The peptide pheromone-inducible conjugation system of *Enterococcus faecalis* plasmid pCF10: cell–cell signalling, gene transfer, complexity and evolution

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Expression of a large set of gene products required for conjugative transfer of the antibiotic resistance plasmid pCF10 is controlled by cell–cell communication between plasmid-free recipient cells and plasmid-carrying donor cells using a peptide mating pheromone cCF10. Most of the recent experimental analysis of this system has focused on the molecular events involved in initiation of the pheromone response in the donor cells, and on the mechanisms by which the donor cells control self-induction by endogenously produced pheromone. Recently, studies of the molecular machinery of conjugation encoded by the pheromone-inducible genes have been initiated. In addition, the system may serve as a useful bacterial model for addressing the evolution of biological complexity.

Keywords: quorum sensing; plasmid biology; gene regulation

1. INTRODUCTION

The antibiotic resistance plasmid pCF10 of *Enterococcus faecalis* is a member of a family of mobile genetic elements whose ability to transfer from a donor to a recipient bacterial cell is controlled by intercellular signalling via a peptide mating pheromone. In the case of pCF10, the heptapeptide signal molecule cCF10 (LVTLVFV) serves as a specific inducer of the pCF10 conjugation genes (Mori *et al.* 1988). To date, this type of plasmid has only been described in enterococci (for reviews, see Clewell & Dunny 2002; Chandler & Dunny 2004). In addition to the biological significance of pheromone-inducible conjugation as a useful paradigm for the study of cell–cell signalling, these plasmids contribute substantially to the increasing importance of the enterococci as opportunistic pathogens, because they disseminate antibiotic resistance determinants and because expression of at least one conjugation protein increases the virulence of the organisms. This form of cell–cell signalling is distinct from many of the quorum-sensing systems described elsewhere in this issue, in that the signalling occurs unidirectionally between two cell types (the donor cells use the signal to monitor recipient cell population density) and that the sensing system is encoded by a genetic element (the plasmid) that is distinct from the chromosomal determinants responsible for signal production. These features have interesting physiological and evolutionary consequences. This paper will review the general properties of the pCF10 system, summarize some recent data on the signalling mechanism and on the control of endogenous

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pheromone activity by the donor cells, and highlight some important questions regarding the evolution of the system in relation to the broader issue of the evolution of biological complexity.

2. OVERVIEW OF SYSTEM

As shown in figure 1, pCF10 is slightly less than 70 kb in size and with at least half of its genetic coding capacity devoted to pheromone-inducible conjugation. A long contiguous region of the plasmid encodes regulatory loci devoted to pheromone sensing and negative control of conjugation functions in the absence of exogenous pheromone, formation of mating aggregates and the conjugative DNA processing machinery (as well as the cognate *oriT* target sequence). Recent DNA sequencing and transcriptional analysis of the pCF10 pheromone response (Hirt *et al.* 2005) suggest that the entire segment from *prgQ–pcfH* is coordinately induced by the addition of exogenous pheromone, while expression of the remaining pCF10 genes and the host chromosomal genes is largely unaffected. An interesting aspect of the system that will be further described below is that, in spite of the fact that all of the conjugation genes are coordinately regulated, they appear to have evolved from at least three different sources (Hirt *et al.* 2005). The segment of pCF10 between *prgN* and *prgQ* encodes both pheromone control and sensing, and plasmid replication and maintenance. Interestingly, pheromone production by the host cell is somehow related to stable maintenance of pCF10 and the replication initiator protein PrgW is a pheromone-binding protein (Leonard *et al.* 1996).

The genetic loci from *prgZ–prgQ* are involved in the initiation of the donor cell response to exogenous pheromone signal and prevention of self-induction of
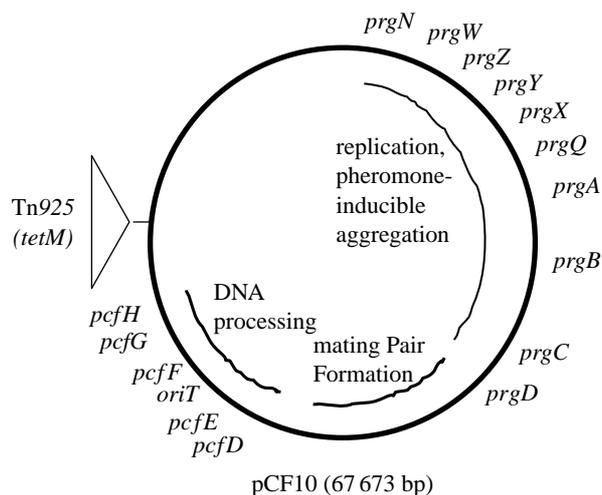
Figure 1. Map of pCF10. The figure shows the approximate positions of a number of genes involved in replication, pheromone sensing and control, and conjugative DNA transfer as described in the text. The curved inner lines depict contiguous segments of pCF10 encoding the biological functions indicated. As described in the text, each of these segments probably originated from a different ancestral source. With the exception of *prgX*, all of the genes from *prgW–pcfH* are transcribed in the clockwise direction. The transposon Tn*925* encodes the tetracycline resistance determinant *tetM*.

the donor cells by endogenously produced cCF10. These genetic functions will be addressed in detail below. The *prgA*, *prgB* and *prgC* genes all encode secreted cell surface proteins, with the product of *prgA* (Sec10 protein) likely mediating surface exclusion (Dunny *et al.* 1985), the Asc10 protein encoded by *prgB* mediating mating aggregate formation (Olmsted *et al.* 1991) and the function of the protein encoded by *prgC* being unknown. Between *prgQ* and *prgA*, there are several genetic determinants that appear to act following initiation of the pheromone response to affect the expression of downstream conjugation genes. These loci encode both positive and negative post-transcriptional regulatory factors, some of which function as small RNAs (Bensing & Dunny 1997; Bensing *et al.* 1997). The function of next block of genes downstream from *prgC* and extending through *pcfD* is largely unexplored, but based on ongoing analysis of amino acid sequence similarity, it might encode a Gram-positive version of a conjugative type IV secretion system involved in formation of the mating channel connecting the donor and the recipient cells. Finally, the segment including *pcfE–pcfH* has been shown to code for the conjugative DNA processing machinery (Staddon *et al.* 2004), with the non-coding region between *pcfE* and *pcfF* containing the functional origin of transfer (Staddon *et al.* 2006). It is noteworthy that the gene order, and the approximate order of expression of the conjugation genes following pheromone induction (Hirt *et al.* 2005), correlates with the order of events in conjugation, i.e. assembly of mating aggregates, followed by formation of functional mating pairs and finally DNA processing and transfer. The system has apparently evolved to produce the minimum amount of conjugation machinery required for effective transfer in response to a pheromone signal,
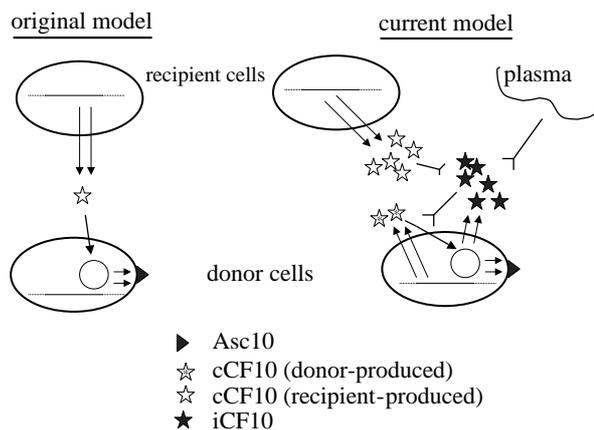

Figure 2. Two models for the role of signalling peptides in expression of pCF10 conjugation functions. The single arrows indicate positive control, the inverted arrows indicate negative control and the double arrows indicate polypeptide synthesis from a plasmid (iCF10, Asc10) or chromosomal (cCF10) gene. Original model: initial studies suggested a simple model whereby a single unidirectional pheromone signal from recipient cells to donors caused induction of the transfer functions, including cell aggregation mediated by Asc10. Current model: further analysis of the system has shown that both donors and recipients can produce pheromone, and that the molar ratio of chromosomally encoded pheromone (cCF10) to plasmid-encoded inhibitor (iCF10) determines the induction state of the donor cell. In a monoculture of donor cells growing in laboratory medium, the balance of these two peptides keeps the transfer system off. The balance can be shifted in favour of pheromone, either by production of pheromone by recipient cells in close proximity (upper left) or by interaction of inhibitor with plasma components (upper right) when the bacteria are growing in the bloodstream of a mammalian host.

while rapidly shutting off the expression of these genes very rapidly following induction (Bae *et al.* 2004).

3. THE INDUCTION STATE OF DONOR CELLS IS DETERMINED BY THE RATIO OF INHIBITOR TO PHEROMONE PEPTIDES

The early studies of pheromone-inducible conjugation suggested a rather simple model, where the interaction of an extracellular signal from a potential conjugative recipient cell with a donor resulted in a mating response (figure 2); it quickly became apparent that plasmid-free recipient cells produced several pheromones, each specific for a cognate plasmid, or family of related plasmids (Dunny *et al.* 1979). This model was supported by conditioned medium experiments where unfractionated culture filtrates of recipient strains induced mating responses by donors (Dunny *et al.* 1978, 1979), and by the subsequent determination of the specific peptide sequences of pheromones specific for several conjugative plasmids (Mori *et al.* 1984, 1986, 1988). Eventually, it was shown that seven to eight amino acid peptides that constitute the mature pheromones are generated by intramembrane processing of cleaved signal peptides from putative secreted lipoproteins (An *et al.* 1999; Clewell *et al.* 2000); cCF10 is produced from the gene product of *ccfA* (Antiporta & Dunny 2002). There are virtually no experimental data available to explain the functions of

any of these lipoproteins, or to indicate whether the pheromones or their signal peptide precursors play any role in the physiology of the producer cell. An intramembrane protease called Eep identified by An and Clewell (An *et al.* 1999) is required for the production of normal levels of several pheromones. Since the mature pheromones are generally more hydrophobic than the signal peptide precursors, Eep proteolysis might be accompanied by active excretion from the cell membrane. In the case of cCF10, a considerable portion of the released pheromone remains associated with the cell wall, and some is also released into the growth medium (Buttaro(Leonard) *et al.* 2000).

It soon became apparent that the 'original' model depicted in figure 2 was not sufficient to explain all aspects of these transfer systems. Since pheromone production is chromosomally encoded, while the response is plasmid determined, it is conceivable that transfer of a plasmid into a new host strain would give rise to constitutive expression of the conjugation genes as a result of self-induction of the newly created donor cell by endogenous pheromone. Since this has never been observed, the plasmid must encode one or more mechanism to avoid such a wasteful self-induction process. In the pCF10 system, as well as other pheromone systems that have been studied, there are two plasmid determinants devoted to preventing self-induction, and genetic disruption of either determinant leads to constitutive expression of the conjugation genes (Hedberg *et al.* 1996; Buttaro(Leonard) *et al.* 2000; Chandler *et al.* 2005*a,b*). One of these determinants, *prgY* in pCF10, encodes a membrane protein whose function is to reduce the level of cCF10 released from the cell membrane. The major portion of PrgY from the amino terminus and extending through nearly 60% of the amino acid sequence appears to lie outside the membrane and is anchored by several C-terminal transmembrane segments. Most mutations that retain protein stability but abolish function map in this extracellular domain. Interestingly, PrgY represents a superfamily of related proteins found in all three biological kingdoms, but not in all species (Chandler *et al.* 2005*a*). No functional analysis of any of these proteins has been completed, except for studies of PrgY and the homologues encoded by two other pheromone plasmids (Weaver & Clewell 1990; Nakayama *et al.* 1994*a*). Since the response of a donor cell to cCF10 requires pheromone import via the concerted activities of the plasmid-encoded PrgZ extracellular pheromone-binding protein and the chromosomal oligopeptide permease system (Leonard *et al.* 1996), one possible way that PrgY could function is to interfere with the import process. However, the cumulative data suggest a different model whereby the extracellular domain of PrgY interacts with nascent cCF10 as it is released from the membrane, and this interaction sequesters, modifies or degrades the peptide; expression of PrgY has no effect on the interaction of *E. faecalis* cells with exogenously added cCF10 (Chandler *et al.* 2005*a*). PrgY control of endogenous pheromone is highly specific for cCF10, and the specificity determinants for recognition by PrgY seem to reside within the mature cCF10 sequence

and not in the signal peptide precursor (J. Chandler & G. Dunny 2006, unpublished data).

Although PrgY is necessary for control of endogenous pheromone activity, it is not sufficient. The donor cells produce reduced but still significant levels of cCF10 in both cell wall fractions and supernatants (Buttaro(Leonard) *et al.* 2000). This residual pheromone activity is neutralized by the production of a plasmid-encoded peptide iCF10 from the *prgQ* locus of pCF10 (Nakayama *et al.* 1994*b*). As described in more detail later, the *prgQ* locus is located at the extreme 5′ end of a long operon encoding many if not all of the conjugation proteins of pCF10. The effects of pheromone induction are: (i) to modestly increase the initiation of transcription from the *prgQ* promoter (this promoter is active at a significant level under all conditions), and (ii) to increase extension of *prgQ* transcripts past a putative termination region such that downstream conjugation functions are expressed (Chung & Dunny 1995; Bensing *et al.* 1997; Bae *et al.* 2004). The only polypeptide coding sequence in the mRNA produced constitutively from *prgQ* transcription is 66 nt Orf encoding a 22-amino acid peptide resembling a signal peptide (without an attached C-terminal secreted protein). The last codons encode the mature iCF10 sequence (AITLIFI); as is the case for cCF10, processing of the full-length precursor to the mature signal molecule occurs concomitantly with peptide export, such that iCF10 must exit the cell and be reimported to function in regulation. High performance liquid chromatography fractionation of culture supernatants of *E. faecalis* strains carrying pCF10 shows that these cells secrete a mixture of iCF10 and cCF10 in a molar ratio of 50–100/1 (Nakayama *et al.* 1994*b*; Hirt *et al.* 2002); this ratio is just sufficient for iCF10 to neutralize the cCF10 biological activity, while allowing the donor cell to remain sensitive to an extremely low (approx. 10^{-11} M) level of exogenous cCF10 as would be produced by the recipient cells in close proximity (Mori *et al.* 1988). Thus, in the 'real world', the induction state of donor cells is dependent on the relative concentrations of the two peptides rather than the absolute cCF10 concentration. Production of iCF10 by the donor cells auto-represses the *prgQ* promoter through an interaction with PrgX (Kozlowicz *et al.* 2004), and the first direct result of induction by exogenous pheromone seems to increase synthesis of iCF10. This probably serves to shut the system back off after a transient period of expression. Consistent with this regulatory model, we recently found that genetic disruption of *prgY* causes an increase in *prgQ* transcription and presumably yielding an increased production of iCF10 to compensate for the elevated levels of cCF10 production by *prgY* mutants (Chandler *et al.* 2005*b*). At first blush, the involvement of two different extracellular peptide signals in controlling pCF10 conjugation functions might seem unnecessarily complicated. However, we recently found that this system is also used by pCF10-containing cells to activate expression of aggregation substance (Asc10) when growing in the mammalian bloodstream (Hirt *et al.* 2002; Chandler *et al.* 2005*b*). A host factor, probably an albumin/lipid complex, selectively sequesters or degrades iCF10, leading to

Table 1. Important properties of *PrgX* and Qa.

property	*PrgX*	Qa
molecular composition	317 amino acid protein	102 nt RNA
source	translated from $3'$ end of P_{Qa} transcript	processed from $5'$ end of P_{Qa} transcript
molecular target	two binding sites in pCF10 DNA	Qs RNA[a]
functions	reduce activity of *prgQ* promoter; positively regulate Qa mRNA processing	inhibit *prgQ* transcription elongation
pheromone sensitivity	$++$	$--$

[a] predicted.

induction (by endogenous cCF10) of conjugation proteins including Asc10. Expression of the latter protein increases virulence of *E. faecalis* in several model systems (McCormick *et al.* 2000), and genetic disruption of either the pheromone-sensing machinery of pCF10 or the ability of the plasmid-containing cells to produce cCF10 abolishes *in vivo* induction. Therefore, it is apparent that the more complex system actually allows for detecting two different kinds of environmental signals (shown in figure 2) that are ultimately of great importance to both the host bacterium and the plasmid.

4. THE MOLECULAR SWITCH CONTROLLING THE INITIATION OF THE PHEROMONE RESPONSE IS A SHIFT IN THE STRUCTURE OF THE C-TERMINUS OF PRGX AS A RESULT OF PHEROMONE BINDING

The transcription of the *prgX/prgQ* region of pCF10 has been studied extensively (Chung *et al.* 1995; Bensing *et al.* 1996, 1997; Bae *et al.* 2000, 2004). The *prgQ* promoter is active in uninduced cells, but the resulting transcripts (termed Qs) are only about 400 nt in length with a $3'$ end in the vicinity of an inverted repeat structure IRS1 that probably functions in transcription termination, mRNA processing or both (Chung & Dunny 1995; Bensing *et al.* 1997; Bae *et al.* 2004). Pheromone induction results in a modest increase (2–5X) in *prgQ* promoter activity, and northern blot analysis reveals the presence of extended forms of mRNAs, the most abundant being QL, which is about 130 nt longer than Qs. Reverse transcriptase polymerase chain reaction analyses show that there is extensive processing of Q transcripts under all conditions, but in induced cells they can extend through *prgB* and beyond (Chung & Dunny 1992, 1995; Bensing *et al.* 1996, 1997). Thus, the most significant result of pheromone induction is to extend transcription from the *prgQ* promoter through the IRS1 region.

The *prgX* gene is adjacent to *prgQ*, but in the opposite orientation. Contrary to initial expectations, *prgX* transcription does not initiate from the region between *prgX* and *prgQ*. Instead, this transcript is produced from a promoter within the *prgQ* locus, but on the non-template strand (Bae *et al.* 2000). Thus, an unprocessed *prgX* mRNA contains several hundred nucleotides of non-coding sequence at its $5'$ end. As is the case for *prgQ* transcripts, the *prgX* message is subject to extensive processing. The most abundant and stable form of this transcript, Qa (Q antisense),

consists of the $5'$ 102 nt (Bae *et al.* 2004). Qa, which is complementary to a segment of Qs, also plays a critical role in regulation of conjugation. Genetic evidence shows that Qa functions to block production of *prgQ* transcripts that extend past IRS1; this activity of Qa was not affected by pheromone. Both Qa and Qs have potential for extensive secondary structure, and it was predicted that pairing of these two RNAs would shift the structure of the $3'$ terminus of Qs into a functional terminator, whereas unpaired Qs would fold in an alternative non-terminating structure (Bae *et al.* 2004). In uninduced cells, the synthesis of Qa and Qs is balanced such that none of the Qs transcripts extend past IRS1. The increase in *prgQ* transcription initiation resulting from pheromone abolition of PrgX repression (next paragraph) essentially titrates all of the Qa and leaves unpaired Qs transcripts that extend past IRS1, ultimately leading to expression of downstream conjugation proteins. This model is currently being examined experimentally.

The other functional product produced from the Qa promoter is PrgX. Genetic analysis of PrgX function showed that this protein represses (albeit modestly) transcription initiation from the *prgQ* promoter, and that repression is sensitive to pheromone (Bae *et al.* 2004); interestingly, PrgX protein also positively regulates its own expression and that of Qa at a post-transcriptional level (Bae *et al.* 2000; Kozlowicz *et al.* 2004). These cumulative results show that the gene products produced by transcription from the Qa promoter affect *prgQ* transcription by two independent mechanisms; once produced, each gene product can function independently of the other, but their mode of synthesis makes them interdependent in wild-type donor cells. Extensive genetic and molecular analyses of PrgX indicate that it is multifunctional. In addition to the functions described previously, PrgX specifically binds pCF10 DNA at two sites in the region between *prgX* and *prgQ*; it exists in *E. faecalis* cells in an oligomeric state, and pheromone addition causes a reduction in the oligomerization state of PrgX (Bae & Dunny 2001). A compilation of the important features of both PrgX and Qa is presented in table 1.

The most recent analysis of PrgX focused on mutants that were deficient in *prgQ* repression, but were not dominant negative and that retained sufficient positive auto-regulatory function to produce normal levels of protein in *E. faecalis* (Kozlowicz *et al.* 2004). Although it was not clear at the outset whether such mutants could be obtained, we were successful in this endeavour. Three phenotypic classes of mutants were
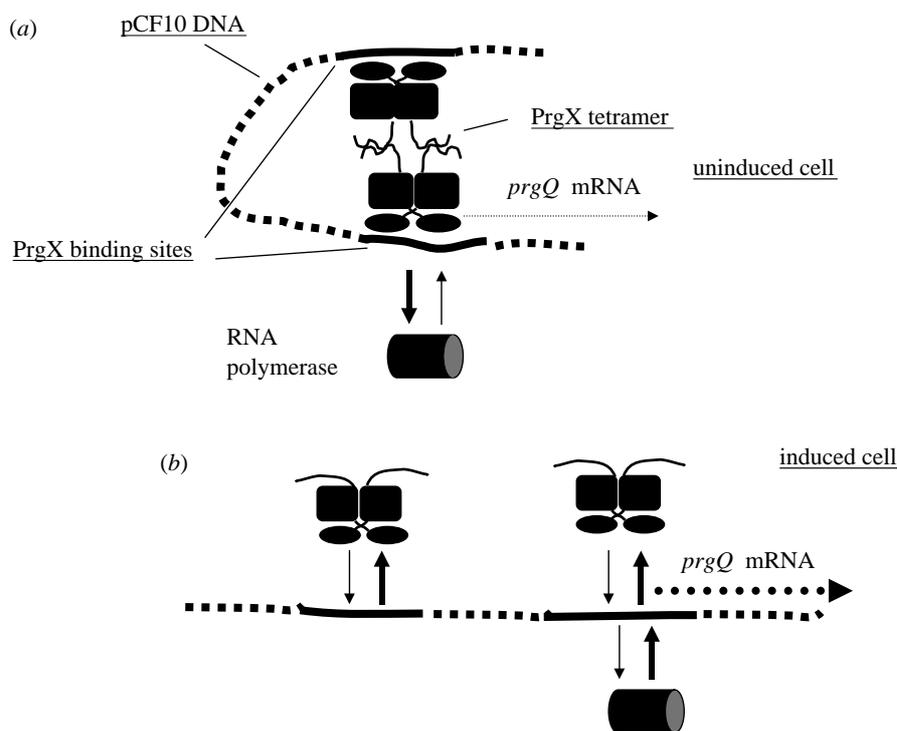
(a)

pCF10 DNA

PrgX tetramer

uninduced cell

prgQ mRNA

PrgX binding sites

RNA polymerase

(b)

induced cell

prgQ mRNA

Figure 3. DNA looping model for PrgX. (*a*) Uninduced donor cell. PrgX molecules bound to each DNA target site interact (the model shown here has each site occupied by a PrgX dimer; the two dimers interact to form a tetramer) to form a DNA loop that stabilizes DNA/protein interactions and increases occupancy of both binding sites. RNA polymerase access to the *prgQ* promoter, which overlaps the lower binding site, is restricted. This reduces *prgQ* mRNA synthesis. (*b*) Pheromone-induced donor cell. Pheromone interaction with PrgX breaks up tetramers, opening the loop, decreasing occupancy of the binding sites and allowing for increased polymerase access to the *prgQ* promoter. This increases production of *prgQ* mRNA.

identified, with most containing substitutions in the C-terminal region of the protein. In the same study, we also confirmed directly that the N-terminus contained the functional DNA-binding domain and we provided biochemical evidence for binding of cCF10 to PrgX. Further analysis showed that all of these mutants were partially defective in DNA binding and/or oligomerization such that *prgQ* repression was impacted more severely than auto-regulation. These results, along with those from previous studies were used to formulate a DNA looping model (figure 3) that accounts for both the regulatory functions of PrgX via a single mechanism. Previous biochemical analyses indicated that the affinity of PrgX binding to pCF10 DNA was not extremely high, with the secondary binding site (which overlaps the *prgQ* promoter and is therefore most important for repression) being particularly weak (Bae *et al.* 2002). We proposed that protein–protein interactions between PrgX molecules attached to the two sites could result in formation of a DNA loop that yields a much more stable complex than those involving only a single binding site in the DNA. We further suggested that the primary effect of pheromone on PrgX could be on the regions involved in protein–protein interactions rather than on the DNA-binding domain; when this model was initially proposed, there was not sufficient evidence to indicate whether each pCF10 DNA-binding site was occupied by a PrgX monomer or a dimer. Structural data described below has demonstrated that each site is bound by a dimer, such that the looped complex contains a PrgX tetramer. Pheromone binding causes disruption of tetramers, destabilizing the loop and ultimately reducing the PrgX occupancy of each DNA target site. While it is easy to envision how formation of this complex could repress the *prgQ* promoter, the mechanism of positive auto-regulation is less obvious. We believe that the looped complex could affect the dynamics of elongation, processing and decay of mRNAs synthesized from the Qa promoter by acting as a roadblock to RNA polymerase molecules. One way that this could work is to block or delay extension of transcription past the secondary PrgX-binding site to allow for folding of the 5' end of the message into a stable structure, i.e. mature Qa, prior to the synthesis of a distal sequence that functions to accelerate $3' \rightarrow 5'$ decay of the entire message. Obviously, this is the most speculative aspect of the model, but there are compelling data that force us to reject simpler alternatives that might appear to be more obvious (Bae *et al.* 2000); further experimental testing of this model is a high priority for current and future work.

A substantial body of published genetic and biochemical evidence (Bae *et al.* 2000, 2002, 2004; Bae & Dunny 2001; Kozlowicz *et al.* 2004) is consistent with the model shown in figure 3, and recent genetic analysis of constructs where the spacing between the two binding sites has been changed to affect their position on the face of the DNA helix (B. Kozlowicz & G. Dunny 2005, unpublished data) also supports the model. In addition, recent determination of high-resolution structures of PrgX and PrgX/cCF10 complexes (Shi *et al.* 2005) has provided physical evidence for crucial features of this model, including
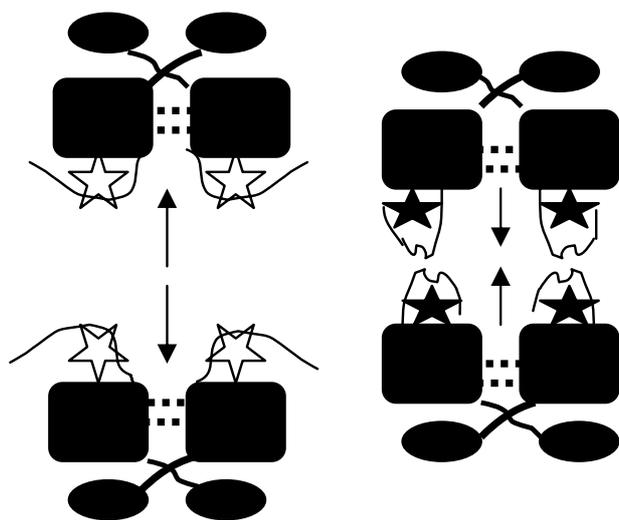
Figure 4. Structural consequences of pheromone and inhibitor binding to PrgX. Extensive intermolecular interactions between amino acid residues in the N-terminal and central domains of PrgX allow the protein to form a stable dimer in all structures examined. In the absence of exogenous peptides, as well as in the presence of iCF10, the PrgX C-terminus assumes a conformation that promotes interaction between pairs of dimers to form stable tetramers; this structure favours formation of DNA loops as shown in figure 3. When complexed with cCF10, the C-terminal arm changes the structure and rotates such that the protein–protein interactions favouring tetramer formation are weakened significantly. Both peptides occupy the same binding cleft formed by a series of parallel and anti-parallel helical domains in the central part of the protein and, in both cases, residues from the C-terminal region interact with the bound peptide. However, iCF10 interacts with different C-terminal PrgX residues than is the case for cCF10.

the direct interaction between PrgX and cCF10, and the effects of cCF10 binding on the PrgX structure and oligomerization state. The structural analysis indicates that a very large portion of each PrgX molecule is α-helical and, consistent with previous biochemical data (Kozlowicz *et al.* 2004), there is a DNA-binding motif near the N-terminus. Extensive atomic interactions between numerous residues in a large central segment of the protein, as well as domain swapping between N-terminal domains of adjacent monomers, promote stabilization of dimers. In PrgX crystals, the molecules are actually in the form of tetramers, where pairs of dimers are held together by a rather small loop region near the C-terminus (figure 4). Pheromone binds to PrgX in a pocket formed by several parallel and anti-parallel helices, and the overall structure of PrgX bound to cCF10 is quite similar to that of the unbound protein. The major difference is that upon occupancy of the binding pocket by cCF10, a major rotation of the C-terminus (approx. 20 amino acid residues) occurs such that a region including residues 296–298 covers the bound pheromone in the pocket, shifts its conformation from a short α-helix to a β-sheet and moves the C-terminal loop involved in stabilization of tetramers such that this interaction is disrupted. It is highly probable that, *in vivo*, these structural changes are sufficient to disrupt the looped structure of the PrgX–pCF10 DNA complex, reducing the occupancy of the operator sites on the DNA and

leading to increased activity of the *prgQ* promoter. Recent genetic, biochemical and structural data (Kozlowicz *et al.* 2006) suggest that the iCF10 inhibitor peptide binds PrgX in the same pocket as cCF10, but that the bound peptide interacts with a different set of amino acid residues near the C-terminus of PrgX, such that the amino acid segment mediating tetramer formation is actually stabilized in a conformation that favours the tetramer-promoting interaction. A significant focus of future investigations will be to examine the interactions of PrgX with itself and with its target DNA in solution, and to develop an *in vitro* transcription system to confirm the molecular mechanisms of regulation suggested by the structural data. In addition, the isolation and characterization of additional mutations in the C-terminal region of PrgX, such as those that give a pheromone-insensitive 'super-repressor' phenotype, should also help to confirm and refine the looping model.

5. MULTIPLE POSITIVE- AND NEGATIVE-REGULATORY LOCI CAN MODULATE EXPRESSION OF DOWNSTREAM CONJUGATION FUNCTIONS FOLLOWING THE INITIATION OF THE cCF10 PHEROMONE RESPONSE

While the molecular mechanism for initiation of the cCF10 pheromone response described previously might seem complex, it is noteworthy that several additional genetic loci located between the Qs-encoding region of pCF10 and the *prgB* gene (whose expression has frequently been used as an indicator of the induction state) have been shown to be involved in positive or negative post-transcriptional control of *prgB* expression (Chung *et al.* 1995; Bensing *et al.* 1997). These loci appear to encode products that act as novel RNAs to affect transcription extension or translation of *prgB* (Bensing & Dunny 1997; Bensing *et al.* 1997). The recent focus of the laboratory on PrgX and the initiation step has precluded analysis of these downstream determinants in the past few years, but our increased level of understanding of the initial stages of the pheromone response should make these downstream events more amenable to further study in the future. Although the results of recent microarray studies of pheromone induction are consistent with the possibility that the entire set of conjugation genes are expressed from transcripts initiating at the *prgQ* promoter (Hirt *et al.* 2005), it is very plausible that there could be separate functional coordinately regulated promoters for the more distal genes in this region, and it will be of interest to address this question experimentally.

6. CAN THE EVOLUTION OF THE pCF10 TRANSFER SYSTEM BY A MODULAR ASSEMBLY PROCESS SERVE AS A MODEL FOR THE EVOLUTION OF BIOLOGICAL COMPLEXITY?

The hallmark of 'intelligent design' for a computer program, an electronic circuit or a mechanical device is that it is the simplest entity that efficiently and accurately performs its given function. In the lore engineering and computer science, there is a term known as the kluge (or kludge) referring to a process by

which a simple and efficient device to accomplish one task gets converted in a stepwise fashion into a Heath Robinson- or Rube Goldberg-like contraption in an effort to adapt the device for alternative uses (http://catb.org/~esr/jargon/html/K/kluge.html). The regulatory circuits controlling expression of pCF10 conjugation functions seem to be much more complex than one might expect to specifically activate the conjugation functions of the donor cells in the presence of recipients. Thus, it could be stated that the system resembles a biological kluge. One explanation for this Byzantine regulation system is that the components have been cobbled together from different ancestral sources and could still be in a fairly early stage of evolution to the optimal system. Alternatively, it is probable that the selective pressures shaping the evolution of the system have been for more functions than simply the optimal regulation of conjugation in response to recipient cell density. Recent experimental data provide support for both of these possibilities.

Complete sequence information is now available for several pheromone-responsive plasmids and predicted pheromone-responsive plasmids (the V583 strain whose genome was sequenced by TIGR (Paulsen *et al.* 2003) contains two large plasmids, each encoding a predicted pheromone-inducible conjugation system, but the functionality of these genes has not been demonstrated). The availability of these data allows for careful comparison of the relatedness of these mobile elements, and for some assessment of their possible modes of evolution. In the case of the three most extensively studied pheromone plasmids, pCF10, pAD1 and pPD1, genetic and molecular analyses strongly suggest that the region of each element corresponding to the *prgN–prgC* segment of pCF10 shown in figure 1, and encoding replication and pheromone-inducible aggregation functions, shows significant overall conservation, consistent with evolution from a common ancestor. However, once the sequencing of these elements was completed, it became clear that this sequence conservation did not extend through the rest of the putative mating pair formation and DNA processing genes, even though all of these genes are coordinately regulated with the conserved upstream genes. In the case of pCF10, the genes predicted to function in formation of the mating channel show much higher relatedness to a putative conjugative element from group B streptococci than to pAD1 or pPD1 (Hirt *et al.* 2005). Furthermore, the pCF10 region encoding the conjugative DNA processing machinery is highly similar to that of the lactococcal conjugative element pRS01 (Staddon *et al.* 2004; Hirt *et al.* 2005) and completely unrelated to the corresponding region of other pheromone plasmids like pAD1 (Hirt *et al.* 2005). From these data, it can be inferred that a primordial pheromone-inducible aggregation module, possibly an autonomously replicating plasmid, became linked in separate evolutionary events to several different mating channels and DNA processing modules to generate the current set of pheromone plasmids. The pheromone-inducible aggregation module has probably been in an enterococcal host for a longer time than the other modules. The fact that there has clearly been a coevolution

between pheromone and at least four components of this segment of pCF10 (PrgX, PrgY, PrgZ and iCF10) is consistent with this idea. It would be of considerable interest to discover a pheromone-inducible (non-conjugative) enterococcal plasmid among current *E. faecalis* strains. Expression of the virulence factor aggregation substance (from *prgB*) is induced during growth of pCF10-carrying *E. faecalis* in blood by endogenously produced cCF10 (Hirt *et al.* 2002). The selective pressure for *in vivo* induction of virulence may have been as important as, or more important than, that for the ability of donor cells or detect recipients in the evolution of the cCF10 sensing system. The relatively recent discovery of the *in vivo* induction phenomenon and its possible role in evolution of the sensing system also illustrates how little we know about the ecology and evolution of cell–cell signalling in bacteria, even for a relatively well-studied organism like *E. faecalis*.

As mentioned previously, the complexity of regulation in this system does not end with the initiation of the pheromone response; several downstream steps are also subject to post-transcriptional control, and we do not have a good idea about why this should be true. However, since both the mating channel and the DNA processing machinery are multi-subunit entities, whose components probably need to be produced in a particular stoichiometry in order to be functional (and to avoid deleterious effects on the host cell), it is not surprising that there could be multiple checkpoints controlling their expression. It is also possible that some components could function in more than one important cellular process (already demonstrated for Aggregation Substance), providing a selective pressure for the evolution of control circuits that allow some conjugation proteins to be produced in higher levels from others encoded within the same transcript.

One of the current challenges for evolutionary biology is to provide more detailed and experimentally testable models for the generation of complexity. In this regard, bacterial structures such as flagella and type III secretion systems have received some attention as useful model systems because it has been suggested that they have certain inherent features, such as 'irreducible complexity' which might impact formulation of models for their genesis by evolutionary processes, while being more amenable to empirical analysis using the rapidly expanding data from microbial genome sequencing and molecular biological analyses of these systems in selected bacteria (Nguyen *et al.* 2000; Paulsen *et al.* 2000). Much of the attention has been focused on the sequence analysis of the protein components of these systems. As we begin to consider the pCF10 pheromone-inducible conjugation system from a molecular evolutionary perspective, it is apparent that both the structural and the regulatory aspects of the sensing and DNA transfer machinery of this system might also prove to be fruitful subjects for more study in relation to the evolution of complexity.

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