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Regulatory circuits controlling enterococcal conjugation: lessons for functional genomics

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Abstract

The regulatory system controlling pheromone-induced plasmid transfer in *Enterococcus faecalis* is the most thoroughly studied genetic system of this species. Transcription initiation from the target promoter is controlled by a pheromone receptor/repressor protein whose activity is determined by its interaction with two peptide signaling molecules that compete for the same binding site, but have opposing effects on the activity of the receptor protein. For the system to function as a sensitive and robust biological switch, several additional levels of post-transcriptional regulation are also required. Expression of important functions encoded within the enterococcal core genome may also be controlled by multilayered regulatory circuitry. The pheromone system may serve as a useful paradigm to guide comprehensive functional genomic analysis of *E. faecalis*.

Introduction

Enterococci are significant members of the normal intestinal flora of humans and numerous animal species, including invertebrates such as insects. They have great medical importance because of their high levels of inherent and acquired antibiotic resistance and their ability to cause opportunistic infections [1]. One of the most important features of these organisms is their inherent ability to survive and propagate under a variety of harsh conditions. Systems for sensing and responding to extra cellular signals undoubtedly contribute to their remarkable survival abilities. Here we summarize the current understanding of the sex pheromone-inducible conjugation system of *Enterococcus faecalis*, a form of intercellular communication where peptide signals inform plasmid-containing cells of the close proximity of potential recipient cells [2]. Recent studies have elucidated the essential nature of multiple levels of regulation for proper function and versatility of the pheromone systems. This review will focus on the pheromone-responsive conjugative plasmid pCF10 [3], and how the experimental tools and knowledge base generated from this research may inform the emerging field of enterococcal functional genomics.

The extracellular phase of pheromone signaling

The sex pheromones are hydrophobic peptides, processed from the signal peptides of secreted lipoproteins [4]. Examination of genome sequences reveals that >50 lipoproteins may be encoded by the *E. faecalis* chromosome, suggesting that the known pheromones represent a small percentage of the potential signals that are produced. While only a handful of plasmids have been analyzed in detail, it seems likely that most of them encode pheromone-sensing systems that evolved from a common ancestor. For a review of common and unique features of various pheromone plasmids, the reader is referred to [5,6].

The mature pheromone cCF10 is a heptapeptide (LVTLVFV) produced by proteolytic cleavage of the signal peptide segment of the putative secreted lipoprotein CcfA [7]. As is the case for several other pheromones [8], the Eep protease is required for the production of wild-type levels of cCF10 [9*]. Eep-mediated endoproteolytic cleavage occurs within the membrane. The pCF10 pheromone-sensing system (Figure 1b) encompasses two signaling molecules, cCF10 and the plasmid-encoded iCF10 inhibitor peptide (AITLIFI) that competitively inhibits the activity of cCF10 [9*]. The amounts of each peptide produced by pCF10-containing cells are tightly controlled (Figure 1b), such that donor cells secrete a mixture of the two peptides in a molar ratio of ~80:1 in favor of iCF10 [10]. This ratio keeps the conjugation system off, but allows for a sensitive response to increases in relative cCF10 activity. The two peptide system is versatile; a change in the ratio of the two signals can occur either by the addition of exogenous cCF10 (when recipients in close proximity contribute to the cCF10 pool) or by depletion of iCF10 (which occurs when donor cells grow in the bloodstream). The latter phenomenon is critical for the production of the protein product (Asc10) of the pheromone-inducible *prgB* gene, which increases enterococcal virulence in several models [11**]. We note that expression of the enterococcal cytolysin toxin is also controlled extracellularly by the balance between antagonistic signaling peptides, which can be altered by interaction with host cells [12*].

Pheromone import, PrgX and action at the *prgQ* promoter

In contrast to peptide signaling molecules whose interaction with the surface of the responder cell transduces a signal across the membrane [5], cCF10 binds to PrgZ on the surface of pCF10-carrying cells and is imported into the cytosol [13], where it interacts with PrgX, derepressing the *prgQ* promoter. Comparison of high-resolution structures of apo-PrgX with those of PrgX::cCF10 and PrgX::iCF10 complexes yielded the model shown in Figure 2 [14*,15**]. Structural, genetic and biochemical studies indicate that in uninduced cells, PrgX/iCF10 complexes form a repressing PrgX tetramer structure that exhibits enhanced occupancy of two operator sites on pCF10 DNA. The lower affinity site overlaps the *prgQ* promoter, and reduces transcription when occupied. Binding cCF10 to PrgX displaces iCF10, altering the structure of the PrgX C-terminus. This is predicted to reduce interaction at the tetramer interface, disrupting the DNA loop and ultimately reducing occupancy of the lower affinity DNA binding site that is critical for repression. Quantitative analysis of *prgQ* transcription under various conditions [16,17] (A. Chatterjee *et al.*, submitted) indicates that repression *in vivo* is never complete. Moreover, the direct effect of pheromone on enhancement of *prgQ* transcription initiation is much lower than the level of induction of expression of downstream loci in the *prgQ* operon, suggesting that post-transcriptional regulation amplifies the pheromone response as described below.

Multiple levels of post-transcriptional regulation resulting from genetic organization

There is extensive evidence for additional levels of control of RNAs transcribed from both the *prgQ* promoter, P_Q, and the *prgX* promoter, P_X [18–21]. A picture of complex, reciprocal regulation between these two operons is beginning to develop. The transcriptional start site of *prgX* mRNA is located downstream of P_Q but oriented in the opposite direction [22**]. This makes transcription from these two promoters convergent for approximately 220 bp, generating transcripts that are complementary in this region (Figure 3). Multiple RNA species are produced from each promoter. P_X generates both the *prgX* mRNA and Anti-Q, a small non-coding RNA corresponding to the 5' 102 nt of the *prgX* transcript. In the absence of pheromone induction, transcription from P_Q generates Qs, a 380 nt RNA that terminates at IRS1, a factor-independent terminator. Qs includes the 66 nt Orf *prgQ*, which produces the iCF10 inhibitor. When induced, P_Q produces increased levels of Qs as well as transcripts

that extend past IRS1, into genes encoding the conjugative transfer machinery (Figure 1a) [22**].

Reciprocal regulation of antagonistic operons by multiple mechanisms

In addition to the terminator, IRS1, Qs contains sequences capable of forming an antiterminator that precludes terminator formation in the nascent transcript. Anti-Q is complementary to a portion of the antiterminator and interacts with nascent *prgQ* transcripts to sequester these sequences, preventing formation of the antiterminator. This allows folding of the terminator helix, which halts transcription at IRS1, attenuating expression of downstream genes [22**]. This mechanism is similar to those that regulate plasmid copy number in other plasmids [23**,24]. As is the case for other systems, Anti-Q and Qs do not form a complete duplex upon interaction. Rather, defined structures within the two RNAs appear to be essential for productive interaction [25**]. Anti-Q can attenuate transcription of sequences downstream of IRS1 *in vitro*, in the absence of RNA chaperones, suggesting that this activity does not require protein factors beyond RNAP [22**].

Transcripts originating at P_x are also post-transcriptionally regulated. Pheromone induction leads to a loss of *prgX* and Anti-Q RNAs [20]. Additionally, experiments using reporter plasmids have demonstrated that transcription from P_x is not directly regulated by PrgX. However, when P_Q is present in *cis*, the PrgX protein is required for transcription of *prgX* [18–20]. Anti-Q levels are modulated in a similar fashion. In the absence of PrgX, a plasmid that contains both P_Q and P_x produces very little Anti-Q (C Johnson, G Dunny, manuscript in preparation). Inactivating P_Q leads to a large increase in Anti-Q expression (C Johnson, G, Dunny, manuscript in preparation). Providing Qs in *trans* from a compatible plasmid results in intermediate Anti-Q expression. Taken together, these data suggest a model in which transcripts from P_x are regulated via multiple mechanisms. Transcription from P_Q in *cis* actively interferes with transcription from P_x , and repression of transcription from P_Q alleviates this interference. Additionally transcripts from P_Q can act in *trans* to downregulate expression from P_x . Recent efforts to apply mathematical modeling and rigorous quantitative analysis to this system suggest that multiple layers of reciprocal regulation between the antagonistic *prgQ* and *prgX* operons are required for the system to function as a sensitive and robust biological switch (A Chatterjee *et al.*, submitted). The convergent and overlapping organization of these operons serves to amplify the relatively subtle direct effect of the pheromone signal. It is also worth noting that the gene organization of the enterococcal cytolyisin locus also features convergent transcription from opposing promoters [26], suggesting that transcription interference provides an additional layer of regulation in this system as well.

Functional genomics

In bacteria, the analyses of regulatory mechanisms controlling gene expression in mobile elements like phages and plasmids have long provided an essential foundation for chromosomal genetics. Experimental tools and mechanistic insights generated from analysis of the pCF10 pheromone response might also enhance comprehensive analysis of genetic regulatory systems in enterococci (Table 1). The increasing medical significance of enterococci, and the advent of new technologies for genome sequencing and expression analysis, has contributed to a remarkable upsurge in functional genomic studies. Here we consider the extent to which the novel regulatory mechanisms controlling the pCF10 pheromone response might function on a genomic scale. We searched the literature and found that about 50 different enterococcal promoters and/or their cognate transcription factors have been analyzed by various combinations of genetic, biochemical, and in a few cases, structural approaches. Studies of pCF10 and other pheromone plasmids demonstrate

that transcription factors very frequently have multiple DNA binding targets (and perhaps RNA targets) and multiple regulatory functions that may not be apparent from focused studies of a single promoter. While PrgX shows very low levels of amino acid sequence relatedness to other transcription factors, recent structural and genetic studies have indicated that PrgX is a member of an emerging superfamily of transcription factors with common structural features [C. Johnson and G. Dunny] that regulate virulence in bacilli [27**] and gram positive cocci [28] in response to extracellular peptide signals. Global effects on host cell physiology mediated by transcription factors targeting multiple operons have already been documented in several cases, such as the CroRS system [29–31] and the Ers system [32–34], and emerging technologies such as RNAseq [35] should enable the definition of the molecular basis for the control of enterococcal physiology on a genomic scale and with high resolution in the future.

Analysis of the pheromone response has shown that extensive secondary structure and processing of transcripts may produce abundant products whose 5' ends do not actually represent sites of transcription initiation. Thus it is important to verify putative initiation sites and their corresponding promoters by molecular methods that can distinguish 5' ends generated by RNA processing from those resulting from true initiation [36], and by careful genetic analysis of the upstream sequences required for proper expression of a gene of interest. Loci encoding specific biological functions frequently are organized as cassettes with the regulated genes and their adjacent cognate regulators are transcribed in the opposite direction. Differentiating between 'back-to-back' divergent promoters and convergent promoters where transcription of the regulatory gene(s) actually initiates from the nontemplate strand within the target operon is critical.

Studies of the pheromone system, of plasmid replication [23**,24], and of the *par* toxin/antitoxin system [37] in enterococci and close relatives suggested that post-transcriptional mechanisms could be as important in gene regulation as promoter control, if not more so. There is growing appreciation of the importance of post-transcriptional gene regulation mediated by antisense RNA interactions, riboswitches, and other RNA-based mechanisms in enterococci on a genomic scale. Competing terminator/antiterminator structures, or structures in which the ribosome binding site of an mRNA is sequestered or free in 5' untranslated regions have been documented for biosynthetic-resistance, catabolic-resistance, and antibiotic-resistance operons in enterococci and related species [38–40]. The choice between alternative secondary structures is controlled by *trans*-acting sRNAs in some cases and by small molecule metabolites in other cases. The *E. faecalis eut* operon encoding the ability to utilize ethanolamine as a nutrient source provides a remarkable new example of multiple forms of post-transcriptional control. This polycistronic locus encodes a two-component regulatory cassette (HK17/RR17 or EutW/EutV; [41**]) that functions by a phosphorelay [42]. However, it appears that the EutV response regulator binds to mRNA and controls transcription termination rather than promoter activity. It is proposed that this system responds to availability of ethanolamine [41**]. Interestingly the operon also contains adenosylcobalamin-responsive riboswitches; the data suggests that these novel features of the mRNAs produced from this locus provide post-transcriptional mechanisms to sense both the availability of the catabolic substrate for the operon and of the adenosylcobalamin cofactor essential for enzymatic activity.

The importance of as yet unidentified antisense RNA regulators in control of essential enterococcal virulence functions is suggested by a recent report that enterococcal RNase J2 is a positive regulator of expression of adhesive pili required for biofilm formation and virulence [43**]. A plausible interpretation of these results is that this RNase degrades an antisense RNA that negatively regulates pilus expression. Further evidence for a global role of sRNAs comes from our genetic screens for promoters activated during biofilm formation

[44] and in a subdermal abscess model infection (K Frank *et al.*, submitted for publication). These studies suggest the existence of differentially expressed antisense transcripts from the 'nontemplate strands' of protein-encoding genes under specific conditions. The fact that extensive antisense transcription has been observed in the phylo-genetically related *Listeria monocytogenes* [45] also supports the likely existence of many antisense regulators in enterococci and related genera. Given the rapid pace of progress in analysis of enterococcal gene regulation during the past few years, we suggest that this type of research will provide new approaches to the control and prevention of lethal infections caused by antibiotic-resistant strains.

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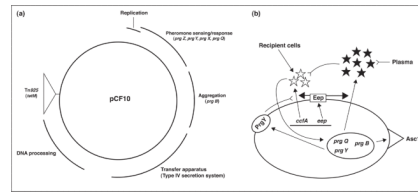


Figure 1.

Genetic organization of pCF10 and the role of extracellular peptide signals in the control of pCF10 conjugation. **(a)** Map of pCF10, based on DNA sequencing [16] and genetic studies [46]. The curved lines outside the circle denote the segments of the plasmid encoding various biological functions as indicated, with specific genes discussed in the text listed below the respective regions in which they are located. **(b)** The induction state of pCF10-containing cells is determined by the molar ratios of cCF10 pheromone (white stars; encoded by the chromosomal *ccfA* gene) to iCF10 inhibitor (black stars; encoded by the *prgQ* gene of pCF10). Both peptides are processed to their mature heptapeptide form from precursor polypeptides in the membrane by the Eep protease (rectangle in cell membrane). The pCF10-encoded PrgY protein (oval in the membrane) reduces the level of cCF10 produced by its host cell; residual cCF10 escaping PrgY is neutralized by iCF10. Both peptides function by internalization and interaction with PrgX, as described in Figure 2 and in the text, with cCF10 causing increased expression of the *prgQ* operon leading to production of Asc10, and other proteins involved in conjugation. The peptide ratio can be shifted by recipient cells in close proximity, or in the bloodstream, where iCF10 is sequestered, resulting in self-induction by exogenously produced cCF10. Single arrows indicate a positive effect of one component of the system on another; double-stemmed arrows indicate the polypeptide product of a gene encoded by pCF10 (oval in the cell) or the chromosome (line in the cell); inverted arrows indicate inhibition or destruction of one component by another.

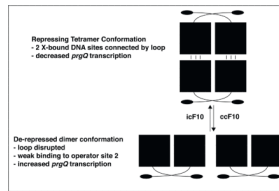


Figure 2.

Pheromone and inhibitor peptides affect PrgX repression of *prgQ* transcription by altering the oligomerization state of PrgX in pCF10-containing cells. PrgX monomers contain an N-terminal DNA binding domain (filled oval) and a C-terminal regulatory domain (filled square) connected by a short linker region (curved line). Domain swapping promotes formation of a stable dimer that can occupy one operator binding site on pCF10; the region upstream from *prgQ* contains two such sites. Interaction of iCF10 with PrgX stabilizes a small-C-terminal loop proposed to function as an interface for protein/protein interactions between pairs of dimers to stabilize a tetramer structure (upper panel) where each dimer occupies one operator site on pCF10, and the two sites are connected by a DNA loop (not shown). Pheromone binding to the same region of PrgX causes a structural change at the extreme C-terminus of PrgX that abolishes the tetramer-stabilizing interaction (lower panel), and indirectly reduces operator site occupancy. This increases transcription initiation at *prgQ* by RNA polymerase.

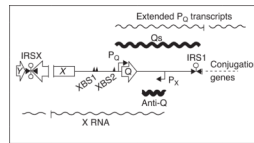


Figure 3.

Genetic organization of *prgX* and *prgQ* regions of pCF10 and reciprocal antisense regulation. Map of the *prgX* and *prgQ* regions is shown with ORFs for *prgY*, *X*, and *Q* indicated by open arrows. The primary and secondary PrgX binding sites are indicated by triangles labeled XBS1 and XBS2, respectively. The promoters P_X and P_Q are indicated, along with transcriptional start sites, by bent arrows. Factor-independent terminators are indicated by lollipops. Wavy lines above and below the map indicate RNAs transcribed from P_Q and P_X, respectively. Transcripts from these two promoters share a 220 bp overlapping complementary region, and published [22^{**},25^{*•}] and unpublished data C. Johnson & G. Dunny, manuscript in preparation demonstrated that each transcript negatively regulates the corresponding complementary transcript by antisense mechanisms.

Table 1

Features of pCF10 regulation applicable to functional genomics			
Mechanism	pCF10 component(s)	Non-pCF10 example(s)	Considerations
Regulation of promoter	PrgX	Many; some positive regulators	Precise mapping of transcription starts, DNA targets. Cofactors and their effects on regulatory proteins
Change in mRNA 28 structure to affect termination translation, or RNA processing	Q/Anti-Q RNAs	T box, <i>S_{MK}</i> box, <i>par</i> RNAs, <i>eut</i> riboswitches	Antisense RNA, other RNA-binding ligands
Convergent transcription	<i>prgQ/prgX</i>	RIVET clones	Confirmation and demonstration of function; <i>cis/trans</i> effects