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Structural analysis of the Anti-Q-Qs interaction: RNA-mediated regulation of *E. faecalis* plasmid pCF10 conjugation

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Abstract

Conjugation of the *E. faecalis* plasmid pCF10 is triggered in response to peptide sex pheromone cCF10 produced by potential recipients. Regulation of this response is complex and multi-layered and includes a small regulatory RNA, Anti-Q that participates in a termination/antitermination decision controlling transcription of the conjugation structural genes. In this study, the secondary structure of the Anti-Q transcript and its sites of interaction with its target, Qs, were determined. The primary site of interaction occurred at a centrally-located loop whose sequence showed high variability in analogous molecules on other pheromone responsive plasmids. This loop, designated the specificity loop, was demonstrated to be important but not sufficient for distinguishing between Qs molecules from pCF10 and another pheromone-responsive plasmid pAD1. A loop 5' from the specificity loop which carries a U-turn motif played no demonstrable role in Anti-Q-Qs interaction or regulation of the termination/antitermination decision. These results provide direct evidence for a critical role of Anti-Q/Qs interactions in posttranscriptional regulation of pCF10 transfer functions.

Keywords

Antisense RNA; Conjugation; Plasmid; Transcription attenuation; RNA structure

1. Introduction

The pCF10 pheromone-inducible conjugative system (Bae et al., 2000, 2004; Bae and Dunny, 2001) is highly regulated with a number of checkpoints. Pheromone import by responsive donor cells triggers the cytoplasmic molecular switch, PrgX, increasing the initiation and processivity of transcription of the *prgQ* operon (Refer to Fig. 1. for genetic map of the pCF10 conjugation regulatory region). In general, the transcripts from the 5' and 3' end of the *prgQ* operon are involved in regulatory and conjugative functions, respectively (Nakayama et al.,

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1994). The major 5' transcript, Qs, is constitutively expressed and encodes a functional peptide, the iCF10 inhibitor (Nakayama et al., 1994). The function of iCF10 is to suppress the expression of the conjugative machinery at low levels of the pheromone, cCF10, as a competitive inhibitor (Hedberg et al., 1996). Upon induction with cCF10, Qs levels increase and antitermination of Qs transcripts leads to formation of a longer mRNA species, Q_L (Chung and Dunny, 1995). The downstream genes of the *prgQ* operon encode the conjugation proteins including aggregation substance (PrgB), surface exclusion protein (PrgA), etc. The small regulatory RNA Anti-Q is proposed to regulate the termination/antitermination decision at the Qs terminator IRS1 by suppressing readthrough into the conjugative structural genes.

Regulatory, or antisense, RNAs were first identified in studies of mobile genetic elements like plasmids, transposons, and bacteriophage, but have been more recently found to be ubiquitous on bacterial chromosomes as well (Brantl, 2002a; Brantl, 2007; Fozo et al., 2008; Gerdes et al., 1997; Gottesman, 2005; Wagner et al., 2002; Weaver, 2007). In general, regulatory RNAs are not translated, although exceptions exist (Benito et al., 2000; Novick, 2003). These molecules regulate gene expression by several different mechanisms including RNA folding interference, translational repression or activation, premature transcriptional termination, and target RNA degradation. Regulation by the majority of antisense RNAs involves complex formation by complementary nucleotide pairing with their target RNAs. These RNAs have evolved several structural characteristics, which allow an accelerated pairing with the target RNA within a short time-frame. The rate-limiting step in antisense-target RNA complex formation is usually an initial base-pairing interaction between two loops, or a loop and a single-stranded RNA (Franch and Gerdes, 2000). The presence of a common structural motif, the U turn motif, was revealed upon comparative sequence analysis of antisense RNAs. Consisting of a conserved 5'-YUNR-3' sequence, the U turn motif was first identified as a structural element found on the anticodon loops of tRNAs (Ashraf et al., 1999; Quigley and Rich, 1976), where they facilitate rapid codon-anticodon interaction. The U-turn motif is considered to be a general binding rate accelerator that promotes initial RNA-RNA pairing in some, but not all, RNA-regulated systems.

According to the current model of Anti-Q-mediated transcription attenuation (Bae et al., 2004), when Anti-Q is in excess it interacts with its complementary region in the nascent *prgQ* transcript and promotes the formation of a terminator structure producing Qs RNA and preventing expression of the downstream conjugative machinery. When Qs is in excess, unpaired *prgQ* RNA favors an anti-terminator structure allowing transcription of the downstream conjugation structural genes. Anti-Q is a *cis*-acting antisense RNA (Brantl, 2007), being transcribed from the opposite strand of its target. However, Anti-Q is unusual in that it is part of a longer transcript that includes the *prgX* gene encoding the transcriptional repressor of *prgQ*. Anti-Q RNA is processed from the full length Anti-Q-*prgX* mRNA and is positively regulated by PrgX by an unknown mechanism.

In this manuscript molecular details of the interaction between Anti-Q and Qs are reported. Structural analysis of Anti-Q revealed that it formed a cloverleaf like structure with three stem-loops. Loop I, the 5' most loop, contains a 5'-YUNR-3' sequence that could provide a U-turn motif for Anti-Q-Qs interaction, but both *in vitro* and *in vivo* results indicated that it is not essential for Anti-Q function. In contrast, structural analysis of Anti-Q-Qs complexes suggested that critical contacts existed at Anti-Q loops II and III. Phylogenetic analysis suggested that, while the majority of the Anti-Q sequence was conserved among related pheromone-responsive conjugative plasmids, considerable variation was observed in loop II, suggesting that it was important for interaction specificity. While mutational analysis confirmed that loop II was important for determining specificity, it was not sufficient. The implications of these results for the overall mechanism of conjugation control in pheromone-responsive plasmids are discussed.

2. Materials and Methods

2.1 Bacterial strains, media and culture conditions

E. coli strain, DH5 α (Invitrogen) was used for constructing the templates for generating *in vitro* transcripts. It was routinely cultured in Luria Bertani (LB) (Sambrook et al., 1989) broth at 37°C. *E. faecalis* strain OG1X (pAD1) (Clewell et al., 1982) was used as a source of pAD1 DNA. *E. faecalis* strain OG1Sp (Fixen et al., 2007) was used for β -galactosidase assays. *E. faecalis* strains were cultured in Todd Hewitt broth (DIFCO) and M9-YE media (Dunny et al., 1985). Erythromycin (Sigma) was used at concentration of 300 μ g/ml.

2.2 Production of *in vitro* transcripts

Plasmid pXPCAT (Bae et al., 2000) was used as a PCR template for generating the pCF10 Anti-Q and Qs *in vitro* transcription templates used in this study. Genomic DNA from OG1X (pAD1) was used as a PCR template for generating mD and Qs-pAD1 *in vitro* transcription templates. The likely starting points for these RNAs (for mD, -121 base pairs from the start codon of ORF-TTS1; for Qs-pAD1, -36bp from the start codon of *iad*) were based on sequence provided by Pontius and Clewell, 1992 and alignment with the pCF10 homologs. Genomic DNA was extracted using the Masterpure™ gram positive DNA purification kit from Epicentre Biotechnologies as per manufacturer's instructions. Primers used for all constructs are listed in Table 1. PCR was performed using PCR supermix Hi fidelity (Invitrogen) according to manufacturer's protocol.

The wild type Anti-Q and Qs templates for *in vitro* transcription were generated using end primers to fuse the T₇ promoter sequence at the 5' end. For generating mutants, either an end primer was used to introduce the mutation (for Anti-Q-U mutant) or the mutant was generated using a three step site-specific mutagenic PCR approach (for Qso/t-U and Anti-Q-pAD1) as previously described (Shokeen et al., 2008). The primers (Table 1) were used at a final concentration of 200nM. All PCR products were sequenced at the University of South Dakota DNA sequencing core facility to confirm the expected DNA sequence.

In vitro transcripts were synthesized using T₇ polymerase (Ambion Megashortscript kit) as per manufacturer's instructions with the following modifications. Approximately 300nM amounts of purified PCR product (using Promega Wizard SV Gel and PCR Clean-Up Kit) was used as a template for transcription reactions. The transcription reactions were incubated in a cabinet incubator for 4 hours at 37°C. The remaining DNA template was digested using DNaseI as per manufacturer's protocol. The transcripts were resolved on a 5% polyacrylamide denaturing (7M Urea) gel. The RNA bands were visualized and excised by placing a Fluor-coated TLC plate (Ambion) under the gel and exposing the gel to a handheld UV lamp (shortwave UV light 254nm). The excised bands were soaked in crush soak solution (10mM Tris.HCl (pH 7.5 at 23°C), 200 mM NaCl, 1 mM EDTA (Winkler, et al., 2002). The RNA was recovered from the crush-soak solution by precipitation with ethanol.

The transcripts were end labeled as per the protocol described by Greenfield et al., 2001. The position of transcripts was visualized by exposing the gel to X-ray film and then the band excised from the appropriate position on the gel and extracted as above.

2.3 Generation of *in vivo* constructs

To make pBK2, the *Pst*I-*Eco*RI fragment was digested from p043lacZ (Kozlowicz et al., 2004) and was inserted into a similarly digested pCI3340 backbone (Hayes et al., 1990). An *Xho*I site was also engineered at *prgQ*-173 (Kozlowicz, B. K., 2005 Ph.D. Dissertation). This fragment contains the *prgX-Q* region with a *lacZ* fusion downstream of IRS1.

pBK2–24, with the U-turn mutation, was generated using the Quikchange™ site-directed mutagenesis technique (Stratagene) using Pfu Ultra II polymerase (Stratagene) and a pBK2 template with primer pair YUNR U9→C Forward (5'-GTTCTCGTTTGTGCAACATTGGTTACAACGT-3') and YUNR U9→C Reverse (5'-AATGTTGCAACAAACGAGAACCGAGTAGAGTTC-3'). The *XhoI-XbaI* fragment containing the mutation was gel purified and cloned into a similarly digested pBK2 backbone.

2.4 Gel Shift Assay

End labeled target RNA was incubated with 10 to 20 molar excess of the appropriate unlabelled binding RNA in TMN binding buffer (Greenfield et al., 2000) at 37°C for 30 min for complex formation. The reactions were stopped by diluting with an equal volume of FD loading buffer (Greenfield et al., 2000) on ice. For positive controls, incubating RNAs were “fully duplexed” by gradually increasing the temperature to 65°C and bringing the temperature back to 25°C in a span of 30 min. Reactions were run on 5% denaturing polyacrylamide gels as described by Greenfield et al., 2000. The gels were dried and exposed to a storage phosphor screen (GE Healthcare) overnight and imaged using a Typhoon 9410 scanner (GE Healthcare). A time course study was carried out to calculate the rates of RNA interaction (Greenfield et al., 2000). The band volumes were calculated to determine the density of each band using Imagequant software (GE Healthcare). These band volumes were used to calculate the percentage of unshifted RNA. The binding rate constants for binding of the RNAs were determined as per Persson et al., 1988, using the exponential slope for each sample. This rate of interaction was calculated as a second-order binding rate constant which serves as a measure of the kinetics of the interaction of these RNAs.

2.5 Secondary structure determination using Pb (II) probing

Pb (II) probing analysis of end-labelled *in vitro* transcripts was carried out as previously described by Greenfield et al., 2001. Pb(II) cleaves any single stranded RNA with no sequence specificity. Reactions were run alongside a ladder produced by partial hydrolysis of Anti-Q RNA with Alkaline hydrolysis buffer (Ambion) and a commercially available size standard marker (Decade Marker, Ambion) to determine sites of cleavage. Fragment sizes are 4 nt larger than coordinates of Anti-Q in Fig. 1 because 4 nt were added to the 5' end of the *in vitro* constructs by the T7 promoter fusion primer.

To determine sites of interaction, Pb(II) cleavage was performed on end-labeled Anti-Q or relevant Anti-Q mutants incubated with 10 molar excess unlabelled Qs or Qso/t in TMN buffer for 30 minutes. Because protection of Loop II from Pb(II) cleavage was the most prominent change upon complex formation, an interaction rate was determined based on a time course of Loop II protection. The binding rate constants for binding of the RNAs were carried out as per Persson et al. (1988).

2.6 β -Galactosidase Activity Assay

β -Galactosidase activity was assayed as described by Miller, 1972, with the following modifications: cells were cultured overnight in M9-YE with selective antibiotics. The overnight culture was diluted 1:5 in fresh medium with or without 50ng/ml cCF10 and grown for 90 minutes to mid-log phase. The cells were pelleted and resuspended in assay buffer (Z buffer-0.06M Na₂HPO₄.7H₂O, 0.04M NaH₂PO₄.H₂O, 0.01M KCl, 0.001M MgSO₄, 0.05M β -mercaptoethanol, pH to 7.0.) to eliminate error due to the effects of different carbon sources in the growth medium on the β -galactosidase enzyme activity.

3. Results

3.1. Secondary structure of Anti-Q

In order to evaluate the mechanism by which Anti-Q interacts with nascent *prgQ* transcripts, it was important first to determine the secondary structure of Anti-Q RNA. Binding of antisense and target RNAs is usually initiated at single stranded regions such as loops and bulges. The computer predicted structure of Anti-Q RNA, as shown in Fig. 2A, suggested a compact clover-leaf structure with three major stem-loops and several bulges. Two potentially important structures were identified in Loop I and Loop II. Loop I contains a potential “U–turn motif” that has been demonstrated to accelerate the interaction between complementary RNAs in other RNA regulated systems (Franch et al., 1999). Loop I is highly conserved in Anti-Q homologs identified on other pheromone responsive plasmids (Fig. 2B). Loop III and the four bulges are fairly conserved with mostly single nucleotide variability except in the case of pPD1 which appears to be more divergent. In contrast, Loop II is highly variable with each molecule showing multiple base substitutions. Based on this bioinformatic analysis, it was hypothesized that Loop II provided the specificity for Anti-Q-Qs interaction while Loop I was important for controlling the rate of the interaction.

To confirm the predicted structure of Anti-Q RNA, secondary structural analysis of *in vitro* produced Anti-Q RNA was performed by partial Pb(II) cleavage. The cleavage pattern of Anti-Q is shown in Fig 3 and Fig. S1 and the sites of Pb(II) cleavage are mapped to the predicted Anti-Q structure in Fig. 2A. As predicted, Loop I and Loop II provided prominent cleavage clusters at nucleotides 9–12 and 46–51, respectively, centrally located between regions of relative insensitivity identifying the stems. In each case, the 5' nucleotide of the loop appeared to be hypersensitive to cleavage. In addition, cleavage was observed at nts 80–83, the predicted position of Loop III. The structures carrying these single stranded loops were designated SL (stem loop) 1, 2, and 3 respectively. Further lead cleavages occurred between the SL1 and SL2 at nts 21–26 and nts 34–37 at predicted single strand bulges, B1 and B2. Two more bulges were observed between SL2 and SL3 at nts 60–65 and nts 71–73 and these bulges were named B3 and B4, respectively.

Thus, the Pb(II) probing data supported the predicted secondary structure of Anti-Q. In particular, Pb(II) cleavage data demonstrated that the putative U- turn motif and specificity loop were exposed on single stranded loops that could provide the primary sites of interaction with nascent Qs.

3.2. Analysis of the Anti-Q/Qs binding mechanism

To further investigate Anti-Q-Qs interaction, complex formation was initially evaluated using gel shift assays. RNA-RNA interactions controlling plasmid functions frequently must occur within a “window of opportunity” in which the target is in the proper conformation to allow interaction with the regulator (Brantl and Wagner, 1998). Once transcription of the target has proceeded beyond this point, interaction is no longer efficient. Since the putative function of Anti-Q is to control the balance between terminator and antiterminator structures, it was possible that full length Qs might not interact efficiently with Anti-Q since the terminator was already formed. Therefore, a shorter transcript without the Qs terminator region was constructed and designated Qso/t. Gel shift experiments with both labeled Anti-Q and each unlabeled Qs construct and labeled full length Qs and Qso/t with unlabeled Anti-Q were performed. In head to head comparisons, a greater proportion of complex was consistently seen with Qso/t rather than full length Qs (data not shown). The proportion of Anti-Q RNA in complex after thirty minutes of incubation with Qs was measured as a ratio of shifted Anti-Q RNA to the total amount of Anti-Q RNA. On average, 27 ± 5 % of the labeled Anti-Q was shifted with Qso/t while only 18 ± 3 % of the labeled Anti-Q was shifted in the presence of 20 molar

excess of Qs. Although this difference was not statistically significant due to high variability between individual experiments, Qso/t was used routinely for further experiments because it consistently shifted more Anti-Q. The rate of interaction of Anti-Q and Qso/t as determined by gel shift was $3 \pm 0.8 \times 10^3 \text{M}^{-1}\text{s}^{-1}$. This is very slow compared to RNA interaction in other antisense RNA regulated systems where second order binding rate constants between $1.0 \times 10^6 \text{M}^{-1}\text{s}^{-1}$ and $5.0 \times 10^6 \text{M}^{-1}\text{s}^{-1}$ are observed (Franch et al., 1999). Furthermore, even after prolonged incubation, complete shifting of Anti-Q was not observed (data not shown). This suggested that the gel shift approach, while clearly showing that the Anti-Q and Qs RNAs interact, may not be optimal for measuring interaction rates. These results also suggested the neither Qs nor Qso/t are the optimal interacting partner with Anti-Q. However, six other truncated Qs products, from Qs266 lacking the entire 3' side of the antiterminator structure to Qs367 lacking only half of the 3' side of the terminator structure, showed no significant difference from Qso/t in their interaction with Anti-Q (data not shown). Further work will be required to determine whether other nascent Qs products interact more strongly with Anti-Q.

To more accurately measure the interaction of Anti-Q and Qs, interaction points in the Anti-Q-Qso/t complex were determined by identifying regions of protection from Pb(II) cleavage in the Anti-Q structure in complex. While results shown in Fig. 4 show Qso/t-mediated protection at the specificity loop (II in Fig. 4), no protection was observed at the Loop I U-turn motif (I). Protection was also observed in some bases of Loop III (III) and bulge 4 (B4). A similar pattern of protection was observed when labeled Anti-Q was incubated with full length Qs (data not shown). The rate of interaction of the two RNAs was determined, based on the kinetics of specificity loop protection, to be $3.6 \pm 0.14 \times 10^6 \text{M}^{-1}\text{s}^{-1}$. This rate of binding is similar to that of other antisense RNA bimolecular interactions.

3.3. Effect of mutation of the U turn motif of Anti-Q on Anti-Q-Qso/t complex formation

The lack of interaction at the U-turn motif was somewhat surprising and worthy of further investigation. To better evaluate the role of Loop I in the interaction between Anti-Q and Qs, an Anti-Q mutant with a defective U-turn motif was constructed. In this mutant, the invariant U at nt 9 of the U-turn motif (see Fig. 2A) was mutated to C. A complementary change was also made in the Qso/t so that the effect of the U-turn structure could be examined without affecting complementarity. To assess the possibility that the interaction at the U-turn motif might be transient yet required for interaction at the other loops, interaction was assessed by gel shift and Pb(II) cleavage structural analysis. No difference in interaction was observed in gel shift experiments (data not shown) and, as shown in Fig. 5, protection was still observed at Loop II and III and bulge 4 in the U-turn loop mutant. To assess the possibility that, while not required for interaction, the U-turn motif might be required for optimal interaction rates, the rate of protection of Loop II was determined. The rates of interaction of the Anti-Q-U-turn mutant with Qso/t U-turn mutant and wild-type Qso/t were determined to be $4.4 \pm 0.21 \times 10^6 \text{M}^{-1}\text{s}^{-1}$ and $4.4 \pm 0.98 \times 10^6 \text{M}^{-1}\text{s}^{-1}$ respectively, not significantly different from the rate of interaction of the wild-type Anti-Q with Qso/t, suggesting that the U-turn motif was not important for this interaction.

These *in vitro* results suggested that the U-turn motif was not important for interaction of Anti-Q with Qs under these conditions and might, therefore, not be required to ensure proper termination of Qs at the T1 terminator. To evaluate the role of the U-turn motif in termination, the U-to-C mutation was introduced in pBK2 (Kozlowicz, B. K., 2005 Ph.D. Dissertation), a pheromone response reporter plasmid with the *lacZ* gene fused downstream of the IRS1 terminator. Since Anti-Q and Qs are transcribed from opposite strands of the same DNA in this construct, the mutant plasmid, designation pBK2-24, retains complementarity of the interacting RNAs but disrupts the U-turn motif. The U-turn motif mutation resulted in a slight increase in uninduced levels of readthrough into the *lacZ* (170 ± 1.41 MU in the mutant vs.

108 ± 36.76 MU in the wild type) and no effect in pheromone-induced cells (734 ± 72.12 MU in the mutant vs. 677 ± 94.75 MU in the wild type). Therefore, both *in vitro* and *in vivo* results support the conclusion that the U-turn motif present in Loop I is not required for Anti-Q-mediated regulation of termination/antitermination of Qs transcription.

3.4. Effect of mutation of the specificity loop of Anti-Q on the Anti-Q-Qso/t complex formation

The lack of an effect of U-turn motif mutations, either *in vitro* or *in vivo*, on the interaction of Anti-Q with Qs indicated that interaction at Loop II may be critical for complex formation. To investigate the role of Loop II in Anti-Q-Qs interaction and its role in determining the specificity of interaction, a construct was generated wherein the specificity loop of the Anti-Q RNA was mutated to incorporate the sequence of the specificity loop of mD RNA, the pAD1 Anti-Q counterpart (Pontius and Clewell, 1992; Tomita and Clewell, 2000). This mutant was named Anti-Q-pAD1. In addition, constructs for *in vitro* production of mD RNA (Pontius and Clewell, 1992; Tomita and Clewell, 2000) and the Qso/t counterpart of pAD1 were also generated. Gel shift assays were carried out to determine the specificity of binding interactions of Anti-Q, Anti-Q-pAD1, and mD with Qso/t and Qso/t-pAD1. The average percent shifted from three experiments is shown in Table 2. Anti-Q and mD interacted well with their cognate Qs targets but poorly with their non-cognate targets. Complex formation was still observed between Anti-Q-pAD1 RNA and Qso/t, although migration of the complex was altered presumably due to an altered conformation of the complex due to the lack of interaction at Loop II (Fig. S2). Pb(II) probing analysis confirmed that interaction at Loop II was lost in this complex and interaction at Loop I did not appear (data not shown). Anti-Q-pAD1 interacted better with Qso/t-pAD1 than did Anti-Q, but not as well as did mD. This was evident both in the percent shifted and the large amount of partially shifted complex that was not observed with mD (Fig S2). And while Anti-Q-pAD1 interacted with Qso/t, mD interacted only with Qso/t-pAD1. These results, as well as those shown in Fig. 5, suggested that the Loop II sequence is important for determining the specificity of Anti-Q-Qs interaction. Other base differences between Anti-Q and mD, e.g. in B4 and Loop III, are likely to also contribute.

4. Discussion

For antisense-mediated regulation of plasmid functions, efficient inhibition frequently relies on a rapid bimolecular interaction between the regulatory RNA and its nascent target in a specific window of opportunity. Binding of the antisense/sense RNAs may initiate by loop-loop or linear region-loop interactions. In general, the interacting loops have a high GC content and comprise 5–8 nucleotides and the stems longer than 10 base pairs must contain bulges to prevent RNase III digestion. These structures allow an efficient binding of the antisense/sense RNAs (Hjalt and Wagner, 1992; 1995). In *cis*-acting antisense RNAs, a U-turn motif frequently stimulates the interaction between regulator and target (Brantl, 2007). Two examples of antisense-mediated regulation of conjugation have been closely examined. Conjugation is regulated at the level of translation in the *E. coli* F plasmid, where interaction with the FinP regulatory RNA blocks the ribosome binding site for the key positive regulator, TraJ (Brantl, 2002b; Gubbins *et al.*, 2003). In contrast, conjugation in the *E. faecalis* plasmid pCF10 is regulated at the level of transcription termination. Based on analyses of *cis*-acting point mutations and RNA analysis, it was suggested that Anti-Q interacts with the complementary region of the *prgQ* transcript and promotes the formation of a terminator structure, which in turn prevents the expression of downstream conjugative loci (Bae *et al.*, 2004).

In order to test the model for Anti-Q function and to further elucidate the interaction between Anti-Q and the nascent *prgQ*/Qs transcript, the structure of Anti-Q and its relevant points of interaction with Qs were determined. Anti-Q forms a cloverleaf structure whose single stranded regions include only three loops and four bulges. Interaction studies demonstrated that the

primary sites of interaction between Anti-Q and a version of Qs lacking the IRS1 terminator stem-loop occurred at Loop II and less prominently at Loop III and bulge 4 (B4). Binding rate analysis using protection of the Loop II nucleotides as an indicator of interaction revealed a binding rate constant of approximately $4 \times 10^6 \text{M}^{-1} \text{s}^{-1}$, consistent with that observed in other antisense RNA controlled systems. Phylogenetic analysis indicated that the Loop II sequence was highly divergent between Anti-Q homologs in other pheromone-responsive plasmids, suggesting that this structure might provide specificity for suppression of conjugation. This conclusion is supported by the strength of interaction at this site. However, the observation that substitution of the Anti-Q for the mD Loop II sequence allowed interaction with Qs RNAs from both pCF10 and pAD1 rather than resulting in a specificity switch, suggests that additional nucleotide differences, for instance at Loop III and B4, may be involved in determining specificity.

The Loop I sequence contains a U-turn motif which has been shown in other systems to be important for RNA-RNA interactions. The disruption of inter-loop interactions by mutating U- turn motifs has shown a significant reduction in binding rate constants in other systems (Dix *et. al.*, 1986; Ashraf *et. al.*, 1999, Franch *et. al.*, 1999). Surprisingly, analysis of the Anti-Q-Qs interaction showed no interaction at Loop I. Furthermore, mutation of the Anti-Q U-turn motif had no effect on either the pattern of interaction or the binding rate constant regardless of whether it was interacted with Qs with or without a complementary mutation. Additionally, *in vivo* analysis in which the U-turn mutation was introduced into the natural Anti-Q-Qs context showed little effect on readthrough of the IRS1 terminator. Although Loop II mutants still interacted with Qs, Loop I contacts still were not observed in this complex. It is possible that Loop I might show interaction with an earlier conformation of Qs than the one used here, this explanation does not account for the *in vivo* results. It is possible that alterations in the balance of Anti-Q and Qs resulting from separating the locus from the rest of pCF10 may obscure subtle effects of Loop I U-turn motif. However, it should be noted that in this construct the Anti-Q and Qs genes are in their natural *cis* context, utilize their own promoters and include all known regulators necessary to provide their normal production levels. Northern analysis indicates that the normal ratio of AntiQ:Qs is maintained in this construct; the only observable difference between pBK2 and pCF10 is an approximate doubling of both RNAs consistent with the two-fold difference in copy number between the two replicons (data not shown). Therefore, the effects of cloning, if any, should be minimal. Cumulatively, these data indicate that the U-turn motif of Anti-Q Loop I is not involved in the interaction with nascent Qs that regulates the termination/antitermination decision. Since the detailed secondary structure of Qs was not determined in this study, the possibility that U-turn motifs in that molecule might be involved in the Anti-Q-Qs interaction cannot be ruled out. It is also possible that, rather than controlling the Qs termination/antitermination decision, the Loop I U-turn motif plays a role in the interaction of nascent Anti-Q with full length Qs to control readthrough into the *prgX* gene thereby controlling post-conjugation shut-down of the pheromone response. This possibility is currently under investigation.

The regulatory interactions occurring in and around the P_Q and $P_{\text{Anti-Q}}$ promoters are multifaceted, balancing the need for constitutive production of iCF10 with that for effective termination at IRS1. In addition to the Anti-Q-Qs interaction, they involve interaction of PrgX with its regulatory binding sites and proper processing of the $P_{\text{Anti-Q}}$ message to produce appropriate amounts of Anti-Q and PrgX. The details of the interaction between Anti-Q and Qs described in this manuscript provide the groundwork for further studies on this complex regulatory system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Ashraf SS, Ansari G, Guenther R, Sochacka E, Malkiewicz A, Agris F. The uridine in “U-turn”: contributions to tRNA-ribosomal binding. *RNA* 1999;5:503–511. [PubMed: 10199567]
- Bae T, Clerc-Bardin S, Dunny GM. Analysis of Expression of *prgX*, a key negative regulator of the transfer of the *Enterococcus faecalis* pheromone-inducible plasmid pCF10. *J. Mol. Biol* 2000;297:861–875. [PubMed: 10736223]
- Bae T, Dunny GM. Dominant negative mutants of PrgX: Evidence for a role of PrgX dimerization in negative regulation of pheromone-inducible conjugation. *Mol. Microbiol* 2001;39:1307–1320. [PubMed: 11251846]
- Bae T, Kozlowicz BK, Dunny GM. Characterization of *cis*-acting *prgQ* mutants: evidence for two distinct repression mechanism by Anti-Q RNA and PrgX protein in pheromone-inducible enterococcal plasmid pCF10. *Mol. Microbiol* 2004;51:271–281. [PubMed: 14651627]
- Benito Y, Kolb FA, Romby P, Lina G, Etienne J, Vandenesch F. Probing the structure of RNAIII, the *Staphylococcus aureus* *agr* regulatory RNA, and identification of the RNA domain involved in repression of protein A expression. *RNA* 2000;6:668–679. [PubMed: 10836788]
- Brantl S. Antisense RNAs in plasmids: control of replication and maintenance. *Plasmid* 2002a;48:165–173. [PubMed: 12460531]
- Brantl S. Antisense-RNA regulation and RNA interference. *Biochem. Biophys. Acta* 2002b;1575:15–25. [PubMed: 12020814]
- Brantl S. Regulatory mechanisms employed by *cis*-encoded antisense RNAs. *Curr. Opin. Microbiol* 2007;10:102–109. [PubMed: 17387036]
- Brantl S, Wagner EGH. Kissing and RNA stability in antisense control of plasmid replication. *Trends Biochem* 1998;23:451–454.
- Chung JW, Dunny GM. Transcriptional analysis of a region of the *Enterococcus faecalis* plasmid pCF10 involved in positive regulation of conjugative transfer functions. *J. Bacteriol* 1995;177:2118–2124. [PubMed: 7536732]
- Clewell DB, Tomich PK, Gawron-Burke MC, Franke AE, Yagi Y, An FY. Mapping of *Streptococcus faecalis* plasmids pAD1 and pAD2 and studies relating to transposition of Tn917. *J. Bacteriol* 1982;152:1220–1230. [PubMed: 6292164]
- De Boever EH, Clewell DB, Fraser CM. *Enterococcus faecalis* conjugative plasmid pAM373: complete nucleotide sequence and genetic analysis of sex pheromone response. *Mol. Microbiol* 2000;37:1327–1341. [PubMed: 10998166]
- Dunny GM, Zimmerman DL, Tortorello ML. Induction of surface exclusion (entry exclusion) by *Streptococcus faecalis* sex pheromones: use of monoclonal antibodies to identify an inducible surface antigen involved in the exclusion process. *PNAS* 1985;82:8582–8586. [PubMed: 3936037]
- Dix DB, Wittenberg WL, Uhlenbeck OC, Thompson RC. Effect of replacing uridine 33 in yeast tRNA^{Phe} on the reaction with ribosomes. *J. Biol. Chem* 1986;261:10112–10118. [PubMed: 2426258]
- Fixen KR, Chandler JR, Le T, Kozlowicz BK, Manias DA, Dunny GM. Analysis of the Amino Acid Sequence Specificity Determinants of the Enterococcal cCF10 Sex Pheromone in Interactions with the Pheromone-Sensing Machinery. *J. Bacteriol* 2007;189:1399–1406. [PubMed: 17098891]
- Fozo EM, Hemm MR, Storz G. Small toxic proteins and the antisense RNAs that repress them. *Microbiol. Mol. Biol. Rev* 2008;72:579–589. [PubMed: 19052321]
- Franch T, Gerdes K. U- turns and regulatory RNAs. *Curr. Opin. Microbiol* 2000;3:159–164. [PubMed: 10744992]
- Franch T, Petersen M, Wagner EG, Jacobsen JP, Gerdes K. Antisense RNA regulation in prokaryotes: rapid RNA/RNA interaction facilitated by a general U-turn loop structure. *J. Mol. Biol* 1999;294:1115–1125. [PubMed: 10600370]

- Gerdes K, Gulyaev AP, Franch T, Pedersen K, Mikkelsen ND. Antisense RNA- regulated programmed cell death. *Ann. Rev. Genet* 1997;31:1–31. [PubMed: 9442888]
- Gottesman S. Micros for microbes: non- coding regulatory RNAs in bacteria. *Trends Genet* 2005;21:399–404. [PubMed: 15913835]
- Greenfield TJ, Ehli E, Kirshenmann T, Franch T, Gerdes K, Weaver KE. The antisense RNA of the *par* locus of pAD1 regulates the expression of a 33-amino-acid toxic peptide by an unusual mechanism. *Mol. Microbiol* 2000;37:652–660. [PubMed: 10931358]
- Greenfield TJ, Ehli Franch T, Gerdes K, Weaver KE. Antisense RNA of the *par* post-segregational killing system: structural analysis and mechanism of binding of the antisense RNA, RNA II and its target, RNA I. *Mol. Microbiol* 2001;42:527–537. [PubMed: 11703673]
- Gubbins MJ, Arthur DC, Ghetu AF, Glover JNM, Frost LS. Characterizing the structural features of RNA/RNA interactions of the F-plasmid FinOP fertility inhibition system. *J. Biol. Chem* 2003;278:27663–27671. [PubMed: 12748195]
- Hayes F, Daly C, Fitzgerald GF. Identification of the minimal replicon of *Lactococcus lactis* subsp. *Lactis* UC317 plasmid pCI305. *Appl. Environ. Microbiol* 1990;56:202–209. [PubMed: 16348092]
- Hedberg PJ, Leonard BA, Ruhfel RE, Dunny GM. Identification and characterization of the genes of *Enterococcus faecalis* plasmid pCF10 involved in replication and in negative control of pheromone-inducible conjugation. *Plasmid* 1996;35:46–57. [PubMed: 8693026]
- Hjalt TA, Wagner EG. The effect of loop size in antisense and target RNAs on the efficiency of antisense RNA control. *Nucleic Acids Res* 1992;20:6723–6732. [PubMed: 1282705]
- Hjalt TA, Wagner EG. Bulged-out nucleotides in an antisense RNA are required for rapid target RNA binding *in vitro* and inhibition *in vivo*. *Nucleic Acids Res* 1995;23:580–587. [PubMed: 7534907]
- Kozlowski BK, Bae T, Dunny GM. *Enterococcus faecalis* pheromone responsive protein PrgX: genetic separation of positive autoregulatory functions from those involved in negative regulation of conjugative plasmid transfer. *Mol. Microbiol* 2004;54:520–532. [PubMed: 15469521]
- Kozlowski, BK. Minneapolis: University of Minnesota; 2005. Ph.D. Dissertation
- Miller, JH. Experiments in Molecular Genetics. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 1972. p. 352-356.
- Nakayama J, Ruhfel RE, Dunny GM, Isogai A, Suzuki A. The *prgQ* gene of the *Enterococcus faecalis* tetracycline resistance plasmid, pCF10, encodes a peptide inhibitor, iCF10. *J. Bacteriol* 1994;176:2003–2004. [PubMed: 8144469]
- Nakayama J, Yoshida K, Kobayashi H, Isogai A, Clewell DB, Suzuki A. Cloning and characterization of a region of *Enterococcus faecalis* plasmid pPD1 encoding pheromone inhibitor (*ipd*), pheromone sensitivity (*traC*), and pheromone shutdown (*traB*) genes. *J. Bacteriol* 1995;177:5567–5573. [PubMed: 7559344]
- Novick RP. Autoinduction and signal transduction in the regulation of staphylococcal virulence. *Mol. Microbiol* 2003;48:1429–1449. [PubMed: 12791129]
- Paulsen IT, Banerjee L, Myers GS, Nelson KE, Seshadri R, Read TD, Fouts DE, Eisen JA, et al. Role of mobile DNA in the evolution of vancomycin-resistant *Enterococcus faecalis*. *Science* 2003;299:2071–2074. [PubMed: 12663927]
- Persson C, Gerhart E, Wagner EGH, Nordstrom K. Control of replication of plasmid R1: kinetics of *in vitro* interaction between the antisense RNA, CopA, and its target, CopT. *EMBO. J* 1988;7:3279–3288. [PubMed: 2460339]
- Pontius LT, Clewell DB. Conjugative transfer of *Enterococcus faecalis* plasmid pAD1: nucleotide sequence and transcriptional fusion analysis of a region involved in positive regulation. *J. Bacteriol* 1992;174:3152–3160. [PubMed: 1315730]
- Quigley GJ, Rich A. Structural domains of transfer RNA molecules. *Science* 1976;19:796–806. [PubMed: 790568]
- Sambrook, J.; Fritsch, EF.; Maniatis, T. Molecular cloning: a laboratory manual. 2nd edition. Vol. Volume 3. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 1989. Appendix B
- Shokeen S, Patel S, Greenfield TJ, Brinkman C, Weaver KE. Translational regulation by an intramolecular stem-loop is required for intermolecular RNA regulation of the *par* addiction module. *J. Bacteriol* 2008;190:6076–6083. [PubMed: 18641135]

- Tomita H, Clewell DB. A pAD1- encoded small RNA molecule, mD, negatively regulates *Enterococcus faecalis* pheromone response by enhancing transcription termination. J. Bacteriol 2000;182:162–1073.
- Wagner EGH, Altuvia S, Romby P. Antisense RNAs in bacteria and genetic elements. Adv. Genet 2002;46:361–398. [PubMed: 11931231]
- Weaver KE. Emerging plasmid-encoded antisense RNA regulated systems. Curr. Opin. Microbiol 2007;10:110–116. [PubMed: 17376732]
- Winkler WC, Cohen-Chalamish S, Breaker RR. An mRNA structure that controls gene expression by binding FMN. PNAS 2002;99:15908–15913. [PubMed: 12456892]

Fig. 1. Genetic map of the pCF10 conjugation regulatory region

The triangular arrows above and below the line represent the *prgQ* (P_Q) and Anti-Q ($P_{\text{Anti-Q}}$) promoters, respectively. P_Q directs the transcription of the Q_S transcript which is terminated at the IRS1 terminator, the first lollipop structure to the right of *prgQ*, in the absence of cCF10. cCF10 induction leads to readthrough of terminators IRS1 and IRS2, production of Q_L and eventually the conjugation structural genes. Transcription from $P_{\text{Anti-Q}}$ generates both the regulatory Anti-Q RNA and the *prgX* mRNA which encodes the negative transcriptional regulator of the system. Anti-Q is processed from this larger transcript by an unknown mechanism. The hatched boxes labeled 1° b.s. and 2° b.s. indicate the primary and secondary binding sites for PrgX which control transcription from P_Q .

Fig. 2 A

Fig. 2 B

Fig. 2. Secondary structure of Anti-Q RNA

A. The RNA secondary structure of Anti-Q was predicted using Sfold analysis (<http://sfold.wadsworth.org/>). Various stem loops (SL1-3), isolated stems (S1 and S2); and bulges (B1-4) were predicted and are as marked. Loop I harbors the U-turn motif, 5'-CUAA-3' corresponding to the 5'-YUNR-3' consensus. Loop II was designated as the specificity loop. Pb(II) cleavage sites are indicated with arrows. The '*' symbol denotes Pb (II) hypersensitive sites. The circled U residue in Loop I was mutated to a C in the YUNR mutant. In the Anti-Q-pAD1 mutant the entire 5'-UCAUAC-3' sequence of Loop II was replaced with the 5'-GGUACGA-3' sequence of the corresponding Loop II of the pAD1 Anti-Q homolog, mD (Pontius and Clewell, 1992; Tomita and Clewell, 2000). **B.** Sequence comparison of Anti-Q with pAD1 mD and other putative related regulatory RNAs. The sequence of mD RNA was as previously published (Pontius and Clewell, 1992; Tomita and Clewell, 2000). Other sequences were derived by examination of analogous regions of other sequenced pheromone responsive plasmids pTEF2 (Paulsen et al., 2003), pAM373 (De Boever et al., 2000), and pPD1 (Nakayama et al., 1995). Alignment was performed using CLC Combined Workbench under default settings. Stem loops and bulges are represented by labeled boxes.

Fig. 3. Secondary structure analysis of Anti-Q using Pb(II) probing

Anti-Q RNA transcript was produced *in vitro* and end labeled. ^{32}P -labeled RNA was subjected to partial cleavage with Pb(II) and fractionated on 10–12% denaturing polyacrylamide gels. Autoradiograph is shown with the concentration of Pb(II) in mM amounts indicated above each lane. A hydroxyl ladder and a size standard marker, Decade Marker from Ambion (10, 20, 30, 40, 50, 60, 70, 80, 90, 100 nucleotides) were also resolved on the same gel. The position of bands of the size standard are marked by the arrows on the left, but the actual ladder is not shown in this image. As mentioned in Materials and Methods, *in vitro* transcription adds 4 nt to the 5' end of the Anti-Q transcript relative to that shown in Fig. 1 so the positions of cleavage sites are modified accordingly. The positions of different structures identified corresponding

to the proposed structure of Anti-Q in Fig. 1 are as indicated. In both SL1 and SL2, the first nucleotide in the loop appears to be more accessible than the others; these bands are indicated by arrows on the right side of the gel. Longer runs showing better resolution of the 3' end structures are shown in Figure S1.

Fig. 4. Structural analysis of complex formed by Anti-Q and Qso/t interactions

Anti-Q RNA transcript was produced *in vitro* and end labeled. ^{32}P -labeled Anti-Q RNA was allowed to interact with 10 molar excess of unlabeled Qso/t transcript at 37°C for 30 minutes and the complex was subjected to partial cleavage with Pb(II). Digestion reactions were run on 10–12% denaturing polyacrylamide gels. End labeled Anti-Q RNA was also subjected to partial Pb(II) cleavage and resolved on the same gel. The autoradiograph of the cleavage pattern at different run lengths is shown in panels, I and II. A hydroxyl ladder (lane OH^-) and a size standard marker (lane M) (10, 20, 30, 40, 50, 60, 70, 80, 90, 100 nucleotides) were also resolved on the same gel. Marker bands are indicated by arrows with their sizes. Due to tightness of space, arrows for the fastest three migrating marker bands are on the right in panel II, and are

10, 20 and 30 nucleotides respectively. Lane 1, Anti-Q- 0mM Pb(II); Lane 2, Anti-Q- 40mM Pb(II); Lane 3, Anti-Q- 120mM Pb(II); Lane 4, Anti-Q-Qso/t- 0mM, Lane 5, Anti-Q-Qso/t- 40mM; and Lane 6, Anti-Q-Qso/t- 120mM. The positions of Loop I (I), Loop II (II), Bulge 4 (B4), and Loop III (III) are boxed and labeled. Note that while all bands of Loop II appear to be protected in complex, the protection at Loop III and bulge 4 is more subtle with prominent bands protected (arrows) above and below fainter bands, respectively.

Fig. 5. Secondary structural analysis of Anti-Q-Qso/t-U turn mutant interactions

Anti-Q-Umut RNA transcript was produced *in vitro* and end labeled. ^{32}P - labeled Anti-Q-Umut RNA was allowed to interact with 10 molar excess of unlabeled Qso/t-U transcript at 37°C for 30 minutes and the complex was subjected to partial digestion with Pb(II). Digestion reactions were run on 10–12% denaturing polyacrylamide gels. End labeled Anti-Q-Umut RNA was also subjected to partial Pb(II) cleavage and resolved on the same gel. The autoradiograph of the cleavage pattern at different run lengths is shown in panels, I, II and III. A hydroxyl ladder and a size standard marker (10, 20, 30, 40, 50, 60, 70, 80, 90, 100 nucleotides) were also resolved on the same gel. Lane 1, Anti-Q-U- 0mM Pb(II); Lane 2, Anti-Q-U- 40mM Pb(II); Lane 3, Anti-Q-U- 120mM Pb(II); Lane 4, Anti-Q-U-Qso/t-U- 0mM, Lane 5, Anti-Q-U-Qso/t-U- 40mM; and Lane 6, Anti-Q-U-Qso/t-U- 120mM. The positions of Loop I, Loop II, Bulge 4, and Loop III are as indicated. While protection of Loop II is clearly apparent in panels I and II, B4 and Loop III bands are obscured by heavy exposure. A longer run more clearly showing

the prominent B4 and Loop III cleavages in uncomplexed Anti-Q and their absence in complex is shown in panel III.

Table 1Primers for *in vitro* Transcription

Construct		Primers (5'→3')
Anti-Q	5' Anti-Q-T ₇	5'-TGTAATACGACTCACTATAGGGGTTGTAA CTAATGTTGCAACAAACGA -3'
	3' Anti-Q	5'-GAACCGACTGCCATAGGACGGCA -3'
Qso/t	5' Qs-T ₇	5'- TGTAATACGACTCACTATAGGGATAGGAGGG GTGTAAATGAA-3'
	3' Qso/t	5'-GAACTGTATACATTCCCCGC -3'
Qs	5' Qs-T ₇	5'- TGTAATACGACTCACTATAGGGATAGGAGGGG TGTAATGAA-3'
	3' Qs	5'- AAAAGCGGGGAACATATACATGAA-3'
Anti-Q-U	5' Anti-QU- T ₇	5'-TGTAATACGACTCACTATAGGGGTTGTAA CCAATGTTGCAACAAACGA -3'
	3' Anti-Q	5'-GAACCGACTGCCATAGGACGGCA -3'
Qso/t-U	5' Qs-T ₇	5'- TGTAATACGACTCACTATAGGGATAGGAGGGG TGTAATGAA-3'
	3' Qso/t-U MP	5'-TGTTGCAACATTGGTTACAACGTATA -3'
	5' Qso/t-U OP	5'-TACGTTGTAACCAATGTTGCA-3'
	3' Qso/t	5'-GAACTGTATACATTCCCCGC -3'
Anti-Q-pAD1	5' Anti-Q-T ₇	5'-TGTAATACGACTCACTATAGGGGTT GTAACAAATGTTGCAACAAACGA -3'
	3' Anti-Q-pAD1 MP	5'-CAATTCATCTGTACCCATGAACTCT -3'
	5' Anti-Q-pAD1 OP	5'-CAATTCATCTGTACCCATGAACTCT -3'
	3' Anti-Q	5'-CATGAATTGTTAACTGC -3'
Qs-pAD1	5' Qs-pAD1-T ₇	5'- TGTAATACGACTCACTATAGGGGTGAAGTTAA GTTTAAATAAG-3'
	3' Qso/t	5'-GAACTGTATACATTCCCCGC -3'
mD	5' Anti-Q-T ₇	5'-TGTAATACGACTCACTATAGGGGTTGTAAGTTAA TGTGCAACAAACGA -3'
	3' Anti-Q	5'-GAACCGACTGCCATAGGACGGCA -3'

^aMP: Mutagenic Primer, OP: Overlapping primer, and EP: End Primer

Table 2

Specificity of binding interactions: Percent shift of labeled Anti-Q/ Anti-Q-pAD1/ mD.

	Anti-Q	Anti-Q-pAD1	mD
Qso/t	42.7 ± 1.27	50.9 ± 13.45	4.46 ± 4.45
Qso/tpAD1	4.61 ± 0.26	58.91 ± 2.92	75.77 ± 3.26

Note: Either the shifted or unshifted counts were used in determining the percent shifted. Partially shifted bands were not included.