig. 6A and B). However, under the same conditions,  $\Delta rprA$ ,  $\Delta ricI$ , or  $\Delta rpoS$  donors displayed up to  $\sim 36$ -fold ( $\Delta ricI$ ) increased conjugation rates (Fig. 6B), similar to those of wild-type strains grown in rich medium (Fig. 6A). These data indicate a restrictive role for RicI during *Salmonella* virulence plasmid conjugation under membrane stress conditions.

Next, we sought to understand how RicI controls pSLT transfer. We first tested whether increased pSLT transfer of  $\Delta ricI$  *Salmonella* was also reflected in a higher rate of F-pili production. To this end, we treated cells with a fluorescently labeled derivative of bacteriophage R17. R17 specifically binds F-like pili and allows for accurate quantification of pili assembly (53). We used flow cytometry to compare pSLT-encoded F-like pili production in wild-type and  $\Delta ricI$  cells. Approximately 5% of wild-type cells displayed F-like pili on their surface, whereas this frequency was increased to  $>20\%$  in  $\Delta ricI$  mutants (Fig. 6C). These data indicate that RicI inhibits production of pSLT-encoded pili and suggests that the increased conjugation rates of  $\Delta ricI$  mutants (Fig. 6A and B) might be a consequence of increased F-like pili formation.

To investigate the gene-regulatory pattern underlying these phenotypes, we tested whether RicI affected expression of *traJ*. In F-family plasmids, TraJ is the major transcriptional activator of the *tra* operon encoding most of the proteins necessary for conjugation (54). Therefore, elevated expression of TraJ could well explain the increased F-pili production of  $\Delta ricI$  cells (Fig. 6C). However, expression of the transcriptional reporters *traJ::lacZ* and *traB::lacZ* [activated by TraJ (7)] remained unchanged in the *ricI*

mutant, whereas levels of both reporters (Fig. S6A and B) were significantly increased in *dam*-deficient cells, which served as a positive control (8). These data indicate that RicI inhibits plasmid transfer through a mechanism independent of TraJ.

Cell fractionation assays showed that RicI localizes to the inner membrane or periplasm of *Salmonella* (Fig. S6C), suggesting that RicI does not act at the gene-regulatory level but rather through interaction with other proteins. To identify interaction partners of RicI, we performed protein coimmunoprecipitation (co-IP) experiments in lysates of *Salmonella* expressing RicI::3 $\times$ FLAG. Visualization of copurified protein by silver staining revealed enrichment of a small protein ( $\sim 18$  kDa) in cells expressing the RicI::3 $\times$ FLAG protein, compared with co-IP in wild-type cells used for control (Fig. S6D). Mass spectrometry identified this protein as TraV, which is a membrane-bound lipoprotein that functions as an anchor of the type IV secretion apparatus (55). To validate the predicted interaction of RicI and TraV, we made use of a yeast-two-hybrid system in which reconstitution of the GAL4 protein through two interacting protein partners is required to drive the expression of the *HIS3* and *ADE2* genes, which are required for cellular growth (56). Indeed, we observed that plasmid-borne expression of neither RicI nor TraV fusion proteins alone would allow growth on selective plates, whereas combination of the two would restore *HIS3* and *ADE2* expression (Fig. 6D). Together, our data indicate that the RprA-activated RicI protein inhibits F-pili production through an interaction with TraV.



**Fig. 6.** RicI inhibits pSLT conjugation in *Salmonella*. (A) Conjugation rates of the pSLT plasmid in the indicated donor strains. (B) Same as A but conjugation was tested in the presence of 4% bile salts. (C, Top) Alexa 488-labeled R17 bacteriophage was used to visualize the pSLT conjugation pili in wild-type and  $\Delta ricI$  cells. (Bottom) Quantification of labeled wild-type and  $\Delta ricI$  cells using FACS analysis. Flow cytometry analysis of Alexa 488 fluorescence intensity and percentages of cells that do not show R17 binding (blue) and cells exhibiting R17 binding (red). Histograms represent the percentages of fluorescent (R17-bound) and nonfluorescent cells. (D) Yeast two-hybrid assays of RicI–TraV interaction. Combination of RicI and TraV fusion proteins restores growth of yeast cells on selective medium, whereas expression of the individual fusion proteins (in combination with the control plasmids pGBKT7 or pGADT7) is insufficient. SlrP–Trx provided a positive control (74), and the negative controls were RicI/TraC and RicI/TrbE.

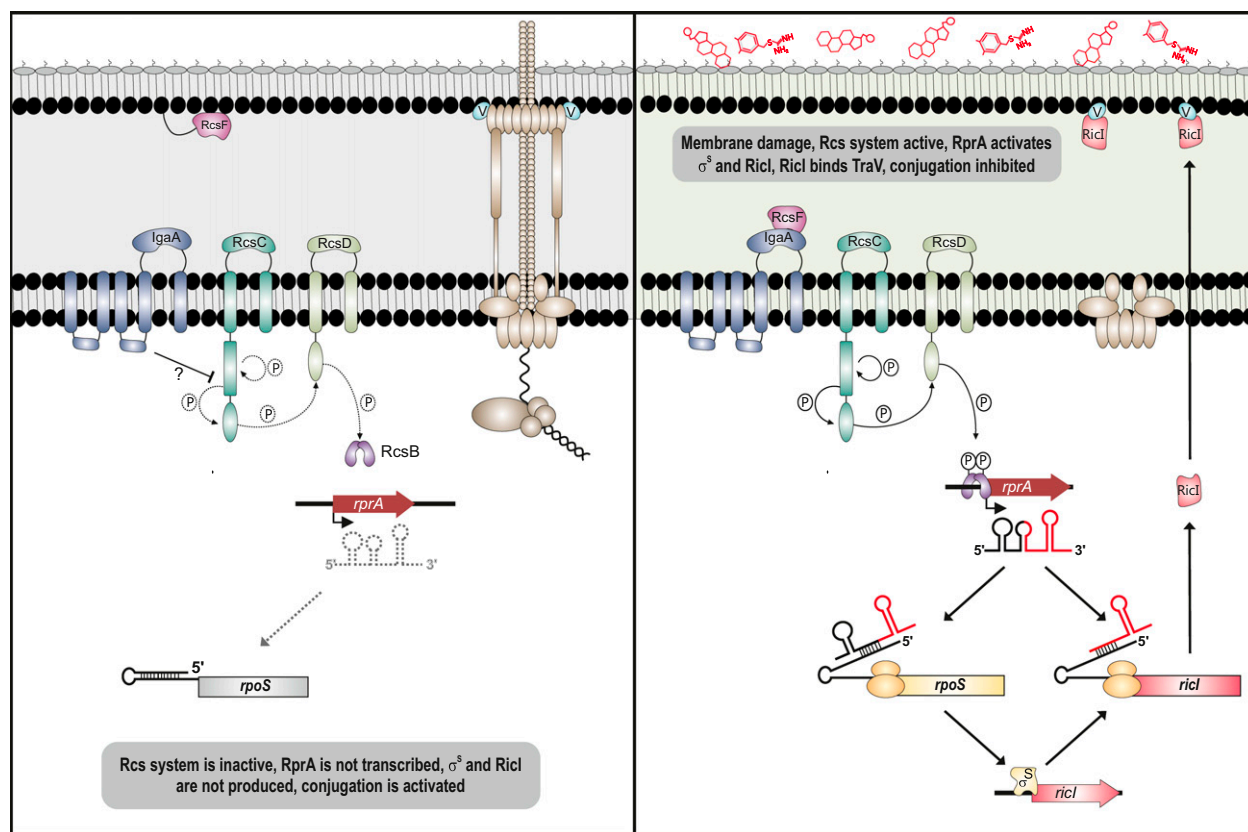
## Discussion

Studies aimed at understanding the interplay of regulatory factors have identified recurring patterns called network motifs (51). Typically, these network motifs are composed of two hierarchically acting transcription factors, but an increasing number of examples suggest that sRNAs can be integral parts of similar regulatory circuits (57, 58). One of the most common network motifs is the FFL wherein one regulator controls another regulator, which both regulate the expression of a third gene (59). When both regulators act in concert, the loop architecture is coherent, whereas opposing regulatory functions define an incoherent FFL (49). In this study, we describe a regulatory circuit paralleling a transcription factor-driven coherent FFL; however, in this arrangement, the RprA sRNA replaces the top-tier transcriptional regulator. RprA acts posttranscriptionally to activate two transcripts: the *rpoS* mRNA encoding the general stress sigma-factor  $\sigma^S$ , and the  $\sigma^S$ -controlled *ricI* mRNA encoding a membrane-associated protein (Fig. 5A). This FFL functions as a regulatory AND-gate whereby both regulators, RprA and  $\sigma^S$ , are essential for *ricI* activation (Fig. 5C and D).

The AND-gate logic of this FFL has important implications for the regulatory dynamics of the circuit. For example, although the *rpoS* and *ricI* mRNAs are both activated through the same sRNA, production of RicI protein significantly lags the synthesis of  $\sigma^S$  (Fig. 5E). This regulatory pattern is also biologically relevant, allowing the FFLs to act as a “persistence detector” (59) in which only sustained activation of RprA, leading to accumulation of  $\sigma^S$  above the critical threshold required for *ricI* transcription, will trigger the FFL. In addition, the system can swiftly respond to OFF-pulses (Fig. S4D) as inhibition of either RprA or  $\sigma^S$  will terminate *ricI* activation.

Other bacterial sRNAs have been documented to be part of FFLs with globally acting transcription factors (60, 61). For example, the Spot 42 sRNA is repressed by the global regulator CRP and itself inhibits the translation of many CRP-dependent mRNAs (60). This multioutput FFL facilitates carbon source transition and minimizes leaky gene expression under steady-state conditions (60, 62). Both Spot 42 and RprA are components of coherent FFLs, and both regulate gene expression through direct base pairing with target mRNAs. However, the regulatory functions of the two sRNAs are conceptually different: Spot 42 serves as an accelerating factor in the regulation of CRP target genes (60), whereas RprA is strictly required for RicI production (Figs. 3A and 5D). In other words, repression of CRP-target genes by Spot 42 creates a regulatory OR-gate, whereas activation of RicI through RprA and  $\sigma^S$  establishes an AND-gate function.

Unlike Spot 42 and most other characterized sRNAs, which exist as a single transcript, RprA is a processed sRNA with two independent seed-pairing domains. The proximal domain base pairs with *rpoS* mRNA, whereas the distal domain targets *ricI* mRNA (Fig. 5B). Importantly, the two forms of RprA have different stabilities: the full-length RprA is cleaved by RNase E and relatively short-lived, whereas the processed RprA is more stable (31, 34). We predict the different stabilities of the two RprA forms to have important implications for the regulatory dynamics of the FFL. On one hand, the rapid turnover rate of full-length RprA might function as a timed “erase function” for the system that will eliminate information from previous signal transduction events. On the other hand, the higher stability of processed RprA will allow activation of *ricI* independent of full-length RprA, given that  $\sigma^S$  production is activated through an alternative pathway. Indeed, expression of processed RprA alone



**Fig. 7.** Model of RicI-mediated conjugation inhibition in *Salmonella*. (Left) Under regular growth conditions (no membrane stress), the Rcs system is inactive and RprA is not produced. Therefore, RprA cannot activate *rpoS* and *ricI* will not be expressed. Expression and assembly of the pSLT conjugation apparatus is permitted. (Right) When the Rcs system is activated (e.g., by bile salts or A22), full-length RprA will activate the *rpoS* mRNA leading to  $\sigma^S$  production.  $\sigma^S$  activates the transcription of *ricI*, and the *ricI* mRNA can be activated by the processed RprA variant. Finally, RicI interacts with TraV to inhibit assembly of the pSLT conjugation pilus.

can be sufficient to activate RicI production in stationary-phase cells when  $\sigma^S$  is present (Fig. 1D).

Why does *Salmonella* limit pSLT transfer when the membrane is damaged? Despite the potential benefit of plasmid transfer at the population level, assembly of the conjugation pilus is a burden for individual donor cells and requires tight control, especially under stress conditions. In fact, synthesis of F-like pili causes bile sensitivity in *E. coli* (63) and bile inhibits transfer of pSLT, the *Salmonella* virulence plasmid (10). Synthesis of RicI inhibits conjugal transfer of pSLT (Fig. 6A), and rationalizing from its interaction with the pSLT-encoded periplasmic protein TraV (Fig. 6D), RicI is likely to directly interfere with pilus assembly (Fig. 7). This view is supported by our observation that adsorption of phage R17 occurs at reduced levels upon RicI production (Fig. 6C). Bile salts are bactericidal (64), and the RicI protein may provide a safety device that protects *Salmonella* from the danger of conjugation apparatus assembly when envelope integrity is in jeopardy. Since synthesis of both RprA and RpoS is activated in the presence of bile, inhibition of pilus formation might protect *Salmonella* against the membrane-damaging activities of bile salts and down-regulate the energy-intensive assembly of transenvelope machineries such as the conjugation apparatus. Interestingly, the CpxAR pathway has been shown to fulfill an analogous function in enteropathogenic *E. coli*: CpxAR-mediated activation of the protease-chaperone pair, HslVU, results in degradation of TraJ, the major transcriptional activator of plasmid transfer genes (65). Because all of these components are also conserved in *Salmonella*, the CpxAR and Rcs pathway might work in concert to control transfer of

pSLT. In fact, two or more redundantly acting pathway could account for the residual inhibition of conjugation observed for bile-treated  $\Delta rprA$ ,  $\Delta ricI$ , or  $\Delta rpoS$  donor cells (Fig. 6B).

The regulatory AND-gate involving RprA illustrates how sRNAs can function as specialization devices in global regulons. *E. coli* and *Salmonella* control the production of  $\sigma^S$  at multiple levels (16, 17), and in turn  $\sigma^S$  controls a large regulon (66, 67). Nonetheless, even though a variety of environmental stresses activate  $\sigma^S$  production, not every of these stress conditions requires repression of plasmid transfer. The strict requirement for posttranscriptional activation of *ricI* through RprA ensures that conjugation is only inhibited when the membrane integrity is compromised and the Rcs or CpxAR pathways are activated. Other environmental factors activating  $\sigma^S$  independent of RprA will not affect RicI expression and plasmid transfer. In *E. coli*, a similar diversification of the  $\sigma^S$  regulon is present with *csgD* and *ydaM*: although  $\sigma^S$  activates these two genes for curl fiber and cellulose production, RprA represses their mRNAs (35, 68), suggesting that RprA promotes certain stress-related functions of the  $\sigma^S$  regulon but inhibits the functions of CsgD and YdaM.

Although our study has focused on understanding the activation of RicI synthesis by RprA, other putative RprA targets predicted here suggest additional roles for this sRNA in the control of pSLT-mediated functions. For example, our pulse expression results predict RprA to repress the pSLT-encoded *traT* mRNA (Fig. 1C). TraT belongs to the group of surface exclusion proteins, which block conjugative transfer of plasmids to cells bearing identical or closely related plasmids (69). Given that bile salts can induce curing of pSLT plasmid (10), repression



of *traT* by RprA could allow the uptake of new plasmids. Other potential targets are the *Salmonella*-specific *SL2594* and *SL2705* loci from prophage regions. Similar to the repression of plasmid conjugation via *RicI*, RprA could inhibit the assembly of phage-derived structures under conditions of envelope stress. This regulatory pattern might further extend into virulence functions of *Salmonella*: invasion of the host cell epithelium requires a type 3 secretion system (T3SS) of virulence proteins into the host cell. The *rtxA* mRNA encodes an activator of this T3SS (70), and its repression by RprA (Fig. 1C) suggests that RprA inhibits the assembly of this T3 secretion apparatus when the bacterial envelope is damaged. The list of RprA targets also suggests additional regulatory circuits. For example, the *yqaE* gene is activated by CpxR (22) and RprA, which may constitute another FFL featuring both transcriptional and posttranscriptional control. For certain target candidates (e.g., *guaA*), we observed opposite regulation by both the full-length and processed RprA variants (Fig. 1C); the causes for such opposite regulations remain to be explored.

To our knowledge, RprA is the first processed sRNA controlling distinct sets of target mRNAs through two different isoforms (Fig. 1C), but additional work will be required to understand its regulatory full scope. We note that, of 64 potential RprA targets, 34 are predicted to be up-regulated (Fig. 1C). This number is unusually high compared with other well-characterized sRNAs, but it shrinks to a single activated target (*ricI*) when only the processed form of RprA is induced. This high number of activated genes could well result from the significant amount of  $\sigma^S$  protein that is produced even after short induction (15 min) of the full-length RprA sRNA (Fig. 1D). Indeed, cross-comparison with a recently published list of  $\sigma^S$ -dependent *Salmonella* genes (66) revealed that 22 of the 34 activated targets are regulated by  $\sigma^S$ ; further experiments will tell which of these genes are directly regulated by  $\sigma^S$ , RprA, or both. This notwithstanding, our study suggests that the widely conserved RprA sRNA has a more complex biological role than previously anticipated.

## Materials and Methods

**Bacterial Strains and Growth.** Bacterial strains and details on their construction are listed in Table S1. Strains were grown at 37 °C in Luria-Bertani (LB) broth or on LB plates. Ampicillin (100  $\mu$ g/mL), kanamycin (50  $\mu$ g/mL), chloramphenicol (20  $\mu$ g/mL), and L-arabinose (0.2%) were added where appropriate. *Salmonella* wild-type (SL1344) or mutant strains were transformed by electroporation.

**Plasmids and Oligonucleotides.** Plasmids and DNA oligonucleotides are listed in Tables S2 and S3, respectively. Details on plasmid construction are provided in *SI Materials and Methods*. Target fusions to *gfp* were constructed as described previously (39).

**Western Blot Analysis, Fluorescence, and  $\beta$ -Galactosidase Assays.** Culture samples were taken according to 1 [OD<sub>600</sub>], centrifuged for 4 min at 16,000  $\times g$  at 4 °C, and pellets were resuspended in sample loading buffer to a final concentration of 0.01 OD/ $\mu$ L. Following denaturation for 5 min at 95 °C, 0.1

OD equivalents of sample were separated on SDS gels. Western blot analyses of  $\sigma^S$ , GFP, FLAG fusion proteins, and fluorescence assays followed published protocols (71). Quantitative Western blot data were obtained using a Fuji LAS-3000 imaging system (GE Healthcare), and band intensities were quantified using the AIDA software (Raytest). Probing for GroEL served as loading control.  $\beta$ -Galactosidase assays were performed as described before (72).

**Northern Blot and Microarray Experiments.** Total RNA was prepared and separated in 5% or 6% (vol/vol) polyacrylamide–8.3 M urea gels (5–10  $\mu$ g of RNA per lane) and blotted as described (73). Membranes were hybridized at 42 °C with gene-specific <sup>32</sup>P-end-labeled DNA oligonucleotides in Rapid-hyb buffer (GE Healthcare). Microarray experiments were carried out as described before (33). Plasmids pKP15 and pKP22 allowed pulse expression of full-length and processed RprA, respectively. Microarray data have been deposited at GEO ([www.ncbi.nlm.nih.gov/geo/](http://www.ncbi.nlm.nih.gov/geo/)) (accession code GSE67187).

**pSLT Conjugation Assays and R17 Phage Labeling.** Conjugation rates of pSLT plasmid were determined as described previously (10). Labeling of conjugation pili followed established protocols (53). For flow cytometry measurements, cells (20  $\mu$ L) and R17 conjugated to Alexa 488 (5  $\mu$ L) were mixed at room temperature for 60 min. Cells and bound bacteriophages were harvested by sedimentation for 4 min at 16,100  $\times g$ . Cell pellets were suspended in 1 mL of PBS. Fluorescent R17 was measured by flow cytometry. Data acquisition and analysis were performed using a Cytomics FC500-MPL cytometer (Beckman Coulter). Gates were drawn to separate cell showing high forward side (cells exhibiting R17 binding, higher cell size), and cells displaying low forward side (cells without R17 binding, smaller cell size). Alexa 488 fluorescence intensity was analyzed within the gates set as high or low forward side. Data were obtained and analyzed with MXP and FlowJo 8.7 software, respectively.

**Yeast Two-Hybrid Assay.** Plasmids pIZ1872 (*ricI*) and pIZ1878 (*traV*) were transformed into strains Y2HGOLD and Y187, and transformants were selected on selective media (SD-Trp for pIZ1872 and SD-Leu for pIZ1878). Y2HGOLD/pIZ1872 and Y187/pIZ1878 were mixed on a YPD plate and incubated for 24 h at 30 °C. Mating mixtures were patched on yeast dropout medium (SD) lacking tryptophan and leucine (Clontech) and were incubated at 30 °C for 2 d before replica plating on agar lacking tryptophan, leucine, histidine, and adenine and on agar plates lacking tryptophan and leucine.

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