

# RNA-Mediated Reciprocal Regulation between Two Bacterial Operons Is RNase III Dependent

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**ABSTRACT** In bacteria, RNAs regulate gene expression and function via several mechanisms. An RNA may pair with complementary sequences in a target RNA to impact transcription, translation, or degradation of the target. Control of conjugation of pCF10, a pheromone response plasmid of *Enterococcus faecalis*, is a well-characterized system that serves as a model for the regulation of gene expression in bacteria by intercellular signaling. The *prgQ* operon, whose products mediate conjugation, is negatively regulated by two products of the *prgX* operon, Anti-Q, a small RNA, and PrgX, the transcriptional repressor of the *prgQ* promoter. Here we show that Qs, an RNA from the 5' end of the *prgQ* operon, represses expression of PrgX by targeting *prgX* mRNA for cleavage by RNase III. Our results demonstrate that the *prgQ* and *prgX* operons each use RNAs to negatively regulate gene expression from the opposing operon by different mechanisms. Such reciprocal regulation between two operons using RNAs has not been previously demonstrated. Furthermore, these results show that Qs is an unusually versatile RNA, serving three separate functions in the regulation of conjugation. Understanding the potential versatility of RNAs and their various roles in gene regulatory networks will allow us to better understand how cells regulate complex behavior.

**IMPORTANCE** Bacteria use RNA to regulate gene expression by a variety of mechanisms. The *prgQ* and *prgX* operons of pCF10, a conjugative plasmid of *Enterococcus faecalis*, have been shown to negatively regulate one another by a variety of mechanisms. One of these mechanisms involves Anti-Q, a small RNA from the *prgX* operon that prevents gene expression from the *prgQ* operon. In this work, we find that Qs, an RNA from the *prgQ* operon, negatively regulates gene expression from the *prgX* operon. These findings have a number of implications. (i) The Anti-Q and Qs RNAs act by different mechanisms, highlighting the variety of ways in which bacteria can regulate gene expression using RNAs. (ii) Reciprocal regulation between operons mediated by small RNAs has not been previously described, deepening our understanding of how bacteria regulate complex behavior. (iii) Additional roles for Qs have been described, demonstrating the versatility of this RNA.

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Regulatory RNAs play an integral role in control of gene expression in bacteria. RNAs that interact with and regulate other RNAs via direct base pairing are referred to as antisense RNAs and may be encoded in *trans*, on a different region of DNA, or in *cis*, on the same region of DNA as their targets. Antisense RNAs encoded in *cis* are best studied in mobile genetic elements (1) and in toxin-antitoxin loci, found both on chromosomes and extrachromosomal elements (2). These RNAs have complete complementarity to their targets and generally do not require the RNA chaperone Hfq for productive interaction (3). RNA sequencing studies have recently revealed many other *cis*-encoded antisense RNAs in bacterial chromosomes, where their role is beginning to be explored (4, 5).

In many cases, antisense RNAs control expression of the target gene by directing degradation of the target message (3, 5). RNase III is an endoribonuclease that can mediate this regulation by cleaving double-stranded RNA (6, 7). Homologs have been identified in all three domains of life and studied well both in eu-

karyotes, where RNase III family members Drosha and Dicer are involved in small RNA maturation (8), and in eubacteria. In bacteria, RNase III has been found to play several roles, including maturation of tRNA and rRNAs, as well as RNA-directed gene silencing via degradation of regulatory RNA-mRNA pairs (7). Unlike RNases such as RNase E, RNase J1, and RNase Y, RNase III can efficiently process substrate RNAs with 5' triphosphate groups (9–11). This allows RNase III to initiate RNA decay by cleaving its target and generating a 5' terminus with a monophosphate, which can then serve as an efficient docking site for other RNases.

pCF10 is a conjugative plasmid of *Enterococcus faecalis*. Conjugation is initiated when cCF10, a peptide pheromone secreted by plasmid-free *E. faecalis* cells, is imported by plasmid-bearing cells (12). Inside the host cell, cCF10 interacts with PrgX, alleviating its repression of the *prgQ* promoter ( $P_Q$ ), located on the plasmid, causing transcription from  $P_Q$  to increase 5- to 10-fold. However, the increase in expression of downstream genes in the *prgQ* operon is more than 100-fold (13). This augmentation is the

result of a cascade of regulatory interactions between the *prgQ* operon and the *prgX* operon, whose products repress conjugation.

Transcription of *prgX* is initiated at a promoter ( $P_X$ ) within the *prgQ* operon, but oriented in the opposite direction (see Fig. S1 in the supplemental material), making transcription of the two operons convergent for 223 nucleotides (nt) (14). Each operon encodes a small RNA within its 5' terminus, which is antisense to 5' sequences within the other operon (15, 16). We have previously demonstrated (17) that Anti-Q, encoded in the *prgX* operon, negatively regulates gene expression from the *prgQ* operon by enhancing termination within a 5' leader sequence. This mechanism can function *in vitro* without host factors beyond RNA polymerase (RNAP) (17). In this work, we find that Qs, an RNA produced from 380 nt at the 5' end of the *prgQ* operon, directs the host-encoded RNase III to cleave X RNA, the *prgX* message, within the region of complementarity. This activity can be reconstituted *in vitro*.

Our cumulative data demonstrate that Qs RNA has at least three distinct functions in the regulation of conjugation. It serves as a leader sequence that can attenuate the expression of downstream genes via termination (17); it acts as an mRNA for *prgQ*, whose polypeptide product is processed to the inhibitor peptide iCF10 (18); and it is a regulatory RNA that directs degradation of X RNA. More strikingly, this work shows that RNAs derived from the 5' end of each operon reciprocally regulate expression of downstream genes from the opposing operon via distinct mechanisms. To the best of our knowledge, such reciprocal regulation using RNAs has not been previously demonstrated.

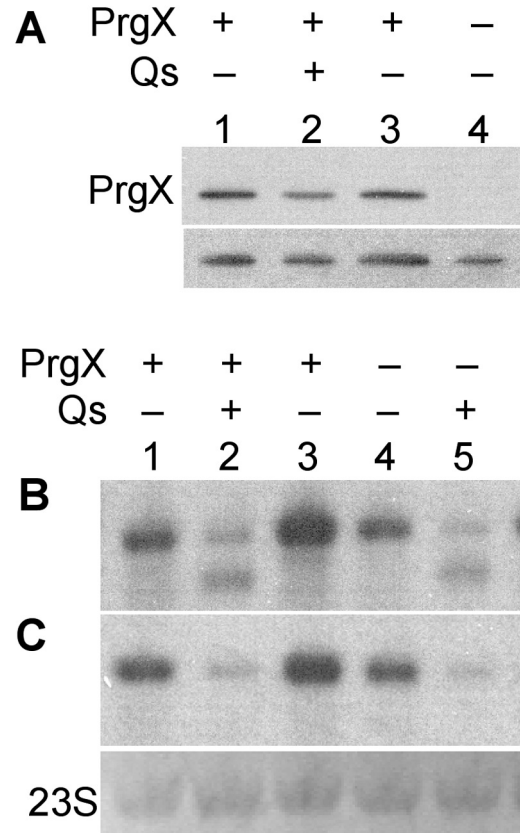
## RESULTS

Cells bearing pCF10 produce X RNA, a 1.4-kb transcript that encodes PrgX, as well as other transcripts initiated at the *prgX* gene, including a 1.2-kb transcript (16) (see Fig. S2 in the supplemental material). Upon pheromone induction, X-RNA levels drop dramatically and PrgX protein levels drop slightly (16). Recent work demonstrated that when  $P_Q$  is not repressed by PrgX, transcription from this promoter directly interferes with transcription from  $P_X$  *in cis*; transcripts from  $P_Q$  also had a *trans*-acting negative effect on expression of transcripts from  $P_X$  (13). We hypothesized that the Qs RNA negatively regulates the expression of PrgX by acting on X RNA via a mechanism involving interaction between complementary sequences on the RNAs.

In order to explore Qs regulation of PrgX and X-RNA expression, we used a previously developed system in which transcripts from the *prgX* and *prgQ* operons are provided separately from compatible plasmids (13, 17). Only one operon is transcribed from each plasmid, the promoter of the opposing operon being inactivated by point mutations within its  $-10$  region (see Fig. S1 in the supplemental material).

To test whether Qs regulates PrgX expression, we generated *E. faecalis* strains bearing the plasmid that transcribes the *prgX* operon, pBK2-26, with and without the plasmid that transcribes Qs, pDM5-25. We then performed Western blotting using a PrgX-specific antibody on whole-cell lysates from these strains (Fig. 1A). We found that providing Qs *in trans* reduced PrgX levels to  $77\% \pm 10\%$  (Fig. 1A, compare lane 2 to lane 1), whereas the empty vector did not (Fig. 1A, lane 3).

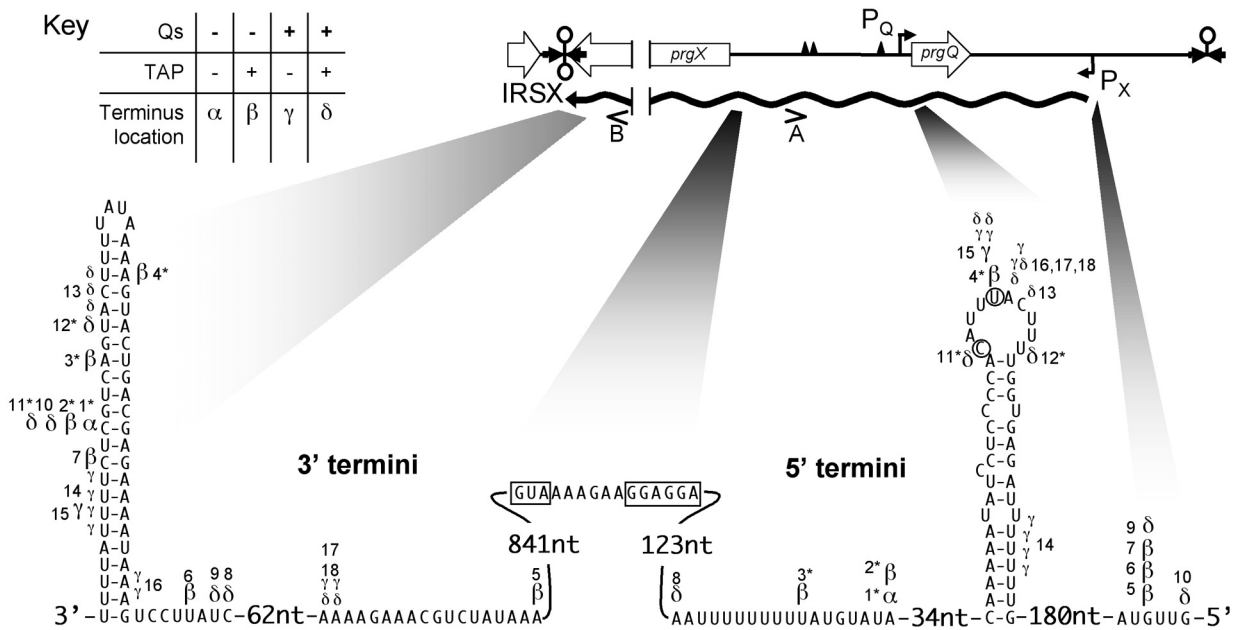
In order to test the effect of Qs on X RNA, we prepared RNA from these strains and analyzed it using Northern blots. We hybridized blots using a probe specific for the *prgX* open reading



**FIG 1** Qs negatively regulates expression of PrgX and its message, X RNA. Western and Northern blots against products of the *prgX* operon produced by *E. faecalis* OG1Sp bearing the following plasmids are shown: pBK2-26 (lane 1), pBK2-26 plus pDM5-25 (lane 2), pBK2-26 plus pAT18 (lane 3), pBK2-126 (lane 4), pBK2-126 plus pDM5-25 (lane 5), and pBK2-126 plus pAT18 (lane 6). pBK2-26 produces X RNA with a wild-type *prgX* ORF. pBK2-126 carries missense mutations in codons 3 and 4 of the *prgX* ORF. pDM5-25 produces Qs. pAT18 is the vector control for pDM5-25. (A) A Western blot for PrgX. The bottom blot shows an unidentified host protein that serves as a loading control (16). (B and C) Northern blots hybridized to a probe specific for the *prgX* ORF (B) or the first 99 nt of X RNA (C). The loading control is an ethidium bromide stain of 23S rRNA.

frame (ORF); in the absence of Qs, we detected a single 1.4-kb band (Fig. 1B, lane 1). This band was the correct size to be a transcript that initiated at  $P_X$  and terminated at IRSX, the *prgX* terminator. When we provided Qs, this band diminished in intensity, and a second,  $\sim 1.2$ -kb band appeared (lane 2). The empty vector had no effect (lane 3). A probe specific for 99 nt of the 5' end of X RNA hybridized to the 1.4-kb band, but not the  $\sim 1.2$ -kb band (Fig. 1C). To test whether this phenomenon was dependent on PrgX, we introduced nonsense mutations into the third and fourth codons of the *prgX* gene, preventing translation of PrgX (Fig. 1A, lane 4) and found that this did not alter the effect of Qs (Fig. 1B, compare lanes 1 to 3 with lanes 4 to 6). Taken together, these data indicate that Qs causes differential expression of *prgX* transcripts, generating a second *prgX* message that is missing the 5' terminus of full-length X RNA, and that PrgX is dispensable for this phenomenon.

We hypothesized that the  $\sim 1.2$ -kb band was generated by nucleolytic removal of about 200 nt from the 5' terminus of the



**FIG 2** Qs directs removal of ~200 nt from the 5' terminus of X RNA. The 5' and 3' termini of single X-RNA transcripts were determined using a coupled 5'-3' RACE protocol. A map of the region is shown, with the full-length X RNA transcript drawn as a thick wavy line. Arrowheads labeled A and B indicate the positions of oligonucleotides pCF10\_8072 and pCF10\_7149R, respectively. A partial sequence and proposed structures within X RNA are shown, along with the number of nucleotides in any gaps. Shaded triangles show the position of given sequences along the X-RNA transcript. The mapped 5' and 3' termini are indicated by letters that correspond to the RNA sample from which that transcript was derived (see key and text). Numbers next to these letters identify the corresponding 5' and 3' termini sequenced from single transcripts. Asterisks indicate that 1 to 6 nt of ectopic sequence was inserted at the junction between the termini. Circled residues were identified as processing points *in vitro* (Fig. 4).

1.4-kb band. In order to test this, we used a coupled 5' and 3' rapid amplification of cDNA ends (5'-3'RACE) protocol to identify both termini of single X-RNA transcripts. We also used tobacco acid pyrophosphatase (TAP) to distinguish 5' termini generated as a result of *de novo* RNA synthesis from those derived by RNA processing.

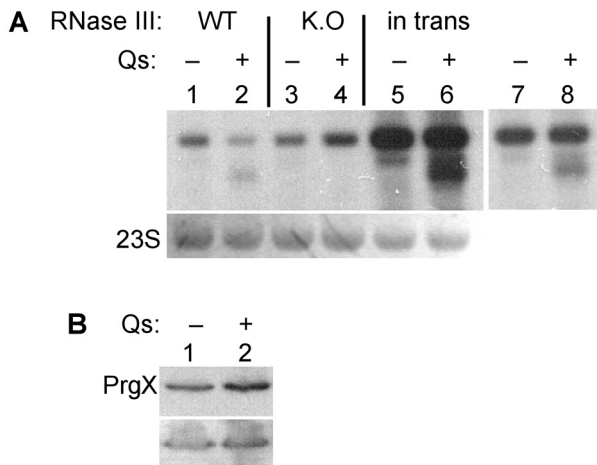
In the presence of Qs, most of the 5' termini clustered in two distinct regions (Fig. 2). One contained the *prgX* transcriptional start site, a pair of closely spaced G residues (16). These 5' termini were detected only in the TAP-treated sample ( $\delta$ ). The other region was about 205 nucleotides downstream, within sequences complementary to Qs. Termini in this region were identified in TAP-treated and untreated samples ( $\gamma$ ), indicating that they were generated as a result of processing, rather than transcript initiation. In the absence of Qs, the most commonly detected 5' terminus was the *prgX* transcriptional start site, detectable only in the TAP-treated sample ( $\beta$ ). In the sample not treated with TAP ( $\alpha$ ), most sequences tested did not contain a bona fide *prgX* 5'-3' junction, but rather nonspecifically amplified sequence. The 3' termini from all samples generally fell within IRSX, though recessed 3' termini were also detected. Taken together, these data indicate that the smaller RNA is generated by removal of about 200 nt from the 5' end of full-length X RNA, rather than the activity of a second promoter, and that there is no coordinated processing of the 3' terminus.

Because X RNA is processed in a region that is complementary to Qs, we hypothesized that RNase III is responsible for the processing. *E. faecalis* V583 has one annotated gene that codes for RNase III, *rnc* (EF-3097; NCBI accession # AEO16830). The predicted protein sequence of this gene is 39% identical to *Esche-*

*richia coli* RNase III and is conserved in *E. faecalis* OG1RF. BLAST searches of the *E. faecalis* OG1RF genome using the predicted protein sequence of EF3097 and the protein sequence of *E. coli* RNase III did not produce any other alignments with an E value below 1.5, suggesting that there is one copy of *rnc* encoded in the *E. faecalis* chromosome. We generated an in-frame deletion of *rnc* in *E. faecalis* OG1RF, generating strain OG1RF $\Delta$ 3097. Under the conditions tested, this strain was not defective for growth in broth culture (see Fig. S3 in the supplemental material).

To test whether RNase III cleaved X RNA in a Qs-dependent fashion, we transformed *E. faecalis* OG1RF $\Delta$ 3097 with the X-RNA-producing plasmid with and without the Qs-producing plasmid. We also provided RNase III *in trans* from a plasmid that transcribed *rnc* under control of P<sub>23</sub> (19), a constitutive promoter. We found that in the absence of RNase III, Qs did not direct processing of X RNA (Fig. 3A, lanes 3 and 4). When RNase III expression was complemented in *trans*, Qs-directed processing of X RNA was restored (lanes 5 to 8). X RNA was expressed at substantially higher levels under these conditions (lanes 5 and 6). The reason for this is unclear. Additionally, when RNase III was deleted, cells bearing the entire pCF10 plasmid no longer produced processed X RNA (see Fig. S1 in the supplemental material).

We then tested whether RNase III was necessary for Qs to repress PrgX expression. We performed Western blotting on lysates from *E. faecalis* OG1RF $\Delta$ 3097 bearing an X-RNA-producing plasmid with a wild-type *prgX* ORF with and without a Qs-producing plasmid. We found that in the absence of RNase III, Qs did not repress PrgX expression (Fig. 3B). In fact, X RNA and PrgX expression appeared to increase in response to Qs (2.1  $\pm$  1.2-fold and 1.6  $\pm$  0.1-fold, respectively). It is possible that in the absence



**FIG 3** RNase III is necessary for Qs-directed cleavage of X RNA *in vivo*. (A) RNA was prepared from the indicated strains of *E. faecalis* with pBK2-126, producing X RNA, with (+) or without (-) Qs from pDM5-25. The *rnc* gene encoding RNase III is either present on the chromosome (wild type [WT]), deleted from the chromosome (knockout [K.O]), or deleted from the chromosome and provided from pCJ9:His-EF3097 (*in trans*). This RNA was analyzed by Northern blotting using a probe specific for the *prgX* open reading frame. Lanes 7 and 8 are identical to lanes 5 and 6 except the film was exposed for a shorter duration to allow better visualization of the bands. The loading control is an ethidium bromide stain of 23S rRNA. (B) Western blotting was performed on total cellular protein from *E. faecalis* OG1RFΔ3097 bearing pBK2-26 providing X RNA with a wild-type *prgX* ORF with and without pDM5-25, providing Qs. The bottom blot shows an unidentified host protein that serves as a loading control.

of RNase III, formation of a duplex with Qs protects X RNA from alternate degradation pathways. This conjecture was not explored further. These data demonstrate that RNase III is necessary for Qs-directed processing of X RNA and repression of PrgX.

To confirm that RNase III mediates cleavage of X RNA, we fused a six-histidine tag to the N terminus of *E. faecalis* RNase III, similar to a method used to purify *S. aureus* RNase III (20) and purified His-tagged RNase III from *E. faecalis* cell lysates (see Fig. S4 in the supplemental material). We then tested this preparation for its ability to cleave both X RNA and Qs in the presence and absence of the other RNAs *in vitro*. We found that RNase III did not cleave X RNA or Qs alone but cleaved both RNAs at several locations in the presence of the complementary transcript (Fig. 4A). We found that 5'-labeled cleavage products were about 200 nt or less in length, indicating that cleavage took place within the region of complementarity between the RNAs. These data indicate that RNase III cleaves X RNA when directed by Qs.

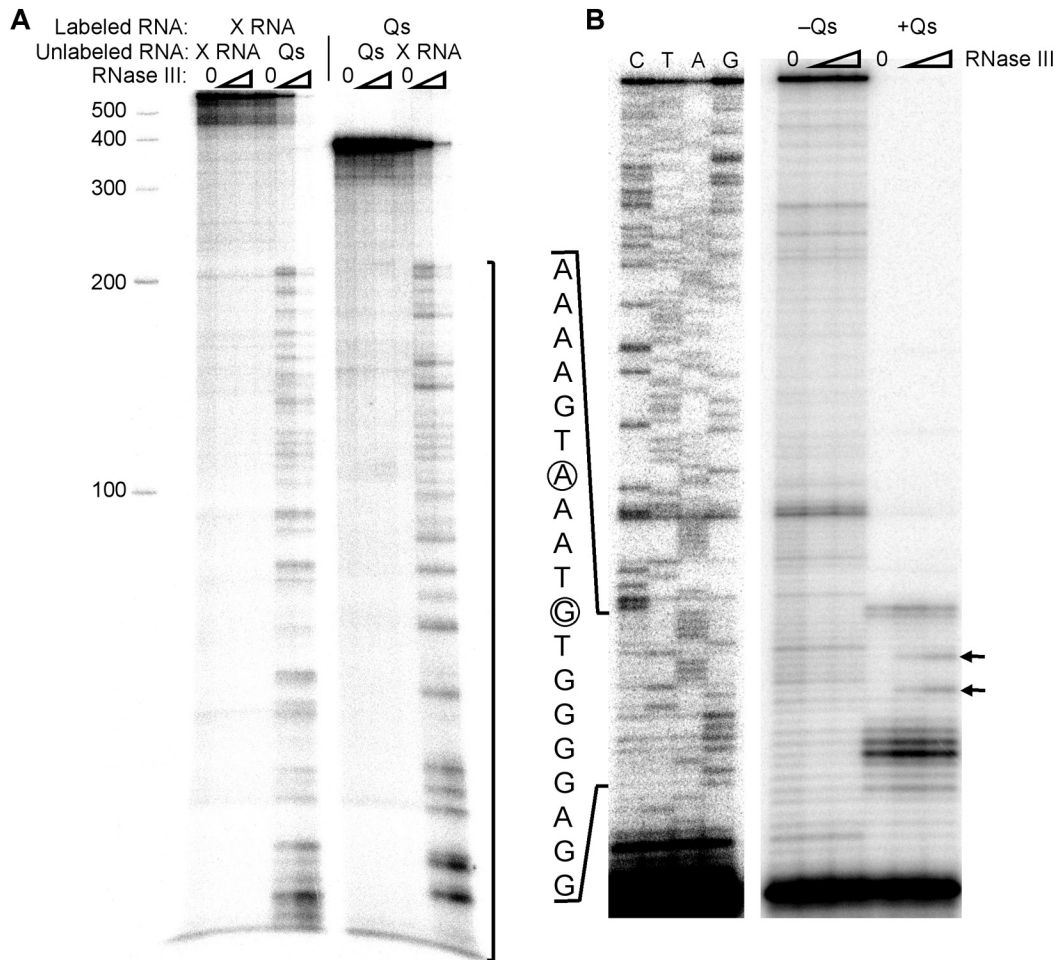
We tested whether RNase III cleaved X RNA *in vitro* at processing sites identified *in vivo*. To do this, we used RNase III to cleave unlabeled X RNA in the presence and absence of Qs and used primer extension to map the cleavage sites (Fig. 4B). In the absence of Qs, we could not detect RNase III-mediated cleavage of X RNA. When Qs was added without RNase III, primer extension generated numerous new bands within the region of complementarity, indicating that the reverse transcriptase had difficulty extending through the RNA duplex. When RNase III was added, two additional bands appeared, both mapped to 5' termini identified by *in vivo* experiments (Fig. 2). These data indicate that RNase III is responsible for the processing of X RNA observed *in vivo*.

To better understand how Qs directs processing of X RNA, we

performed a deletion analysis of Qs. We found that the only aspect of Qs necessary to direct processing of X RNA is any sufficiently long portion of Qs between positions +1 and +110, which is entirely complementary to X RNA; however, no particular sequences within this region were needed to direct processing, though processing appears to be directed to the region complementary to the Qs sequences provided. Qs sequences that are complementary to X RNA but outside this region do not direct processing (see Fig. S5 in the supplemental material). We used sfold (<http://sfold.wadsworth.org> [21]) to analyze the region of X RNA processing and identified a possible stem-loop structure (Fig. 2). To test whether this predicted structure directed processing of X RNA to a precise location, we generated a mutation predicted to disrupt the stem structure in each RNA (Mut48) and a compensatory mutation that restored the stem structure in Qs (Mut49) (Fig. 5A). We transformed *E. faecalis* OG1Sp with plasmids bearing wild-type or mutant X RNA and Qs alleles and assessed processing of X RNA using Northern blots. Consistent with the deletion analysis, these mutations did not prevent processing (Fig. 5B). When either Qs or X RNA carried the stem-disrupting mutation, the processing site was altered (Fig. 5B, compare lane 1 to lanes 2 and 4). This was not corrected if the predicted stem structure was restored in Qs by further mutation (lane 5). If both RNAs carried the stem-disrupting mutation, the site of processing shifted back to its wild-type location (lane 3). We then used a 5' RACE protocol to identify the processing site when both RNAs were wild type (lane 1) or both carried the stem-disrupting mutation (lane 3). The primary location of processing was identical in both cases (Fig. 5A). We conclude that, under the conditions tested, the predicted stem-loop structure is dispensable for directing processing to the wild-type location and that the location of the processing site is instead dependent on complementarity between the two RNAs. Furthermore, because the Qs alleles that are missing the *prgQ* ribosome binding site (RBS) and initiation codon can direct processing, translation of *prgQ* is dispensable for processing (Fig. S5).

**Conclusion** This is the first report of two opposing operons that each use a small RNA to reciprocally regulate downstream gene expression from the other operon. Notably, each RNA acts via a different mechanism; Qs, derived from the 5' terminus of the *prgQ* operon, directs posttranscriptional processing of X RNA of the *prgX* operon by the host factor RNase III. Previous work demonstrated that Anti-Q, an RNA derived from the 5' terminus of the *prgX* operon, negatively regulates transcription readthrough from the *prgQ* operon without the assistance of host-encoded proteins (16, 17, 22). These regulatory effects are embedded within a larger network of interactions between the *prgQ* and *prgX* operons, which will be discussed below.

The results of experiments presented in this work suggest a model for Qs regulation of X RNA presented in Fig. 6A. X RNA is transcribed from the *prgX* operon. When not interacting with Qs, X RNA does not present any suitable target sites for RNase III. Qs is transcribed from the opposite strand of DNA as X RNA. The 5' region of Qs interacts with complementary sequences in the 5' region of X RNA, forming a duplex over 100 bp in length. This double-stranded heteroduplex is cleaved in multiple locations by RNase III; the cleavage sites closest to the 3' region of X RNA generate the new 5' termini detected *in vivo*. Processed X RNA may serve as a substrate for subsequent degradation, as discussed below.

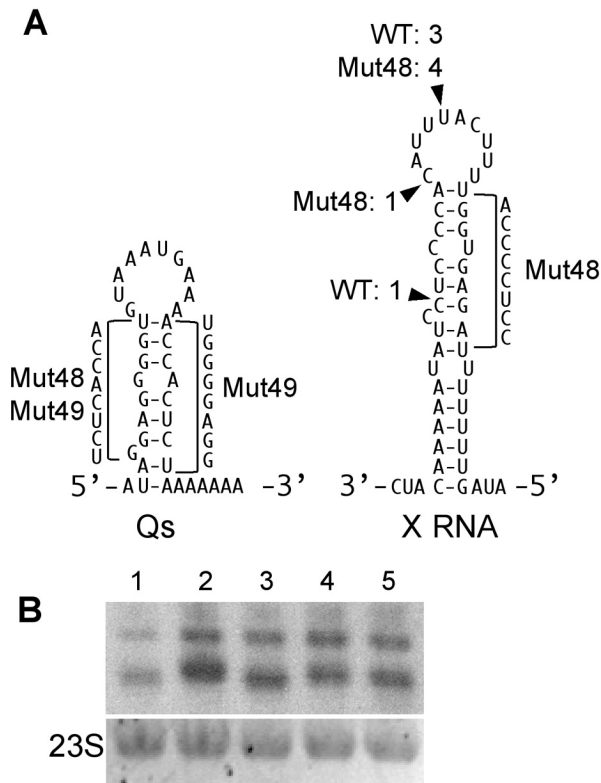


**FIG 4** RNase III mediates Qs-directed cleavage of X RNA *in vitro*. (A) Full-length Qs and a 550-nt 5' fragment of X RNA were transcribed *in vitro*. One RNA was 5' labeled with  $^{32}\text{P}$  and incubated with or without the unlabeled antisense RNA and with or without increasing levels of RNase III. The amount of RNase III is indicated above the gels by 0 or by the height of the white triangle above the gel. The products were electrophoresed on a denaturing 6% polyacrylamide-urea gel and examined using a phosphorimager. Products digested in the overlapping region between the RNAs are indicated by the bracket to the right of the gel. The far left lane is a size standard with the length in nucleotides indicated. (B) Primer extension reaction done on an unlabeled 5' X-RNA fragment incubated with and without Qs and with and without RNase III. The DNA oligonucleotide was 5' labeled with  $^{32}\text{P}$  and used to prime a reverse transcription reaction. The products were electrophoresed on a denaturing 10% polyacrylamide-urea gel and examined using a phosphorimager. A chain termination ladder is shown in the left gel. The ladder is from the same gel as the extensions, but with contrast and brightness adjusted to visualize the bands.

These findings also reveal that Qs plays at least three roles in the regulation of conjugation, acting as an attenuating leader sequence, an mRNA, and a regulatory RNA that can act in *trans*. Such versatility is uncommon, though RNAIII of *Staphylococcus aureus*, *pel* of *Streptococcus pyogenes*, and SgrS of *E. coli* are all bifunctional, acting as regulatory RNAs and mRNAs (23–25).

Transcription of the *prgQ* and *prgX* operons is convergent for 223 nt. One consequence of this arrangement is that when  $P_Q$  is derepressed, transcription of the *prgQ* operon represses transcription from  $P_X$  via transcriptional interference (13). Another consequence is that RNAs transcribed within the overlapping region are perfectly complementary to transcripts from the other operon, giving rise to at least two antisense regulatory interactions. These interactions allow one operon to become dominant at the expense of the other; pheromone-sensitive repression of the *prgQ* promoter by PrgX ultimately determines the relative levels of transcription of the two operons. Our current model of regulation

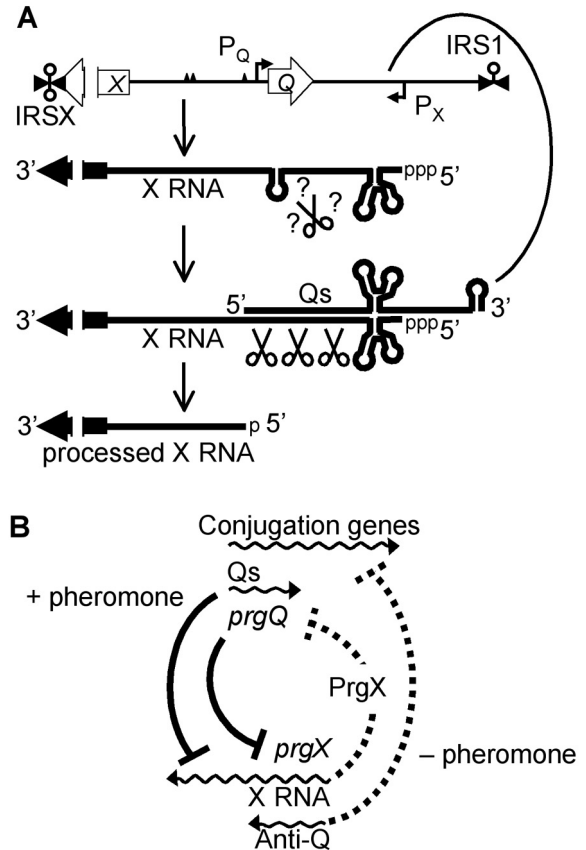
between the *prgQ* and *prgX* operons is depicted in Fig. 6B. In the absence of cCF10, the products of the *prgX* operon are dominant and prevent conjugation (broken lines). Transcription from  $P_Q$  is repressed by PrgX. Repression is leaky, allowing transcription of the *prgQ* gene. Our previous report showed that the Anti-Q small RNA (sRNA) attenuates the expression of downstream conjugation genes by interacting with nascent *prgQ* transcripts to favor formation of a factor-independent terminator within these transcripts. This terminated transcript is Qs, which encodes iCF10, an inhibitory peptide that interacts with PrgX to maintain repression (18, 26). Plasmid-free *E. faecalis* cells secrete the pheromone cCF10. When this is imported into the host cell, products of the *prgQ* operon become dominant (solid black lines). cCF10 interacts with PrgX, alleviating repression of  $P_Q$ . The increase in transcription from  $P_Q$  directly represses transcription from  $P_X$ , reducing expression of products of the *prgX* operon. Additionally, substantially more Qs is produced, which directs processing of X



**FIG 5** A predicted stem-loop structure is not necessary to direct processing of X RNA. (A) The sequence and a predicted structure of Qs and X RNA in the region of processing. The RNA is indicated below each diagram. Mutations tested are shown. The black arrowheads indicate the location of 5' termini of processed X RNA identified by 5' RACE with the number showing how many times each terminus was identified when both Qs and X RNA were wild type (WT) or when both carried mutation 48 (Mut48). (B) RNA was prepared from *E. faecalis* OG1Sp carrying plasmids that produce the X RNA and Qs alleles indicated below and then analyzed by Northern blotting using a probe specific for the *prgX* open reading frame. Lane 1, WT X RNA and WT Qs; lane 2, WT X RNA and Mut48 Qs; lane 3, Mut 48 X RNA and Mut48 Qs; lane 4, Mut48 X RNA and WT Qs; lane 5, WT X RNA and Mut49 Qs. The bottom gel was stained with ethidium bromide to show 23S rRNA.

RNA, decreasing PrgX expression. The increase in transcription from  $P_Q$  eventually titrates Anti-Q levels, allowing transcription to extend past the transcription terminator IRS1, into genes functionally involved in conjugation. This regulatory pathway allows sensitive detection of the pheromone signal and a robust switch-like response to changes in the ratio of cCF10 to iCF10 (13, 27).

Unlike *trans*-encoded antisense RNAs, *cis*-encoded antisense regulatory RNAs have extended regions of complementarity to their targets. Despite this, it is often the case that only specific regions interact to mediate the regulatory effects (28–31). Complementarity within these regions is essential, but dispensable for the rest of the RNA, which generally serves a structural role, positioning the interacting motifs for optimal pairing. Under the conditions tested, Qs does not require any particular sequence or structure in order to target X RNA for processing. Instead, any sufficiently large portion of the 5' end of Qs can direct processing within complementary sequences within X RNA. Such general requirements would allow this mechanism to function without constraining evolution of the *prgQ* peptide-coding region, which is within the portion of Qs that directs processing. Additionally,



**FIG 6** Qs-directed processing of X RNA is part of a regulatory circuit. (A) A model of how Qs directs cleavage of X RNA as described in the text. A map of the *prgQ* or *prgX* region of pCF10 is shown as a thin black line with features. RNA is indicated by thick black lines that are labeled X RNA or Qs. Scissors represent RNase III, while “ppp” and “p” indicate that an RNA has a 5' triphosphate or monophosphate group, respectively, at the 5' terminus. (B) A model for mechanisms of reciprocal regulation between the *prgQ* and *prgX* operons, which control the pheromone response, as described in the text. The presence or absence of pheromone is shown. *prgQ* and *prgX* indicate transcription initiated at the respective promoters, RNAs are indicated by wavy lines, inhibitory relationships are indicated by thick black lines with a bar at the end, broken lines indicate regulatory mechanisms employed by the *prgX* operon, and solid black lines indicate regulatory mechanisms employed by the *prgQ* operon. The PrgX protein is indicated.

this mechanism may allow processed or incompletely transcribed Qs RNA to contribute to the regulation of X RNA. Recent modeling suggests that collision of RNA polymerases from the convergent *prgQ* and *prgX* promoters produces truncated RNA fragments (13). Indeed, pCF10 produces *prgQ* fragments, detectable by Northern blotting, that are shorter than Qs (15, 22). We note that under the conditions tested, X RNA and Qs were provided in *trans* at a high gene copy number. Within the context of pCF10, the gene copy number is lower and the RNAs are transcribed from the same DNA molecule. These differences may mask subtle but biologically meaningful contributions of RNA structure or the *cis* configuration to Qs-directed processing of X RNA.

RNase III mediates the effects of many antisense RNAs, both encoded in *cis*, such as the R plasmid *hok-sok* system (32), and encoded in *trans*, such as RNAIII-*spa* (20). RNase III is able to cleave double-stranded RNA helices greater than 20 bp, although some nucleotide combinations act as “antideterminants” to pre-

vent cleavage at certain sites (33). These features are apparent for *E. faecalis* RNase III from analysis of our *in vitro*-reconstituted system (Fig. 4). The labeled RNAs show a laddering pattern that suggests cleavage occurs at several distinct locations along the duplex, with RNase III recognizing the entire duplex but being guided to certain sites by the sequences within the duplex. *In vivo* results, however, suggest that a complete duplex between complementary sequences of Qs and X RNA may not form. Qs RNA from positions +120 to +223 is complementary to X RNA from positions +1 to +103 but does not direct RNase III cleavage. These complementary RNA regions are both highly structured. It is possible that kissing loops within this region of X RNA and complementary structures within Qs interact, imposing torsional constraints on the RNAs that prevent formation of a full duplex and RNase III target sites within this region.

RNase III cleavage of X RNA is upstream of the *prgX* ribosome binding site and open reading frame and is not coordinated with processing of the 3' terminus, leaving processed X RNA with all of the components necessary for PrgX translation. Preliminary findings suggest that translation is suppressed from processed X RNA (C. M. Johnson and G. M. Dunny, unpublished data). It is also possible that processing initiates decay of X RNA. The 5' terminus of unprocessed X RNA has a triphosphate group and extensive secondary structure, both of which protect RNA from degradation (34). RNase III cleavage generates a 5' monophosphate and may allow X RNA to serve as a substrate for docking and activation of other RNases, such as RNase J1. RNase III cleavage has been shown to initiate decay of several mRNAs in *E. coli* (35, 36). We are currently investigating subsequent steps in X RNA degradation and possible roles for processed X RNA in the regulation of pCF10 conjugation.

## MATERIALS AND METHODS

**Strains, plasmids, and reagents.** *E. coli* was cultured in LB broth and plated on LB plates with 1.5% agar. Antibiotics were used at the following concentrations for *E. coli*: erythromycin, 200  $\mu$ g/ml; chloramphenicol, 20  $\mu$ g/ml. *E. faecalis* was cultured in M9-YEG(37) or THB (Todd-Hewitt Broth-Difco) broth and plated on THB plates with 1.5% agar. Antibiotics were used at the following concentrations for *E. faecalis*: erythromycin, 10  $\mu$ g/ml; chloramphenicol, 20  $\mu$ g/ml; spectinomycin, 1,000  $\mu$ g/ml. Strains and plasmids are listed in Table S1. Oligonucleotides used in this study are listed in Table S2. X RNA was provided from plasmids with a chloramphenicol resistance marker and a pCI305 replicon. Qs was provided from plasmids with an erythromycin resistance marker and a pAM $\beta$ 1 replicon. pCI305 and pAM $\beta$ 1 have copy numbers of  $11 \pm 4$  and  $61 \pm 25$  plasmids/chromosome, respectively, in *E. faecalis* (D. A. Manias and G. M. Dunny, unpublished observations).

**Cloning.** Mutations were introduced into plasmids using standard molecular biology techniques, as summarized in Table S3. The construction of the expression vector pCJ9 is shown in Fig. S6.

*E. faecalis* OG1RF $\Delta$ 3097 was generated by using a previously described allelic exchange system (37) to replace the EF3097 open reading frame in strain OG1RF with the sequence ATGAAACAAGGTGAAATAAAAAAAGTATTCCTCAGTAA. The sequence of the mutation was verified by Sanger sequencing reactions primed with EF3097\_UpSeq and EF3097\_DnSeq.

**Northern blots.** *E. faecalis* strains were cultured and RNA was prepared using a Qiagen RNeasy kit with modified enzyme lysis procedure as previously described (17, 38). One microgram of RNA was electrophoresed on a denaturing 1% agarose gel in morpholinepropanesulfonic acid buffer, transferred to a nylon membrane, cross-linked, and hybridized with digoxigenin-labeled probes as previously described (16, 17).

Digoxigenin-UTP body-labeled RNA probes were prepared as previ-

ously described (17). Templates for the probes used in this study were generated with the following primer sets: for the *prgX* ORF probe, pCF10\_7002\_T7 and pCF10\_7983R; for the probe specific for the 5' terminus of X RNA, pCF10\_8298F\_SP6 and pCF10\_8395R; for the Qs probe, pCF10\_8599R\_T7 and pCF10\_8177F; for the Qs from positions +34 to +120 (Qs +34 to +120) probe, pCF10\_8286R\_SP6 and pCF10\_8211\_BsaI; for the Qs +121 to +223 probe, Qa\_RNA\_F\_SP6 and Qa\_RNA\_R.

**5'-3' RACE.** RNA was prepared as described above for Northern blots. The coupled 5'-3' mapping protocol was performed essentially as previously described (4), except that RNA was treated with a Turbo-DNA free kit (Ambion). Tobacco acid pyrophosphatase (TAP) treatment allows processed RNAs to be discerned from unprocessed RNAs because the 5' termini of unprocessed bacterial RNAs carry a triphosphate group, preventing them from serving as a substrate for T4 RNA ligase. RNAs that have been processed by a RNase or treated with TAP do not carry this group, allowing their 5' termini to be ligated to other nucleic acids by T4 RNA ligase. Transcription initiation sites are those 5' termini that are present in TAP-treated samples and absent from TAP-untreated samples (14). Briefly, RNA was divided into aliquots to be treated with TAP to remove 5' triphosphate groups or mock treated and then circularized with T4 RNA ligase. cDNA was generated by reverse transcription using Superscript III primed with oligonucleotide pCF10\_8072 and then PCR amplified using oligonucleotides pCF10\_8072 and pCF10\_7149R. The reaction products were ligated into pGEM-T Easy (Promega), which was used to transform *E. coli* DH5 $\alpha$ . Vector insertions were PCR amplified from individual colonies and the *prgX* 5'-3' junctions were sequenced.

**5' RACE.** RNA was prepared as described above for Northern blots. The 5' termini of processed RNAs were determined as previously described (14) except no TAP-treated samples were prepared. The oligonucleotide ligated to 5' termini was RNA\_oligo. The reverse transcription (RT) reaction was primed with pCF10\_7922, and the PCR was primed with pCF10\_8072 and P1. PCR products were ligated into pGEM-T Easy, which was used to transform *E. coli* DH5 $\alpha$ . Vector insertions were PCR amplified from individual colonies, and the *prgX* 5' termini were sequenced.

**RNase III digest.** RNAs used for *in vitro* experiments were transcribed from PCR templates with T7 promoters using T7 RNA polymerase as previously described (17). Qs was prepared using primers pCF10\_8177\_T7 and Qs\_RNA\_R. The X-RNA 5' fragment was prepared using primers Old\_Anti-Q\_RNA\_T7 and pCF10\_7845. *In vitro*-transcribed RNAs were dephosphorylated using calf intestinal alkaline phosphatase and then 5' labeled using [ $\gamma$ - $^{32}$ P]ATP and polynucleotide kinase as previously described (39). Qs and X RNAs were independently incubated at 60°C for 10 minutes in a dry heat block, which was then turned off, and the RNAs were allowed to cool to 37°C. The labeled (0.5 pmol) and unlabeled (2.5 pmol) RNA were mixed in 10  $\mu$ l TMN (TMN contains 20 mM Tris-acetate [pH 7.5], 10 mM magnesium acetate, 100 mM sodium chloride, and 1 mM dithiothreitol) and incubated at 37°C for 5 minutes. This was mixed with 12 or 30 ng of RNase III in TMN and incubated at 37° for 1 min. The reactions were stopped with phenol-chloroform. RNA was ethanol precipitated from the aqueous phase, resuspended in 20  $\mu$ l of gel loading dye II (Ambion), electrophoresed on a 6% denaturing urea-polyacrylamide gel, and examined using a phosphorimager.

**Primer extension.** Oligonucleotide pCF10\_8137 was 5' labeled using [ $\gamma$ - $^{32}$ P]ATP and polynucleotide kinase (New England Biolabs) following the manufacturer's instructions. Primer extension reactions were performed using avian myeloblastosis virus (AMV) reverse transcriptase as described previously (40).

**SDS-PAGE and Western blotting.** SDS-PAGE and Western blotting of PrgX were performed using a polyclonal antibody as previously described (16).

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## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00189-11/-/DCSupplemental>.

- Figure S1, PDF file, 0.1 MB.
- Figure S2, PDF file, 0.6 MB.
- Figure S3, PDF file, 0.1 MB.
- Figure S4, PDF file, 0.3 MB.
- Figure S5, PDF file, 0.5 MB.
- Figure S6, PDF file, 0.1 MB.
- Table S1, DOC file, 0.1 MB.
- Table S2, DOC file, 0.1 MB.
- Table S3, DOCX file, 0.1 MB.

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