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Pheromone-inducible conjugation in *Enterococcus faecalis*:

A model for the evolution of biological complexity?

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Abstract

Pheromone-inducible transfer of the plasmid pCF10 in *Enterococcus faecalis* is regulated using a complicated network of proteins and RNAs. The plasmid itself has been assembled from parts garnered from a variety of sources, and many aspects of the system resemble a biological kluge. Recently several new functions of various pCF10 gene products that participate in regulation of plasmid transfer have been identified. The results indicate that selective pressures controlling the evolution of the plasmid have produced a highly complex regulatory network with multiple biological functions that may serve well as a model for the evolution of biological complexity.

Keywords

Peptide signal molecule; Horizontal gene transfer; Genetic regulation

Introduction

Evolution does not proceed with a goal in mind; it does not produce a complex structure or regulatory pathway from scratch. Rather, it tinkers in a random fashion with a preexisting structure or pathway, pounded by the relentless pressure of selection, until what emerges is new, improved, or with a different, additional function. Thus, it does what engineers and later computer hackers have referred to as “kluging”, i.e., an engineer may have designed a machine to carry out a specific function. Later, it becomes clear that a small modification may generate a completely new function. And yet again and again until what has emerged bears little, if any functional resemblance to the original object. Evolution most likely progresses in a similar fashion (Jacob, 1977; Lenski et al., 2003).

The analogy is, of course, imperfect; the engineer knows what his goal is; evolution does not. Selection replaces intent. Nevertheless, what emerges at the end of both processes is a product, which while perfectly functional, may be far more complex and contrived than had it been created with its final function in mind at the outset of the process.

The initial studies of pheromone-induced plasmid transfer in *Enterococcus faecalis* suggested that a small molecule produced by recipient cells might induce a simple physiological response by donors that resulted in increased expression of genes encoding cell aggregation and DNA transfer functions. In theory, it would seem that a system whose sole function is plasmid transfer could be controlled in a simple fashion by a positive or negative regulatory protein acting at a single promoter and responsive to either the signal molecule itself, or to a second signal transduced from the membrane upon binding of the signal to the responder cell surface. In

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reality, the phenomenon of pheromone-inducible conjugation in *E. faecalis* is a remarkably complex, and to all appearances contrived system of cell-cell communication controlling the horizontal genetic transfer of conjugative plasmids. It has emerged as a result of an extended co-evolutionary process between two autonomous genetic entities, a bacterium and a self-replicating mobile genetic element. A unique feature of this system is the fact that the genetic determinants for production of the signal molecule are encoded by the host, whereas those encoding the signal detection and response machinery reside entirely on the plasmid (Fig. 1A). Recent studies of the pheromone-inducible pCF10 plasmid have shed new light on the system, revealing additional remarkable features (Chandler et al., 2005b; Kozlowicz et al., manuscript in preparation). These include the fact that the conjugation genes of pCF10, which are all coordinately regulated by pheromone, are comprised of modules that have been assembled from different sources distinct from the corresponding genes of other enterococcal pheromone plasmids (Hirt et al., 2005). In addition it has also been discovered that the regulatory circuit controlled by the peptide pheromone also plays a critical role in the stable maintenance of the plasmid, as well as in the expression of virulence in mammalian hosts (Meloni et al., submitted). Thus, the argument can be made that the complexity of the regulatory system derives from its stepwise assembly from many genetic components into a system that controls multiple aspects of the biology of the organism.

Overview of the pCF10 pheromone-responsive regulatory system

Control of endogenous pheromone activity requires two pCF10 genes with distinct functions

In contrast to many quorum-sensing systems that regulate populations of a single cell type in a density-dependent fashion, enterococcal mating pheromones mediate communication between two different cell types, essentially allowing the donor cells to regulate expression of conjugation ability in response to recipient cell density. Both donors and recipients carry genes for pheromone production on their chromosomes, so in the evolution of the plasmid-encoded pheromone sensing machinery, a mechanism for avoiding self-induction by endogenous pheromone while remaining sensitive to the same molecule from recipients has necessarily emerged. This mechanism utilizes two gene products (Fig. 1A). The PrgY membrane protein reduces the production of endogenous pheromone activity by donor cells (Buttaro et al., 2000; Chandler et al., 2005a), without affecting the donor cell interaction with exogenous cCF10. The iCF10 inhibitor peptide neutralizes the residual excreted cCF10 from donor cells that escapes PrgY control. As will be discussed in the following section, the gene conferring iCF10 production is located at the 5' end of the pheromone-inducible *prgQ* operon (Fig. 1B), which also encodes the pCF10 conjugation machinery. In a sense it seems overly complex to employ a regulatory peptide such as iCF10 when a more efficient PrgY that would completely abolish endogenous pheromone in donors would seemingly accomplish the same goal. However, recent data discussed below indicate that the more complex system may allow for a more sensitive mating response to donor cells, and can also be exploited to induce virulence factor expression in a mammalian host (Fig. 1A).

The pheromone-sensitive switch and initiation of a mating response

At the simplest level, regulation of pCF10 transfer is controlled by a balance of two different peptide signals, pheromone (cCF10) and inhibitor (iCF10). The peptides are both internalized, and bound by the negative regulatory protein PrgX (Fig. 1C) (Kozlowicz et al., manuscript in preparation; Shi et al., 2005). PrgX functions as a reversible molecular switch; its activity is modulated depending upon which peptide is bound. Pheromone binding signals a release of the modest level of repression occurring at the *prgQ* promoter (P_Q), resulting in the expression of downstream conjugation genes. Recent evidence suggests that inhibitor peptide binding signals the system to reset itself by reducing *prgQ* transcription back to the uninduced level (B.K. Kozlowicz and G.M. Dunny unpublished results).

PrgX faces two fundamental problems with regards to how it should regulate pCF10 transfer. The first is to control expression of conjugation factors such that they are only produced when a plasmid-free recipient cell is nearby. Expressing conjugation factors at any other time would be energetically costly for the cell and could select against maintenance of the plasmid in the long term. The second problem that PrgX faces is allowing the expression of iCF10 while repressing P_Q. As mentioned above, the inhibitor peptide required to avoid self-induction is encoded at the 5' end of *prgQ* (Fig. 1B). This complicates the issue of P_Q repression by PrgX. If repression functions very efficiently, then iCF10 will not be produced (Fig. 1C). Alternatively, if PrgX does not repress the system, or leaves it in a completely "on" state, iCF10 will not only be constitutively produced, but the conjugation factors will be as well. Therefore, PrgX has evolved as an conditional weak repressor of P_Q that allows for a low level of constitutive expression from P_Q and depends on an RNA counterpart to block readthrough of transcription into downstream genes (Fig. 1B and C).

PrgX is only one of two critical cytoplasmic negative regulators of pCF10 conjugation functions. Both regulatory molecules are produced from one transcript that initiates at the Q_a promoter (P_{Q_a}, Fig. 1B) (Bae et al., 2000). A 5' fragment of the message comprises the 102 nt Q_a (antisense to *prgQ*) RNA (Fig. 1B) (Bae et al., 2000,2004). A direct interaction between the abundant *prgQ* transcript Q_s and Q_a is believed to prevent transcripts initiating at P_Q from reaching downstream genes, thereby providing a roadblock to transcription beyond the *prgQ* gene (Bae et al., 2004). The presence of Q_a RNA allows PrgX to prevent expression of conjugation while only partially repressing expression of *prgQ*, enabling production of iCF10. This modest level of repression coupled with the ability of PrgX to respond to pheromone leaves the system poised to respond to a slight change in the balance of the pheromone and inhibitor peptides, thus insuring a high level of sensitivity towards the presence of recipient cells.

PrgX was long known to be required for negative regulation of pCF10 conjugation (Hedberg et al., 1996), but the molecular basis of its function and the existence of Q_a RNA has been elucidated more recently. PrgX binds pCF10 DNA cooperatively at two sequences in the *prgX-Q* intergenic region (Bae et al., 2002) and recently has been shown to form a DNA loop by binding to both of its operators (Kozlowicz and Dunny, manuscript in preparation). Crystallographic data indicates that PrgX exists as a stable dimer, and likely forms a tetramer while looping the DNA (Shi et al., 2005). Pheromone-binding has previously been shown to disrupt oligomerization, and crystallographic evidence illustrates that this occurs via reduction of the PrgX tetramer to dimers (Shi et al., 2005). The breaking of tetramers also breaks the DNA loop, and likely reduces the affinity of PrgX for its DNA operator, thus allowing for greater rates of transcription at P_Q. Q_a RNA is a pheromone-insensitive factor, and therefore an increase in the amount of *prgQ* transcripts is predicted to lead to a titration of the Q_a RNA "roadblocks", to extension of *prgQ* transcription into downstream genes, and to expression of the transfer machinery (Bae et al., 2004).

The elaboration of the DNA loop revealed an additional level of complexity inherent in the system. PrgX protein was known to be required for proper post-transcriptional processing of the Q_a-*prgX* transcript to produce Q_a RNA (Bae et al., 2004). Additionally, PrgX is an essential positive regulator of its own expression, although it does not act on its own promoter (P_{Q_a}) (Bae et al., 2000). Interestingly, PrgX was found to require the DNA loop in order to regulate its own production and that of Q_a RNA; indicating that the DNA loop regulates transcription from both strands of pCF10 DNA. There are many examples of regulatory proteins that utilize DNA loop formation for control of multiple promoters (Anderson et al., 1981; Lobell and Schlieff, 1990). PrgX, however is the first example to our knowledge of a repressor that simultaneously controls transcripts produced from both strands of its target DNA loop by acting on one promoter, while participating in a post-transcriptional event in the opposite direction.

The regulatory process detailed above represents one possible solution to the problem of regulating pCF10 transfer. This process is complex, and finely tuned to take advantage of several aspects of the host environment. However, there are far simpler mechanisms one could invoke if setting out to design this type of responsive regulation from scratch. Gene duplication to produce a second *prgQ* gene, and thus a second source of iCF10 would be one solution to reducing the complexity of the system. If there were two *prgQ* genes, one could be controlled by a constitutive low-level promoter, and the second could be gated by PrgX functioning as a strong, pheromone-inducible repressor that acts without a DNA loop or the requirement for Qa RNA.

The PrgX protein itself could have arisen by evolutionary kluging. It is comprised of three domains: an N-terminal DNA binding domain, a central dimerization and pheromone-binding domain, and a C-terminal regulatory domain responsible for discerning which peptide is bound and tetramerization interactions. These domains are organized linearly in the gene and it is conceivable that each module of the protein was selected for independently of the others, resulting in the multifunctional PrgX that we now study (Kozłowicz et al, 2004; Bae et al., 2004). We can imagine a scenario in which the original system contained a PrgX that had only a DNA-binding domain. This protein could function to regulate P_Q without any sensitivity to pheromone. Selection for regulation in response to a signal could result in growth of PrgX to include the central region of the protein that is able to bind pheromone and dimerize. This step would allow pCF10 to hijack a cellular waste product (a fragment of the *ccfA*-encoded signal sequence which corresponds to cCF10) and use it as an indicator. However, the formation of a PrgX dimer may only provide a small level of sensitivity to the pheromone signal. Finally, PrgX could grow to include the C-terminal regulatory arm that mediates tetramer formation, the DNA loop forms, and the sensitivity of the system towards the signal increases. This imagined scenario is, of course, only an illustration of the evolutionary pathway that may have been taken. It is presented here as an example of how each functional aspect of the PrgX protein could have been selected for at a different time, resulting in assembly of the final protein. At each step we must keep in mind that the plasmid as a whole was also evolving and subjected to selective pressures encountered in the environment of its bacterial hosts, which certainly varied over time.

Downstream events in the mating response

While the PrgX/Qa system for controlling the initiation of the pCF10 pheromone response is quite complex in its own regard, it is noteworthy that this regulatory circuit comprises only part of the machinery controlling expression of conjugation functions. Space considerations do not permit a detailed description of genetic regulatory loci downstream (in terms of both the location of the relevant genes and the temporal sequence of molecular events in pheromone induction) from Qs. However, it is clear that multiple genetic determinants affect the processivity of RNA polymerase beyond the Qs region in induced cells, and that expression of downstream conjugation genes, such as *prgB* (encoding the Asc10 aggregation protein), is regulated both at transcriptional and translational levels (Bensing et al., 1997). The data indicate that several small RNAs are involved in several downstream regulatory events (Bensing and Dunny, 1997; Bensing et al., 1997; Chung and Dunny, 1995). The reasons for the existence of these apparent checkpoints in the induction process are not yet apparent. Recent transcriptional profiling studies (Hirt et al., 2005) using microarrays and RT-PCR methods indicate that pheromone induction activates transcription of a contiguous segment of over 25 kb of pCF10 DNA, but it is not yet known whether all of this transcription originates from the *prgQ* promoter or if there are additional pheromone-inducible promoters in the intervening region.

The pCF10 conjugation system: an assembly of gene modules from different sources integrated into a single regulatory circuit and controlling multiple biological functions

Genetic organization of pCF10

An interesting outcome of the sequence analysis of pCF10 was the discovery that the region encoding stable plasmid maintenance and pheromone-inducible conjugation functions was assembled in a modular fashion from evolutionarily distinct sources. As described in detail by Hirt et al. (2005), a contiguous ~15 kb segment of pCF10 encoding plasmid replication functions, pheromone sensing, and production of the aggregation substance protein Asc10 shows considerable relatedness to the corresponding regions of other pheromone plasmids. Even within this conserved region there is significant sequence divergence, probably related to the unique specificities of the various plasmids to sense different peptide pheromones. Nonetheless, it can be concluded that these genes in the various pheromone plasmids represent genetic entities comprised of a plasmid replicon/pheromone-inducible aggregation module that probably evolved from a common ancestor. This module is not sufficient for conjugative plasmid transfer. Immediately downstream of this module in pCF10 are over a dozen genes which have not been functionally characterized; sequence analysis predicts that several of the proteins encoded by these genes are secreted, and there is limited similarity of some of these genes to type IV secretion/conjugation systems of gram-negative bacteria. We believe that these genes may encode the pCF10 mating channel apparatus. They actually show more relatedness to putative conjugative elements from pathogenic streptococci than to the other enterococcal pheromone plasmids (Hirt et al., 2005), suggesting that they evolved from a distinct source. Finally at the distal end of transfer genes, we have identified the genes encoding DNA processing machinery, including the conjugative relaxase gene *pcfG*, and the functional origin of transfer sequence (Staddon et al., 2004). This latter group of genes is most closely related to the pRS01 conjugative element of *Lactococcus lactis*, also studied in our laboratory (Staddon et al., 2004); other pheromone plasmids such as pAD1 use completely different DNA processing genes. Thus even though the entire set of genes required for efficient transfer of pCF10 is coordinately regulated by the cCF10 pheromone, at least three distinct modules were used to assemble the system. We conclude that an ancestral module encoding pheromone sensing has been linked with several different mating channel and DNA processing modules in the evolution of the various pheromone plasmids.

Pheromones, plasmid maintenance and induction of virulence

Recent studies of the pCF10 system suggest that in its evolution the plasmid has appropriated the chromosomally encoded cCF10 pheromone peptide to control additional functions. It has been observed that the pCF10 PrgW protein, which functions as the initiator of plasmid replication, binds cCF10, and that the ability of an enterococcal cell to stably maintain wild-type pCF10 seems to depend on endogenous pheromone production (Meloni et al., submitted); note that the induction of conjugation by endogenous pheromone is blocked by two pCF10 gene products as described above. The details of this relationship between cCF10 production and pCF10 maintenance are still under investigation. In addition to promoting conjugative plasmid transfer, pheromone induction also increases the virulence of the plasmid-containing bacteria. In particular, the expression of Asc10 increases adherence and internalization of mammalian host cells, resistance to phagocytosis and production of large cardiac vegetations in experimental endocarditis (Hirt et al., 2002). High-frequency conjugation and Asc10 expression are induced through the pheromone-sensing system when *E. faecalis* cells carrying pCF10 are grown in an animal host, or in plasma (Hirt et al., 2002). Recent evidence indicates that in vivo induction is mediated by the endogenous pheromone produced by the donor cells themselves when a mammalian host component sequesters or degrades the iCF10 produced by

these same cells (Chandler et al., 2005b). In a pure culture of donors growing in laboratory medium, a precise balance between iCF10 and cCF10 activities is maintained; a very slight alteration of this balance in favor of cCF10 results in induction. This prevents wasteful expression of Asc10 and other conjugation proteins when they are not required, but leaves the donor cell positioned optimally to respond to the presence of recipient cells or to growth [incom]in the mammalian bloodstream by synthesis of the advantageous conjugation proteins. Based on the modular organization of pCF10 described above, we suggest that the pheromone-sensing system could have arisen originally to promote colonization or survival in an animal host, with the conjugation ability added at a later time. The machinery encoding the initiation of the pheromone response, involving two peptides and several other components, is certainly not the simplest possible mechanism to control conjugation, but it clearly has at least one additional distinct and important function, namely the activation of expression of virulence in a mammalian host. The evolution of the current system was thus driven by selection for at least two different functions, which provides a rationale for the degree of complexity.

Conclusions

An important principal in the intelligent design of a mechanical device or a computer program is that the simplest design that effectively performs the desired function is the best. Nevertheless, as our level of understanding of the nature and details of the regulatory circuit controlling the conjugative plasmid transfer in *E. faecalis* becomes increasingly sophisticated, simplicity does not emerge as one of its qualities. In fact, we could conclude that the systems are unnecessarily complex and assembled in a haphazard fashion, analogous to a type of Rube Goldberg contraption.

When considering the evolution of a complex process or structure, one might argue either that the complexity is essentially non-functional and has evolved as a result of evolutionary tinkering, or that the complexity, per se, is functional and is a reflection of subtleties or of additional functions of which we are ignorant. The evidence we have presented here inclines us to favor the former possibility. We propose that the components of the system have been appropriated from a variety of other systems as a part of an evolutionary process that has gradually increased the sensitivity and adaptability of the circuit. Likewise, a kluge can be defined as a means of accomplishing a task that consists of various unrelated parts and mechanisms cobbled together. Thus, the pCF10 system appears to be a representative biological kluge.

The plasmid itself is composed of DNA from a variety of sources and, while this is not unusual for plasmids, it may speak to evolutionary pressures that cause such patchwork genetic elements to arise. Assembly of these pieces into the final structure of pCF10 allows the gleaning of such functions as pheromone-inducible control of plasmid transfer and induction of virulence in vivo. The components that regulate transfer, and PrgX specifically, can also be described as kluged because they represent a rather clunky and complicated system that seems excessive for accomplishing the task of pheromone-inducible regulation. We have recently been able to understand the various roles of each component of the pheromone induction system and in doing so we have revealed that the gene products controlling the system, as well as those controlled by the system have multiple functions for the host bacterium. Each new function reveals yet another selectable step in the assembly of this complex system, and therefore pCF10 may prove an excellent model system for studying the evolution of complexity within biological systems.

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Fig. 1. Regulation of pCF10 transfer. (A) Schematic illustrating contributions of PrgY (Y), iCF10 (black triangle), cCF10 (grey and white hearts), and PrgX (X) toward regulation of pCF10 plasmid transfer. 1., PrgY and iCF10 act to control the effects of endogenous pheromone (grey heart). PrgX represses conjugation genes. 2., Exogenous pheromone binds PrgX and disrupts its activity as negative regulator. Plasmid transfer and virulence factors are then expressed. 3., Induction in vivo resulting from sequestration of iCF10 by a plasma component. (B) Diagram of *prgX-prgQ* region of pCF10. PrgX protein (X) is shown at its binding sites, promoters are indicated with flags that point in the direction of transcription, transcripts are indicated by wavy lines, that is likely to be degraded is indicated by the dotted line, and mature Qa RNA is

indicated by the folded RNA structure. (C) Flow chart illustrating effects of peptides on *prgQ* RNA production, iCF10 production, and gene expression. Black triangles = iCF10, white hearts = exogenous cCF10, grey heart = endogenous cCF10.