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MINIREVIEW

Pheromone-Inducible Conjugation in *Enterococcus faecalis*: Interbacterial and Host-Parasite Chemical Communication

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Conjugation is a major mechanism of horizontal genetic transfer in gram-positive cocci and the genes transferred often include antibiotic resistance and virulence determinants (9, 14, 15, 40). A number of enterococcal conjugative plasmids can transfer very efficiently (frequencies as high as 5×10^{-1} [17]) in liquid cultures. Expression of this highly efficient transfer system (which to date has been shown to be present only in the enterococci) in donor cells is induced by a peptide signal (pheromone) produced by recipient cells.

Most work in this area has focused on two plasmids, the hemolysin-bacteriocin plasmid pAD1 (62), which has been described in detail in recent reviews by Clewell (10, 11) and Wirth (69), and the tetracycline resistance plasmid pCF10 (16). These plasmids share many common features at the molecular and genetic levels and exhibit some very interesting differences. This minireview will focus on the cell surface receptors involved in mating pair formation and on the molecular mechanisms by which the pheromone acts as a chemical signal to induce expression of the transfer functions, with emphasis on pCF10. We will also briefly discuss possible roles in host-parasite interactions for the signalling systems and for the surface components involved in mating aggregate formation.

OVERVIEW OF PHEROMONE-INDUCIBLE CONJUGATION

In the generally accepted model for pheromone-inducible plasmid transfer, recipient cells excrete the peptide pheromone molecule into the medium, where it can diffuse to a potential donor cell. Plasmid-encoded gene products allow the donor cell to bind the pheromone with high affinity, and the specific recognition of this signalling molecule serves as a means of communicating the presence of a recipient cell in close proximity. The bound pheromone somehow initiates a response resulting in activation of expression of transfer functions, including the synthesis of aggregation substance (AS), which can promote attachment to recipients via a complementary receptor called enterococcal binding substance (EBS). The close cell-to-cell contact resulting from AS-EBS binding allows for subsequent formation of some sort of mating channel between the two cells that enables transfer of the plasmid from the donor to the recipient. Since the new donor cell created by this event generally shows a pheromone-inducible transfer pattern identical to that of the original donor (18, 19) (even though it has the genes for both production and response to pheromone), the plasmid must encode functions to prevent the donor cell from responding to its own pheromone.

PHEROMONES

Enterococcus faecalis recipient cells excrete a variety of pheromones, which are encoded by the chromosome (19), although the genes involved have not been identified. Different plasmids encode responses to distinct pheromones, and the specificities of pheromone response generally correspond to the incompatibility groups of pheromone-inducible conjugative plasmids (70). Biochemical identification and analysis of pheromones and pheromone inhibitor peptides (see below) carried out in the laboratory of A. Suzuki at the University of Tokyo in collaboration with the Clewell group and with our group have provided the molecular structures of several of these molecules, as reviewed in references 10 and 11. The pheromones are short, hydrophobic peptides excreted into the medium in fairly small amounts (10^{-8} to 10^{-9} M). In the case of cCF10 (sequence, Leu-Val-Thr-Leu-Val-Phe-Val), the synthetic peptide displays biological activity at concentrations of $<5 \times 10^{-12}$ M, which corresponds to one to five molecules per responder cell under the conditions of the assay (44). The amino terminus is especially important (44, 45, 58) for recognition.

TRANSFER GENE PRODUCTS, MOLECULAR ORGANIZATION, AND REGULATION

The transfer genes of both pAD1 and pCF10 are in a contiguous region of about 25 to 30 kb with the major regulatory sequences at one end and the genes encoding products that carry out the transfer process making up the remainder of the region. Figure 1A shows a simplified map of the transfer region of pCF10, which illustrates this organization.

Cell surface components involved in mating aggregate formation. Genes involved in production of EBS, AS, and entry exclusion or surface exclusion (SE; prevention of plasmid transfer between aggregated donor cells) proteins have been identified. By convention, the surface proteins are named after both their putative function and the plasmid by which they are encoded (25); thus, Asc10 refers to the aggregation substance (encoded by the *prgB* gene) of pCF10 (37). The pCF10 structural gene, *prgA*, encodes SE protein Sec10 (20, 37). Both the AS and SE proteins have structural features characteristic of many surface proteins of gram-positive cocci, including N-terminal signal sequences and C-terminal wall-spanning and "membrane anchor" regions (23). A comprehensive analysis of sequence relatedness among a variety of enterococcal pheromone-responsive plasmids by Galli and Wirth (26) showed a highly conserved region spanning the genes encoding AS and SE in nearly all plasmids tested.

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The internal amino acid sequences of the AS and SE proteins have marked structural differences. Sec10 is predicted to be highly α-helical, particularly in the amino-terminal portion, and showed some similarity to bacterial and eucaryotic proteins known to have a high degree of helical secondary structure (37); interestingly, a region between amino acid residues 150 and 175 of Sec10 showed a very high degree of similarity to the receptor-binding domains of several colicins (64). Conversely, the AS proteins are predicted to have a random coil structure for virtually the entire length of the extracellular portion of the molecule (71), suggesting a rather amorphous configuration on the cell surface. Recent field emission electron microscopic studies (48) indicated that the Sec10 protein may form a fibrillar matrix apparent on the surfaces of induced wild-type donor cells, with Asc10 distributed as an amorphous material within the Sec10 matrix. These findings are generally similar to those of previous studies of the pAD1 system by Wanner et al. (27, 32, 65), with the possible exception that some of the extracellular material reported to be Asa1 protein might be interpreted to also contain Sea1, in view of the more recent results (48). Relatively little is known about the specific domains of the AS molecules that directly interact with recipient cells; the recent review of Wirth (69) contains a summary of work in this area and suggests that the amino-terminal half of Asa1 contains sequences crucial for binding to donors.

Donor cells expressing AS attach to recipients via a receptor (EBS). Cell wall lipoteichoic acid (LTA) may be EBS or a component thereof. The results of experiments showing that purified LTA inhibited AS-mediated aggregation (21) and transposon mutagenesis (63) and molecular cloning (4) of chromosomal genes involved in expression of EBS also support the notion that LTA could be EBS. However, the genetic basis for EBS synthesis and assembly is very complex, and all the *ebs* genes sequenced thus far appear to encode products such as regulatory proteins, cell wall hydrolases, and secretion proteins (4). This suggests that EBS could be structurally complex or that interaction of EBS with other cell envelope components could affect its expression or function. "Reversed receptor experiments" in which aggregate formation was dependent on binding of AS on the recipient cell to EBS on the donor (49) indicated that AS-EBS binding serves as a molecular grappling hook that brings the mating cells into close contact and allows subsequent formation of a mating channel dependent on additional plasmid-encoded gene products. These results and previous data (12, 20) also suggest that the surface exclusion mechanism may operate at a step in the channel formation process subsequent to AS-EBS binding. As yet, there are no data available to indicate a mechanism for surface exclusion, the precise role of the SE proteins, or the possible involvement of other gene products.

Control of surface protein expression. The expression of AS has been studied in some detail as an indicator of the pheromone response. It appears that the genes encoding the AS proteins are controlled by two regulatory circuits, one positive and one negative. In the case of plasmid-carrying cells grown in the absence of pheromone, a negative-control circuit prevents transcriptional activation of the genes encoding AS. As a result of pheromone binding to donor cells, the negative control is (temporarily) abolished, and a distinct positive-control mechanism upregulates AS expression at the transcriptional level.

In the pCF10 system, the region required for positive control of *prgB* expression was localized between the *prgX* gene and the *prgA* gene as illustrated in Fig. 1A. The positive-control system functions at distances of 10 kb or more upstream of the target gene, but only in *cis*, and when the genes are in the same orientation as that of the *prgB* target (6). The data indicated that one or more *cis*-acting positive-control elements might track along the DNA to reach their target, presumably the *prgB* promoter region (6). Both genetic experiments (8) and physical analysis of the *prgB* mRNA (6) indicate that *prgB* transcription initiates a short distance upstream from the ATG start codon for the gene rather than from within the positive-control region. Activation of *prgB* expression requires *prgQ* and *prgS* (8). A constitutively synthesized mRNA hybridizing with a *prgS* probe has been detected (7). The *prgQ* gene encodes a 22-amino-acid polypeptide which is processed to generate the heptapeptide iCF10, which functions as a competitive inhibitor of cCF10 (46). The *prgQ* transcript is much longer, however, with a very abundant, constitutive message (Q_S) of about 430 nucleotides present in cells carrying pCF10. Following pheromone induction, a second message (Q_L) representing a 3′ extension of about 100 bp is observed in addition to Q_S; the Q_L message is produced constitutively, concomitantly with expression of Asc10, in *E. faecalis* strains carrying subcloned fragments of pCF10 in which the negative-control regions have been deleted (7). The data generated thus far indicate that the functional gene product(s) of the *prgQ* region responsible for positive control is likely RNA, rather than protein. Q_S is apparently required for transcription of the *prgS* to *prgA* regions, which are expressed constitutively in wild-type donor cells, and *prgS* is required for Q_L. Q_L, in turn, is required for transcription of *prgB*, resulting in production of Asc10. When negative control is abolished by pheromone induction or by genetic alterations, a product of *prgS* promotes Q_L formation, either by enhancing stability or by antitermination. The features of this model that have been described thus far do not explain the *cis*-acting, orientation-dependent nature of the system. The corresponding positive-control mechanism in the pAD1 system (24, 52, 60) shares some common features with the pCF10 system, including the existence of a gene called *iad*, which encodes an inhibitor peptide and short and long RNA species resembling the Q RNA species (24, 60). In contrast to pCF10, pAD1 contains a gene called *traE1*, which acts in *trans* to activate transcription of the *asa1* gene (24, 60). This positive-control gene is encoded by a region of pAD1 corresponding to the *prgR-prgS* region, which shows no sequence similarity to pCF10. It is possible that both positive-control mechanisms utilize similar RNA molecules, but pAD1 has acquired an extra regulatory protein that enhances the interaction of the regulatory RNAs with their target(s).

The negative-control regions of pCF10 and pAD1 shown in Fig. 1B encode at least four very important functions. These functions include prevention of expression of AS and other transfer gene products by host cells growing in the absence of exogenous pheromone, a related function that entails prevention of self-induction of donor cells by internally produced pheromone, the ability to bind exogenous pheromone and transmit the induction signal, and interestingly, the functional replication machinery for the plasmid.

Ruhfel et al. used a pheromone-binding assay to identify a gene called *prgZ* which encoded the binding function (54). The PrgZ protein is similar in sequence to oligopeptide-binding proteins of *Salmonella typhimurium* and *Bacillus subtilis* (31, 50, 53), with about 40% identical amino acid residues, and is highly related (75% overall identity, over 90% in the amino-terminal portion) to the deduced product of *traC*, encoded by pAD1 (59). Because none of the DNA within several kilobases of *prgZ* or *traC* resembles the sequences of integral membrane proteins and ATPases found in other bacterial oligopeptide transport systems (50), the chromosome may encode genes whose products interact with the plasmid-encoded binding proteins.

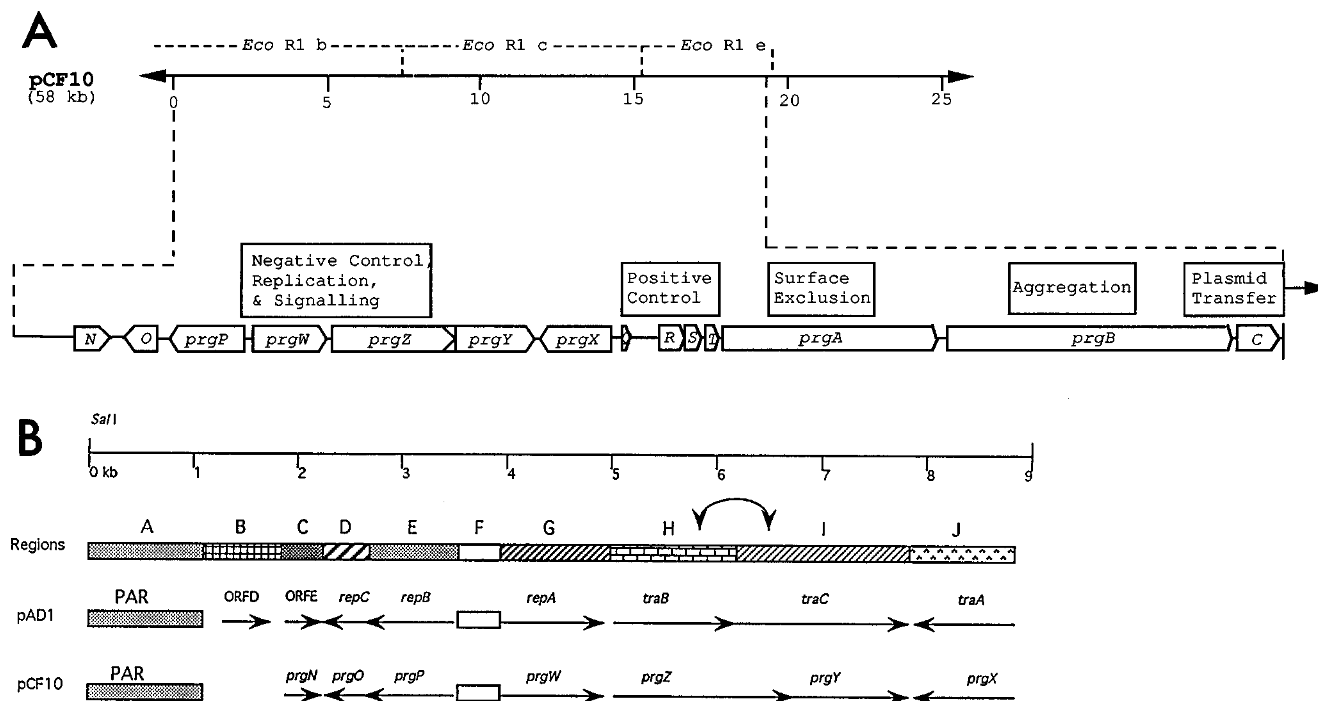

FIG. 1. Organization of plasmid-encoded genes involved in pheromone-inducible conjugation. (A) Illustration of the transfer genes of pCF10, with the positions of *Eco*RI restriction enzyme cleavage sites indicated at the top, functions in the middle, and genes identified by DNA sequencing and other analyses on the bottom. (B) Comparison of the molecular and genetic organization of regions of pAD1 and pCF10 involved in pheromone signalling, negative control, and plasmid replication. The large letters across the top refer to arbitrary regions whose genetic functions and sequence relatedness are summarized below, based on published data and recently obtained results from the Dunny, Clewell, Wirth, and Weaver groups (10, 11, 69). Region A: there is 98% nucleotide sequence identity in this region; in pAD1, a partitioning function has been localized here. Region B of pAD1 is made up of 770 bp, including an ORF (*orfD*) of unknown function, and is absent in pCF10. There is 86% sequence identity in region C; *prgN* has been implicated in negative control. There is no significant sequence homology in region D; functional similarity is not yet known. There is significant sequence similarity in region E; *repB* has been implicated in pAD1 copy control. In pAD1, there are an extra 200 nucleotides in region F and repeated iteron sequences that are not found in pCF10. There is significant protein similarity in region G, which contains the apparent location of *oriT* in pAD1. The H and I regions of the two plasmids are reversed; *prgY* and *traB* are involved in control of endogenous pheromone. For region I, TraC and PrgZ are both pheromone-binding proteins with high similarity. There is no significant homology in region I, but there are probably similar functions in negative control for each plasmid.

Biochemical and genetic studies have indicated that three mechanisms appear to operate in concert to prevent constitutive expression of the transfer functions of cells carrying pheromone-inducible plasmids. Culture supernatants of *E. faecalis* cells carrying pAD1 contain a biological activity that can competitively inhibit the pheromone activity produced by recipients (34, 43). This activity is mediated by a peptide called iAD1, encoded by the *iad* gene of pAD1 (13). It has been suggested that the inhibitor iAD1 could reduce the sensitivity of donors to exogenous pheromone from recipients, so that the system would be turned on only when recipients are very close to donors (11). The lack of pheromone cAD1 in the same supernatants has been ascribed to the product of a "shutdown" gene called *traB* (3, 66). In addition, disruption of a third pAD1 negative regulatory gene, *traA*, results in increased expression of transfer functions even in the presence of active inhibitor and shut-down genes (51, 66), indicative of a negative-control function distinct from inhibitor and pheromone shutdown.

Interestingly, pCF10-containing cells excrete a mixture of cCF10 and iCF10 in a ratio that essentially neutralizes both activities in culture fluids (46). As noted above, iCF10 is encoded by the *prgQ* gene whose RNA products have been implicated in positive control; the sequence of the *prgQ* region is very similar to that of the *iad* region of pAD1 (13). We have recently found that very high levels of pheromone activity can be detected in membrane fractions, apparently trapped during cell lysis, from appropriate *E. faecalis* strains (39). Membranes from plasmid-free cells had titers of pheromone activity at least 100-fold higher than the activity in the supernatant. No activity was present in membranes from wild-type donor cells, whereas intermediate activity was detected if either *prgQ* or *prgY* were deleted or disrupted. The latter gene does not have a high degree of similarity to any pAD1 gene, but it is in a similar location, and its protein product shares some predicted structural features, including a predicted membrane association, with the *traB* gene product (3). Our data suggest that the *prgY* gene product may function to prevent self-induction by degrading or sequestering intracellular pheromone and that iCF10 encoded by *prgQ* could inhibit any residual pheromone that escapes the PrgY mechanism, including the pheromone in the culture fluid.

In the pCF10 system, the *prgN* gene and the *prgX* gene are also involved in negative regulation (30). While the pheromone-binding protein PrgZ is not involved in negative control, inactivation of either *prgW* or *prgY* also abolished negative control. The latter findings may be indirect results of the respective mutations on self-induction rather than direct effects on negative control, since *prgY* is required to prevent self-induction and *prgW* is likely required for transcription of an operon containing *prgZ* and *prgY*.

The same region of pCF10 required to encode both negative control and signalling is also sufficient to support replication of the plasmid. Portions of this region are very similar to the

replication region of pAD1. The *prgW*, *-P*, and *-O* genes are homologous to *repA*, *-B*, and *-C* genes of pAD1; in addition, a putative noncoding partitioning region that is very similar to the PAR region of PAD1 recently described by Weaver et al. (67, 68) has been identified. The identification of potential multifuctional gene products in this region, such as PrgW, is interesting to consider in light of previous findings linking pheromones to incompatibility (70) and replication as well as transfer (67).

The ultimate question with regard to pheromone induction is how the binding of the molecule to the donor cell actually results in the response. Several lines of recent evidence (39) suggest that the pheromone likely operates by becoming internalized and binding to intracellular targets. These include experiments indicating that labelled pheromone is taken up by donor cells, that a pheromone coupled to a large particle (Sepharose bead) maintains PrgZ-specific binding ability but cannot signal donors, and that genetic inactivation of *prgZ* results in a donor strain that can still respond to pheromone (receptor bypass) but is 50- to 100-fold less sensitive than wild-type donors (39). It is likely that a low-affinity, chromosomally encoded peptide transport system may promote pheromone internalization in the absence of PrgZ. In addition, affinity chromatography experiments with pheromone coupled by either the amino or carboxy terminus has identified several specific binding molecules from cell lysates, icluding PrgZ, PrgW, several host proteins, and interestingly, *prgQ* RNA (39). These recent data link the negative- and positive-control circuits, in addition to providing the first strong evidence for pheromone internalization. The data suggest a model for regulation in which pheromone is internalized by an Opp transport system which allows for binding to intracellular regulatory molecules and inactivation of negative control, allowing the positive-control system to activate expression of transfer functions. It is interesting to consider the results obtained in regulatory studies of pCF10 in comparison to those of pAD1 (Fig. 1B). The general organizations of the two regulatory regions are quite similar, and some genes (*prgZ* and *traC*, *prgQ* and *iad*, and *prgW* and *repA*), are almost identical. In contrast, other genes (*prgX* and *traA*; *prgY* and *traB*) probably have similar functions but are not related at the sequence level.

CELLULAR COMPONENTS INVOLVED IN CONJUGATION AS POTENTIAL VIRULENCE FACTORS

The pathogenicity of enterococci has received increasing attention recently because of the very high prevalence of the organism in nosocomial infections (41, 56) and because the inherent and acquired antibiotic resistance of these organisms has resulted in situations where there is no effective antimicrobial chemotherapeutic agent available to treat some cases of life-threatening enterococcal infections (42). Interestingly, there is substantial evidence indicating that several components of pheromone-inducible conjugation may affect the virulence of the organism.

An association between pheromone plasmid-encoded hemolysin and virulence in systemic natural infections (33, 35), as well as in experimental endophthalmitis (36, 57) and endocarditis (5), has been shown. The AS proteins have also been found to enhance the adherence (38) and invasiveness (47) of enterococci and to enhance virulence in experimental endocarditis (5). The effects of host factors on the expression of potential enterococcal virulence factors have also been examined by several groups. There are numerous lines of evidence for enhancement of expression of such factors by growth in serum (1, 2, 28, 29, 38); it is not yet known whether the pheromone signalling mechanism is involved in response to the signalling molecules in serum. Finally, it has been found that several *E. faecalis* peptides identified as pheromones or inhibitors have neutrophil chemotactic activity (22, 55) that appears to function via the N-formyl peptide (F-Met-Leu-Phe) receptor (61) of the neutrophil. The significance of this activity with respect to virulence is not yet known.

CONCLUSIONS

E. faecalis has evolved a complex but efficient chemical communication system that plays an important role in the dissemination of genes, including those encoding antibiotic resistance and virulence traits. Molecular and genetic analyses of this system have elucidated many of its important biological features and have also facilitated the development and adaptation of experimental methods that can be applied to other important aspects of this organism, especially its pathogenicity. Preliminary studies suggest that many of the important components of the pheromone-inducible conjugation system may also play a role in host-parasite interactions involving this organism.

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