Caught at its own game: regulatory small RNA inactivated by an inducible transcript mimicking its target

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A relevant, yet little recognized feature of antisense regulation is the possibility of switching roles between regulatory and regulated RNAs. Here we show that induction of a Salmonella gene relies on the conversion of a small RNA from effector to regulatory target. The chiP gene (formerly ybfM), identified and characterized in the present study, encodes a conserved enterobacterial chitoporin required for uptake of chitin-derived oligosaccharides. In the absence of inducer, chiP is kept silent by the action of a constitutively made small RNA, ChiX (formerly SroB, RybC), which pairs with a sequence at the 5’ end of chiP mRNA. Silencing is relieved in the presence of chitooligosaccharides due to the accumulation of an RNA that pairs with ChiX and promotes its degradation. Anti-ChiX RNA originates from an intercistronic region of the chb operon, which comprises genes for chitooligosaccharide metabolism and whose transcription is activated in the presence of these sugars. We present evidence suggesting that the chb RNA destabilizes ChiX sRNA by invading the stem of its transcription terminator hairpin. Overall, our findings blur the distinction between effector and target in sRNA regulation, raising the possibility that some of the currently defined targets could actually be inhibitors of sRNA function.

[Keywords: Antisense regulation; chitoporin; chitobiose; Hfq; small RNA]

Received March 31, 2009; revised version accepted July 9, 2009.
E. coli and Salmonella is devoted to modulating the protein composition of the outer membrane, the compartment most directly involved in exchanges with the environment (Guillier et al. 2006; Vogel and Papenfort 2006; Valentin-Hansen et al. 2007). All of the major porins and several outer membrane proteins (OMPs) are targets for regulation by one or more sRNAs. The genes encoding these sRNAs are generally regulated at the transcriptional level, and expressed under specific conditions (Mizuno et al. 1984; Chen et al. 2004; Douchin et al. 2006; Figueroa-Bossi et al. 2006; Papenfort et al. 2006; Bossi and Figueroa-Bossi 2007; Thompson et al. 2007; Udekwu and Wagner 2007; Rasmussen et al. 2009).

An interesting feature in the working mechanism of some sRNAs emerged from the study of RyhB, an sRNA that accumulates in response to iron limitation and down-regulates a number of genes for iron-binding proteins (Masse and Gottesman 2002). RyhB was found to be “consumed” when active in regulation. Apparently, upon pairing with the target mRNA, RyhB sRNA is no longer protected by Hfq and becomes susceptible to degradation by RNase E (Masse et al. 2003). Thus, the RyhB-mediated response relies on the continuous production of the sRNA, which permits rapid reversal of the response when iron concentration increases. The same study showed other sRNAs to act by a similar stoichiometric mechanism (Masse et al. 2003).

In the present study, we identify a novel OMP, ChiP, involved in the uptake of chitin-derived oligosaccharides. The protein, required for Salmonella to grow on chito-oligosaccharides as sole sources of both carbon and nitrogen, is only synthesized in the presence of its substrates. We show that ChiP synthesis results from destabilization of a constitutively made small RNA, ChiX, that normally represses ChiP synthesis by pairing with a sequence in the 5’ untranslated region (UTR) of chiP mRNA. This regulatory response occurs by a novel mechanism involving the production of an RNA sequence that can act as an alternative pairing partner for ChiX sRNA. This alternative base pair interaction is ill fated for ChiX sRNA as it targets the sRNA for nucleolytic decay.

Results

Novel sRNA-regulated OMP in Salmonella

To identify potential targets for regulatory sRNAs in Salmonella, in a previous study we screened a library of random MudK (lac) chromosomal insertions, searching for isolates whose lacZ levels varied in the presence or absence of Hfq. One fusion, strongly up-regulated in the Δhfq background, had the MudK element inserted in the uncharacterized ybfM locus (Fig. 1A; Figueroa-Bossi et al. 2006). Analysis of Salmonella OMP preparations in poly-acrylamide gels showed that the extract from the Δhfq strain included a band that was missing in the wild-type sample (Fig. 1B). When eluted from the gel and subjected to MALDI-TOF analysis, the protein was found to correspond to YbfM. Subsequent analysis (described below) revealed that YbfM is a chitoporin, hence, it was renamed ChiP.

A strain carrying chiP::MudK is phenotypically Lac+ and becomes Lac− when Hfq is inactivated. We reasoned that mutations affecting other cell components involved in chiP silencing might similarly result in a Lac− phenotype. Such mutants were sought using growth on lactose as positive selection. The Lac+ mutations mapped at three different loci: (1) the hfq gene; (2) the chiP::MudK locus, and (3) the gene encoding the Salmonella ortholog of SroB (RybC) sRNA (Vogel et al. 2003; Zhang et al. 2003). The latter findings suggested that SroB, renamed ChiX, might participate together with Hfq in chiP down-regulation.

ChiX sRNA is readily detectable in Northern blots of RNA from wild-type cells [Fig. 1C]. In contrast, the sRNA is not detected in cells lacking Hfq. Disappearance of ChiX in the hfq mutant correlates with the appearance of two chiP-related RNAs: a longer species extending to the end of the ybfM gene (renamed chiQ) downstream from chiP [Fig. 1A], and a shorter species terminating within the intercistronic region (ICR) between chiP and chiQ. The same two species accumulate in a RNase E temperature-sensitive (ts) mutant incubated at the restrictive temperature [Fig. 1D]. Taken together, these data allow us...
to infer that normally, ChiX activity leads to RNase E-dependent degradation of chiP transcripts.

ChiP is an inducible chitoporin

In an attempt to identify additional players involved in ChiP regulation, we searched for mutations conferring a conditional Lac\(^+\) phenotype. The lac fusion used in these experiments, chiP91::pCE40[lac], carries the lacZ coding sequence of plasmid pCE40 [Ellermeier et al. 2002] joined to the 9th codon of chiP. This construct expresses lacZ at much higher levels than the original chiP::MudK fusion. Mutants selected at 30°C were screened for the presence of isolates that did not form colonies on lactose plates at 42°C. Two such mutants were identified. Conceivably, they carried alleles of a factor involved in the regulation of chiP, or chiX, or both. Genetic mapping and DNA sequence analysis identified this factor as ChbR, the AraC-type activator of the chitobiose operon, chb [Keyhani and Roseman 1997]. The chb operon (chbBCARFG) includes genes for transport and utilization of chitobiose, along with the chbR regulatory gene (Keyhani and Roseman 1997; Plumbridge and Pellegrini 2004). Chitobiose (the dimer of N-acetylglucosamine) and other chitooligosaccharides [e.g., chitotriose] are produced by the enzymatic breakdown of chitin, the main component of the exoskeleton of arthropods. The involvement of ChbR in chiP regulation, combined with the outer membrane localization of the ChiP protein, suggested that the latter was a porin dedicated to the uptake of chitin-derived oligosaccharides. Consistent with this hypothesis, we observed that both chitobiose and chitotriose effectively induce chiP mRNA synthesis as well as ChiP protein accumulation in the outer membrane [Fig. 2A,B]. Furthermore, the chiP gene is absolutely required for Salmonella to be able to grow on chitotriose as the sole source for both carbon and nitrogen [Fig. 2C]. A similar requirement is observed with chitobiose, but only at low concentrations, suggesting the existence on an additional, lower affinity uptake system for the disaccharide [data not shown]. Existence of this system could explain why chitobiose, but not chitotriose, induces expression of the chiP-lac fusion in spite of chiP being disrupted by the lac sequence (Fig. 2D). In contrast, both sugars are equally efficient at inducing expression of a chiQ-lac fusion, provided that chiP is functional [Fig. 2E]. The latter data indicate that chiQ and chiP genes are coordinately regulated. ChiQ, however, appears dispensable for growth on either chitobiose or chitotriose [Fig. 2C; data not shown].

In chiX\(^+\) strains, chiPQ levels are negligible in the absence of inducers and rise >200-fold upon induction [Fig. 2A–C]. In contrast, in strains deleted for the chiX gene, chitobiose exposure causes chiPQ expression to increase approximately fourfold over an already high basal level [Fig. 2D]. The increase depends on the ChbR protein, and may reflect a direct stimulation of chiPQ transcription. Thus, ChiX tightens the response and amplifies the regulatory range. Failure of chitobiose or chitotriose to induce the chiPQ operon in a strain in which the chbR gene is disrupted by a transposon insertion [Fig. 2D] confirms the ChbR requirement for induction. ChbR is thought to undergo an allosteric change upon binding to a chitobiose-derived metabolite.

Figure 2. Regulation of the chiPQ Operon. [A] Northern blot analysis of chiPQ RNA. Cultures from strains MA3409 [wt], MA8933 [ΔchiX], and MA9816 [rne-3071] were grown in LB or in LB supplemented with chitobiose (2 mM; added at an OD\(_{600}\) of 0.2) to an OD\(_{600}\) of 0.35. RNA was extracted and analyzed as described in the legend to Figure 1C. MA9816 cultures were shifted to 43°C 15 min prior to RNA extraction. RNA blots were hybridized to \(^{32}\)P-labeled oligonucleotides ppB68 [chiPQ and chiP] and pp813 [ssrA]. [B] OMP patterns in the absence and presence of chitotriose. Salmonella OMPs were prepared from 2 mL cultures of strains MA3409 [wt] and MA8933 [ΔchiX] grown overnight in LB without or with 2 mM chitotriose. [C] Genetic requirements for Salmonella growth on chitotriose. Cultures from strains MA3409 [wt], MA9131 [chiP::scar], MA9654 [chiQ::scar], and MA9843 [chbR::Tn5-TPOP] were grown overnight in NCE medium supplemented with 0.2% glycerol were spotted on an NCN plate containing 2 mM chitotriose. Strains MA9131 and MA9654 carry small deletions in the 5′-end deletions of chiP and chiQ genes, respectively. [D] Expression of chiP91-lacZ fusion. Cultures were grown overnight in NCE 0.2% glycerol medium without or with 2 mM chitobiose or 2 mM chitotriose added. β-Galactosidase activity was measured in strains MA9132 [chiP-lacZ], MA9184 [chiP-lacZ ΔchiX], MA9844 [chiP-lacZ chbR::Tn5-TPOP], and MA10049 [chiQ-lacZ ΔchiX chbR::Tn5-TPOP]. [E] Expression of chiQ92-lacZ fusion. Strains used were MA9655 [chiQ-lacZ], MA9841 [chiQ-lacZ ΔchiX], and MA9923 [ΔchiP chiQ-lacZ].
Conceivably, the two conditional ChbR alleles described above (S135L and N238Y) may mimic the allosteric change at low temperature in the absence of inducers.

Mutational analysis of ChiX sRNA

The Lac-based screen proved suitable to a genetic dissection of ChiX sRNA function. Lac+ mutants arising spontaneously, or generated by mutagenizing the chiX gene with the PCR, were isolated from the strain carrying the chromosomal chiP::MudK(lac) insert. The effects of mutations on chiP expression were quantified measuring chiPQ mRNA in Northern blots and assaying β-galactosidase activity in strains with the chiP91::pCE40[lac] fusion.

Several chiX mutations affected a 12-base segment complementary to a sequence spanning the Shine-Dalgarno motif of the chiP gene [Fig. 3A]. This suggests a base pair interaction to be responsible for ChiX-mediated repression. Most of the changes relieve repression completely, while some have less pronounced effects [Fig. 3B,C]. The differences can be ascribed to the nature of the change [i.e., A51G replaces a U:A base pair by a U:G pair] or to its position in the duplex. Mismatches in the middle of the segment [U47C, U49C] appear less detrimental to regulatory proficiency than the ones on the sides [e.g., U45C]. Mutants with a weaker phenotype express significant LacZ activity and yet show little or no chiPQ RNA in the Northern blots [Fig. 3, cf. B and C]. This discrepancy could originate from RNA cleavage events occurring in between rounds of translation initiation. Even though some ribosomes can reach the end of the message [yielding a protein product and measurable LacZ activity], no chiP RNA molecule ever exists in its full length. Northern blot analysis of processing intermediates from the 3' end of the chiQ gene supports this interpretation [Supplemental Fig. S2].

Two chiP mutations obtained in the initial Lac+ selection fall within the presumptive ChiX pairing window [Fig. 3A]. The change in chiP C74A is predicted to restore base-pairing if combined with ChiX G50U. Results in Figure 3D show that chiP C74A disrupts regulation when present alone but not in combination with the ChiX...
G50U allele. Similar compensatory effects were observed with other chiP changes obtained by site-directed mutagenesis [Fig. 3E; data not shown]. These data provide evidence that repression by ChiX sRNA involves base-pairing with chiP mRNA. Likely, formation of the RNA duplex inhibits chiP translation initiation by interfering with ribosome binding.

In chiX mutants carrying changes outside the pairing interval, altered chiP regulation correlates with a decrease in ChiX sRNA levels [Fig. 3A–C]. Most of these mutations disrupt the Rho-independent terminator hairpin, a structure thought to be important for stability in prokaryotic RNAs [Mott et al. 1985; Aiba et al. 1991; Guarneros and Portier 1991]. Therefore, the depletion of ChiX sRNA in these mutants is likely due to increased decay. Two alleles with a weaker phenotype, C10U and A29G, are located in the 5’ half of the molecule. We envisaged that these mutations might affect ChiX–Hfq binding. This idea was tested in vitro comparing the affinities of wild-type and mutant sRNAs for purified Hfq in a gel retardation assay. Results confirmed that both C10U and A29G impair the sRNA’s ability to bind Hfq [Fig. 4A]. Allele A29G falls within an AU-rich sequence [AAUAAUAUA], which includes two overlapping AAYAA motifs proposed to play a role in Hfq recognition [Soper and Woodson 2008]. Given that the mutation disrupts both repeats [AAUGAUAAUA], the data in Figure 4A further substantiate the role of this sequence in Hfq–RNA interactions.

The C10U mutation causes the replacement of a G:C base pair by a G:U base pair in a putative secondary structure at the 5’ end of ChiX sRNA [Fig. 3A]. Structural analysis [Fig. 4B] reveals that the mutation affects the reactivity of surrounding bases as well as the spacing of bands in the sequence ladder [cf. OH− lanes in Fig. 4B]. These results might indicate that the C10U change destabilizes the top portion of the hairpin structure, causing the expansion of the apical loop. Such a perturbation might interfere with the binding of Hfq protein to the adjacent recognition site.

**ChiX sRNA is destabilized during chiPQ induction**

Interestingly, ChiX sRNA levels are drastically reduced in cells growing in the presence of chitobiose [Fig. 5A]. Considering that this decrease could be the basis of chiP regulation, we set out to investigate the underlying mechanism. Our initial hypothesis—that activated ChbR protein acted as a repressor at the chiX promoter—was ruled out by the analysis of a lacZ gene fusion to the chiX promoter, which showed lacZ expression to remain unchanged in the presence of chitobiose [data not shown]. We then postulated that the accumulation of chiPQ mRNA during induction might be sufficient to titrate out ChiX sRNA and stimulate its decay [Masse´ et al. 2003]. However, ChiX depletion was still observed in a strain where chiPQ transcription cannot take place due to a promoter deletion [Fig. 5A]. Nonetheless, the above line of reasoning led us to test whether the ChiX depletion was in some way related to the sRNA’s ability to base-pair. Analysis of two of the pairing mutants described earlier [G50A, U45A] revealed ChiX sRNA levels to be significantly less affected in the presence of the G50A allele [Fig. 5A]. This suggested that ChiX sRNA undergoes degradation during induction as a result of its participation in a base pair interaction. Having ruled out chiP mRNA involvement, we searched elsewhere for an alternative pairing target. We discovered that the ICR
between the first two genes of the chb operon, chbB and chbC, encodes a sequence complementary to most of the pairing segment of ChiX sRNA (Fig. 5B). These findings were all the more interesting as the chb RNA is expected to accumulate in the presence of chitobiose, and thus it could easily become in excess of ChiX sRNA.

Transcription of chbBC ICR leads to ChiX sRNA destabilization

A 37-base-pair (bp) segment covering the chbBC ICR portion complementary to ChiX sRNA from the chbΔ37 mutant. RNA was extracted from strain MA10004 [chbΔ37] and processed as in A. (C) Northern blot analysis of ChiX sRNA from the chbΔ37 mutant. RNA was extracted from strain MA10004 [chbΔ37] and processed as in A. (D) Effect of chbΔ37 on the regulation of the chiP91-lac fusion. β-Galactosidase activity was measured in strain MA10074 [Δchb-37 chiP-lac] as described in Figure 2D.

Titration effects were observed upon raising chiP mRNA levels artificially, as in a strain with a chromosomal pBAD-chiP fusion grown in the presence of arabinose or in a chiP “promoter-UP” mutant isolated in the Lac selection above (data not shown).

The chb promoter was replaced by a module comprising the araC gene and the PBAD promoter (Fig. 5B). Analysis of the resulting strain showed ChiX sRNA levels to sharply decline when cells were grown in the presence of arabinose [Fig. 6A]. In contrast, a similar construct with the PBAD promoter controlling chiPQ transcription had only a modest effect on ChiX sRNA levels (Fig. 6A). Finally, a 186-bp DNA fragment spanning the entire chbBC ICR (chb-186) was inserted in place of the structural portion of the ara operon. (C) Northern blot analysis of ChiX sRNA from the chb-186 strain grown in the presence of chitobiose (Fig. 5B). The resulting strain reproduced the effect of arabinose on ChiX sRNA levels (Fig. 6A), confirming the chbBC ICR to be solely responsible for ChiX sRNA decay.

As expected, arabinose induction of the pBAD-chb fusion constructs leads to chiPQ derepression. However, the chiPQ increase is significantly less dramatic than normally observed following chitobiose exposure. Ascribing this difference to the lack of ChbR activation, the induction experiments were repeated in the presence of
chitobiose [in the strain carrying the entire chb operon fused to the P<sub>BAD</sub> promoter]. Results confirmed that maximal chiPQ derepression is attained when both sugars are concomitantly present [Fig. 6B]. These data further support the idea that ChiP induction normally results from coupling ChiX sRNA decay to the stimulation of chiP expression.

To gather information on the chbBC ICR transcript, the RNA blot in Figure 2A was rehybridized to a probe covering the segment pairing to ChiX. This analysis detected an ~300-base RNA species accumulating during chitobiose induction [solid arrow in Fig. 6C]. Interestingly, this species is not present in the chiX deletion mutant, suggesting that its formation is consequent to pairing with ChiX sRNA. Failure to detect the 300-base RNA in the rns ts mutant, together with the accumulation of a high molecular RNA band in this strain [open arrow in Fig. 6C], points to the involvement of RNase E in the pathway leading to the 300-base RNA.

The chbBC ICR RNA invades ChiX sRNA transcription terminator

The data in Figure 6A suggest that ChiX sRNA is far more susceptible to degradation when pairing with the chbBC ICR than with the chiP 5' UTR. A peculiarity of the chbBC spacer element is that the region of complementarity to ChiX sRNA extends two nucleotides into the transcription terminator hairpin of the sRNA [Fig. 7A]. This raises the possibility that the pairing interaction with chbBC ICR RNA disrupts the bottom portion of ChiX terminator stem, causing the stem to become 2 bp shorter. Given the importance of this region for the overall stability of ChiX sRNA [see mutant analysis above] one expects that the loss of 2 bp would dramatically accelerate sRNA decay. The hypothesis predicts that affecting the ability of the chbBC sequence to base-pair with the terminator portion should prevent its degradation. This prediction was tested by changing either of the two C residues involved to G residues. Results in Figure 7B show that either change attenuates ChiX sRNA decay following activation of chbBC ICR transcription. Concomitantly, either mutation completely abolishes chiP derepression [Fig. 7B]. Therefore, these observations confirm that the first 2 bases on the 5' side of ChiX attenuator stem participate in chbBC ICR pairing. Shortening of the stem might make ChiX sRNA more susceptible to degradation.

Discussion

In this study, we describe the existence of a ChiP in Salmonella, as well as the mechanism by which synthesis of this protein is regulated as a function of substrate availability. ChiP synthesis is induced in the presence of chitoooligosaccharides as a result of two combined events: stimulation of chiP gene transcription and relief of chiP translational repression by ChiX small RNA. Both events are triggered by the transcriptional activation of the chb operon, which encodes the components for chitobiase [and chitotriose] phosphorylpyruvate-dependent phosphotransferase system [PTS] along with the ChbR regulator protein [Plumbridge and Pellegrini 2004]. Binding of a chitobiase/chitotriose-derived metabolite to ChbR causes this protein to activate chb transcription, thus increasing ChbR levels. Inducer-bound ChbR stimulates chiP gene transcription. Concomitantly, an RNA sequence processed from the polycistronic chb transcript [the chbBC ICR element] targets ChiX sRNA for degradation. Altogether, these coordinate events may allow coupling ChiP-mediated uptake of chitoooligosaccharides to their transport across the inner membrane [Fig. 8].

The mechanism by which the chbBC ICR RNA promotes the destruction of ChiX sRNA is unprecedented. ChiX degradation appears to result from chbBC ICR RNA pairing with the portion of the sRNA that makes up the
that the regulatory action of chbBC ICR element must depend on continuous chb operon transcription. Conceivably, this allows the system to quickly readjust once the inducer concentration falls and, with it, the requirement for ChiP activity. One might wonder about what makes such a complex transcriptional-translation control to be preferred to a more classical regulation mediated by the ChbR activator protein only. Particularly counterintuitive in the current mechanism is that chip repression under uninduced conditions relies on both chip and chiX genes to be continuously transcribed. Perhaps the answer lies in the timing of the induction response. Having the chip mRNA already present when ChiX sRNA concentration begins to fall (concomitant with the appearance of the chbBC ICR RNA) might allow ChiP synthesis to start before the chb operon is fully induced, namely, before accumulation of the ChbR activator. The ChiX component of the regulatory mechanism could therefore allow for more efficient priming of the induction switch.

The ChiP protein is highly conserved in enteric bacteria with sequence identities exceeding 90% in the Escherichia and Shigella orthologs. The protein belongs to the OpfD family and is phylogenetically unrelated to the chitinoporin from Vibrio species (Keyhani et al. 2000). Chitin is the most commonly occurring nitrogen-containing polysaccharide in nature. Since Salmonella and E. coli do not naturally secrete chitinases (Francetic et al. 2000), these organisms probably rely on other microorganisms producing the ChiP substrates in the environment. This could occur through the activity of other, chitinase-proficient microorganisms or metabolic processes in eucaryotic hosts. It is relevant in this respect that an increasing number of chitinases have been identified recently in a variety of mammalian species including humans (Escott and Adams 1995; Zhu et al. 2004).

After this work was completed, a report has appeared describing the silencing of ChiP (YbfM) by ChiX (named MicM) in E. coli (Rasmussen et al. 2009). Rasmussen et al. (2009) presented evidence for the base-pairing interaction between the sRNA and the chip 5' UTR and substantiated the importance of Hfq as a catalyst of the interaction. However, the study did not elucidate the function of ChiP nor the basis for ChiX (MicM)-dependent regulation. At the same time, a separate report identified ChiX (named RybC) as an sRNA down-regulating the dpiB gene for the sensor component of a two-component system [DpiA/B] required for citrate fermentation and linked to various phenotypes including SOS induction and plasmid stability in E. coli (Mandin and Gottesman 2009). While the role of ChiX in any of these phenotypes remains elusive, the presence of citrate carrier/utilization genes, citA/citB, immediately adjacent to the chipQ operon in Salmonella [see Fig. 1A] may suggest a link between ChiP and citrate metabolism. [Note that no citA/citB orthologs exist in the E. coli, where, somewhat confusingly, CitA/B is an alternative designation for the DpiA/B system] (Kaspar and Bött 2002). We notice that pairing of dpiB 5' UTR with ChiX RNA extends 1 base (2 bases allowing a G:U pair) into the ChiX transcription terminator [Mandin and Gottesman 2009]. This raises the

\[\text{Figure 7. Analysis of mutations affecting pairing of chbBC ICR RNA with bases in ChiX sRNA transcription terminator stem. (A) Model for chbBC ICR pairing to ChiX sRNA. chbBC ICR bases are numbered starting to the right of the translation stop codon [UAA] of the chbB gene. (B) Analysis of the mutations on ChiX sRNA levels and on chipQ regulation. RNA and \(\beta\)-galactosidase measurements were carried out under the same conditions as in Figure 6. Strains used in the Northern blot analysis of ChiX sRNA (top and middle panels) and for the \(\beta\)-galactosidase determinations were MA10043 [\(\text{PBAD-}\{\text{chb-186}\}\) \(\text{chiP-lac}\)], MA10044 [\(\text{PBAD-}\{\text{chb-186 C17G}\}\) \(\text{chiP-lac}\)], and MA10045 [\(\text{PBAD-}\{\text{chb-186 C18G}\}\) \(\text{chiP-lac}\)]. Strains used for Northern blot analysis of chipQ mRNA were MA10051 [\(\text{PBAD-}\{\text{chb-186}\}\)], MA10052 [\(\text{PBAD-}\{\text{chb-186 C17G}\}\)], and MA10053 [\(\text{PBAD-}\{\text{chb-186 C18G}\}\].]
possibility that in addition to, or rather than, being a regulatory target, the dpiB 5' UTR is itself a down-regulator of ChiX sRNA. Transcription of dpiB (citA) in E. coli is activated by citrate during anaerobic growth (Yamamoto et al. 2009). Under these conditions, citrate utilization depends upon the presence of an oxidizable cosubstrate, usually a carbohydrate (Lutgens and Gottschalk 1980). Perhaps raising the basal levels of ChiP protein helps meet such a requirement in the event that bacteria come in contact with chitooligosaccharides.

An implication from our findings is that sRNA targets identified on the basis of sequence complementarity may actually include modulators or competitive inhibitors of sRNA function activity (Seitz 2009). It seems reasonable to predict that additional sRNAs subjected to this type of control will be found in the future. Best candidates are sRNAs that, like ChiX, are synthesized constitutively or semiconstitutively under standard growth conditions. A number of such molecules have been identified in global searches and are still awaiting characterization (Wassarman et al. 2001; Vogel et al. 2003; Zhang et al. 2003; Padalon-Brauch et al. 2008).

Materials and methods

Strains and growth conditions

Strains used in this study were all derived from Salmonella enterica serovar Typhimurium strain MA3409, a strain LT2 derivative cured for the Gifsy-1 prophage (Figueroa-Bossi et al. 1997). The genotypes of the relevant strains used are shown in Supplemental Table S1. The construction of these strains is detailed in the Supplemental Material. Bacteria were cultured at 37°C in liquid media or in media solidified by the addition of 1.5% Difco agar. LB broth (Bertani 2004) was used as complex medium. Carbon-free medium (NCE) and carbon-free and nitrogen-free medium (NCN) (Maloy and Roth 1983), supplemented with the appropriate carbon and/or nitrogen sources, were used as minimal media. Typically NCE medium was supplemented with 0.2% glycerol. Antibiotics (Sigma) were included at the following final concentrations (in LB): chloramphenicol, 10 μg/mL; kanamycin monosulfate, 50 μg/mL; sodium ampicillin 75 μg/mL; spectinomycin dihydrochloride, 80 μg/mL; tetracycline hydrochloride, 25 μg/mL. LB plates containing 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-gal; from Sigma), 40 μg/mL, were used to monitor lacZ expression in bacterial colonies. Liquid cultures were grown in New Brunswick gyrotyro shakers and growth was monitored by measuring the optical density at 600 nm with a Milton-Roy Spectronic 301 spectrophotometer.

Enzymes and chemicals

Restriction enzymes, T4 polynucleotide kinase and Taq DNA polymerase were from New England Biolabs, Pfu-Turbo DNA polymerase was from Stratagene, T4 DNA ligase was from USB. DNA oligonucleotides were obtained from Sigma Aldrich. A list with all oligonucleotides used in this work is in Supplemental Table S2. Acrylamide-bisacrylamide (30%, 29:1) and other electrophoresis reagents were from BioRad. Agarose was from Invitrogen. Hybond-N + membranes and hybridization buffer used for Northern blot analysis were from GE Healthcare and from Applied Biosystems-Ambion, respectively. Megashort-Script T7 kit, Kinase Max kit, RNases A and T1, yeast tRNA were all from Applied Biosystems-Ambion.

Figure 8. Model for chiP regulation. (A) In the absence of inducers, the chiX gene (black arrow) and the chiPQ operon (green arrows) are transcribed constitutively. Pairing of ChiX sRNA with the 5' UTR of chiPQ mRNA inhibits ChiP synthesis, promoting cleavage of the chiP mRNA by RNase E. The chb operon (blue arrows; only three genes shown) is expressed just to the level needed to prime the system. (B) In the presence of chitooligosaccharides, inducer binding to ChbR causes this protein to activate transcription of the chb operon and to further stimulate chiPQ transcription. Processing of the chb transcript (initiated by RNase E) releases chbBC ICR RNA. This RNA base-pairs with ChiX sRNA making it susceptible to the action of a ribonuclease. The drop in ChiX levels, combined with the increase in chiPQ mRNA, relieves chiP repression leading to a burst of ChiP synthesis. ChiP assembles in the outer membrane resulting in more inducer uptake.
**Genetic techniques**

Generalized transduction was performed using the high-frequency transducing mutant of phage P22, HT 105/1 int-201 (Schmieger 1972) as described [Lemire et al. 2008]. Chromosomal engineering was carried out by the λ red recombination method [Datsenko and Wanner 2000; Yu et al. 2000] as implemented by Datsenko and Wanner [2000] and Uzzau et al. [2001].

Donor DNA fragments were generated by PCR using plasmid or chromosomal DNA templates. Preparation of recipient bacteria, DNA electroporation, and isolation and processing of recombinant clones were carried out as described [Lemire et al. 2008].

**β-Galactosidase assays**

β-Galactosidase activity was measured in toluene-permeabilized cells as described by Miller [1992], and is expressed in Miller units throughout this work.

**OMP preparation**

*Salmonella* OMP fraction was purified by the method of Santiviago et al. [2003] and analyzed by polyacrylamide gel electrophoresis as described previously (Bossi and Figueroa-Bossi 2007).

**RNA extraction and Northern analysis**

RNA was prepared by the acid-hot-phenol method from exponentially growing cells (OD600 of 0.35) as described previously (Bossi and Figueroa-Bossi 2007). RNA was separated on a 1.3% gel and transferred to Hybond-N agarose-formaldehyde gel or on an 8% polyacrylamide/8 M urea gel (Bossi and Figueroa-Bossi 2007). RNA was separated on a 1.3% gel and transferred to Hybond-N agarose-formaldehyde gel or on an 8% polyacrylamide/8 M urea gel (Bossi and Figueroa-Bossi 2007). RNA extraction and Northern analysis were carried out as described previously [Bossi and Figueroa-Bossi 2007].

**In vitro RNA synthesis, purification, and labeling**

A template DNA carrying the *chiX* gene fused to the T7 promoter was generated by PCR from genomic DNA. To produce wild-type and A29G mutant templates, amplification was performed using primers ppC19 and ppC20 on genomic DNA from wild-type *Salmonella* hfg and purified by 8% PAGE. After extraction from gel, RNA was phenol-extracted using the KinaseMax kit (Ambion AM1520) Labeled RNA was end-labeled with [γ-32P]ATP (3000 Ci/mmol from Perkin-Elmer) using the KinaseMax kit [Ambion AM1520]. Labeled RNA was purified by 8% PAGE. After extraction from gel, RNA was phenol-extracted, ethanol precipitated, and resuspended in nucleic-acid free water. Before use in binding and structural studies, RNA was heated in refolding buffer (50 mM Tris at pH 8, 0.1 M NaCl, 0.1 M KCl, 1 mM MgCl2) for 3 min at 85°C, followed by 20 min at room temperature, and finally placed on ice.

**Hfq protein purification**

The *Salmonella* hfg gene was cloned into plasmid pNFB28, a pET-16b [Novagen] derivative designed to obtain either C-terminal or N-terminal (7×) His-tagged fusions to genes of interest. C-terminally tagged Hfq protein was purified from *E. coli* BL21 codon+ cells carrying the plasmid clone. Bacteria were grown in 1 L of LB medium supplemented with ampicillin (100 mg/L) at 37°C until OD600 of 0.2, 1 mM isopropyl-1-thio-D-galactopyranoside was added and cells cultured for a further 3 h. Bacteria were harvested by centrifugation at 10,000 g for 20 min at 4°C. Pellets were frozen at −20°C for several hours prior to resuspension in 50 mL of 50 mM Tris-HCl (pH 7.8), 0.3 M NaCl, 10% Glycerol, 0.1% Triton X-100, 100 μg/mL lysozyme, and protease inhibitor cocktail from Sigma. Cells were sonicated by 15 rounds of 10-sec pulses/10-sec intervals, on ice. Lysate was incubated for 10 min at 75°C and centrifuged at 11,800 g for 40 min. The supernatant was loaded on a 5-mL HiTrap Chelating HP prepacked column from GE Healthcare activated according to the manufacturer's instructions. Column was equilibrated with 25 mL of 50 mM Tris-HCl (pH 7.5), 0.3 M NaCl, and the protein eluted with the same buffer supplemented with 0.5 M imidazole. Following SDS-PAGE electrophoresis of elution aliquots, protein containing fraction was incubated for 10 min at 65°C. Buffer exchange and concentration steps were performed with Centricon 10K Amicon, ultrafiltration unit [Millipore]. Protein was kept at 4°C in 50 mM Tris-HCl (pH 7.8), 0.3 M NaCl, 10% glycerol, and 0.1% Triton X-100. The concentration was determined by Bradford assay.

**RNA structural analyses**

End-labeled RNA was refolded as described above. Enzymatic treatments were performed in 10 μL of reaction mix containing 0.2 pmol of RNA, 1 μg of yeast tRNA, 1× Structure buffer [Ambion], and 0.01 ng of RNase A [1 mg/mL; Ambion AM 2274] or 0.1 U of RNase T1 [1 U/mL; Ambion AM 2283]. Incubation was for 12 min at 37°C. Reactions were stopped by addition of 20 μL of completed Precipitation/Inactivation buffer from the same manufacturer. Partial alkaline hydrolysis was performed according to Ambion’s protocol as follows: 10 μL of reaction mix containing 0.2 pmol of RNA, 1 μg of yeast tRNA, 1× Alkaline Hydrolysis buffer, were incubated for 8 min at 95°C, placed on ice, and 20 μL of completed Precipitation/Inactivation buffer [Ambion] added. After recovery from precipitation, all samples were run on a 10% sequencing polyacrylamide gel in 0.5× TBE buffer for 3 h at 4°C at constant current of 15 mA. Results were analyzed by phosphorimaging using ImageQuant software. RNA structural analyses were performed by the following methods: 10 ng of RNA, 1 μg of yeast tRNA, 1× Structure buffer [Ambion], and 0.1 ng of RNase A [1 mg/mL; Ambion AM 2274] or 0.1 U of RNase T1 [1 U/mL; Ambion AM 2283]. Incubation was for 12 min at 37°C. Reactions were stopped by addition of 20 μL of completed Precipitation/Inactivation buffer from the same manufacturer. Partial alkaline hydrolysis was performed according to Ambion’s protocol as follows: 10 μL of reaction mix containing 0.2 pmol of RNA, 1 μg of yeast tRNA, 1× Alkaline Hydrolysis buffer, were incubated for 8 min at 95°C, placed on ice, and 20 μL of completed Precipitation/Inactivation buffer [Ambion] added. After recovery from precipitation, all samples were run on a 10% sequencing polyacrylamide gel in 0.5× TBE. Results were analyzed by phosphorimaging.

**Acknowledgments**

We thank Nicolas Villagra and Francesca Fiorini for participating in the experiments in Figures 2B and 4, respectively. Furthermore, we thank Francesca Fiorini for purifying Hfq and for technical assistance with the in vitro RNA analyses. We are extremely grateful to Jackie Plumbridge and Pepe Casadesús for critical reading of the manuscript and for insightful feedback. This work was made possible by a grant from the French National Research Agency [ANR; BLAN07-1_187785].
Udekwu KI, Wagner EG. 2007. Small RNAs controlling outer membrane porins. Curr Opin Microbiol 10: 152–155.

Vogel J. 2009. A rough guide to the non-coding RNA world of Salmonella. Mol Microbiol 71: 1–11.

Vogel J, Papenfort K. 2006. Small non-coding RNAs and the bacterial outer membrane. Curr Opin Microbiol 9: 605–611.

Vogel J, Bartels V, Tang TH, Churakov G, Slagter-Jager JG, Huttenhofer A, Wagner EG. 2003. RNomics in Escherichia coli detects new sRNA species and indicates parallel transcriptional output in bacteria. Nucleic Acids Res 31: 6435–6443.

Wassarman KM, Repoila F, Rosenow C, Storz G, Gottesman S. 2001. Identification of novel small RNAs using comparative genomics and microarrays. Genes & Dev 15: 1637–1651.

Waters LS, Storz G. 2009. Regulatory RNAs in bacteria. Cell 136: 615–628.

Yamamoto K, Matsumoto F, Minagawa S, Oshima T, Fujita N, Ogasawara N, Ishihama A. 2009. Characterization of CitA–CitB signal transduction activating genes involved in anaerobic citrate catabolism in Escherichia coli. Biosci Biotechnol Biochem 73: 346–350.

Yu D, Ellis HM, Lee EC, Jenkins NA, Copeland NG, Court DL. 2000. An efficient recombination system for chromosome engineering in Escherichia coli. Proc Natl Acad Sci 97: 5978–5983.

Zhang A, Wassarman KM, Ortega J, Steven AC, Storz G. 2002. The Sm-like Hfq protein increases OxyS RNA interaction with target mRNAs. Mol Cell 9: 11–22.

Zhang A, Wassarman KM, Rosenow C, Tjadec BC, Storz G, Gottesman S. 2003. Global analysis of small RNA and mRNA targets of Hfq. Mol Microbiol 50: 1111–1124.

Zhu Z, Zheng T, Homer RJ, Kim YK, Chen NY, Cohn L, Hamid Q, Elia JA. 2004. Acidic mammalian chitinase in asthmatic Th2 inflammation and IL-13 pathway activation. Science 304: 1678–1682.
Erratum

Genes & Development 23: 2004–2015 (2009)

Caught at its own game: regulatory small RNA inactivated by an inducible transcript mimicking its target
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For the above paper, the French Centre National de la Recherche Scientifique [CNRS] has requested that the name of Francesca Fiorini, whose involvement in this study is described in the acknowledgment section of the above article, be included in the author list.

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Genes Dev. published online July 28, 2009
Access the most recent version at doi:10.1101/gad.541609

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Published online July 28, 2009 in advance of the full issue.

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