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The *par* toxin-antitoxin system from *Enterococcus faecalis* plasmid pAD1 and its chromosomal homologs

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Abbreviations: TA, toxin-antitoxin; PSK, post-segregational killing; SD, Shine-Dalgarno

The *par* post-segregational killing locus present on *Enterococcus faecalis* plasmid pAD1 was the first Type I toxin-antitoxin system described in Gram-positive bacteria. Translation of the 33 amino acid Fst toxin, encoded on RNA I, is suppressed by a 66 nucleotide regulatory RNA, RNA II. RNA I and RNA II are transcribed convergently and interact at dispersed regions of complementarity, establishing a stable complex that accumulates in plasmid-containing cells. RNA II is slowly removed from the complex, allowing translation of RNA I in plasmid-free segregants. Intramolecular structures are also important for regulating translation of RNA I. The Fst toxin contains a putative transmembrane domain and is believed to exert its function at the bacterial cytoplasmic membrane, although its precise target and mode of action have yet to be determined. Numerous chromosomal homologs of pAD1 *par* have been identified in Gram-positive bacteria suggesting that this locus may play important roles in cellular function.

Introduction: par_{pAD1} and Its Relatives

The pAD1 *par* determinant was originally identified as a locus required for maximal stability of the plasmid's basic replicon.[1] The first indication that *par* might be a toxin-antitoxin (TA) system came from the investigation of a serendipitously isolated pAD1 mini-plasmid that triggered host cell death when induced with cAD1, a peptide pheromone usually required for induction of plasmid conjugation functions.[2] Later work showed that this phenomenon resulted from the fortuitous fusion of a pheromone-inducible promoter to the toxin-encoding mRNA of the *par* locus, RNA I.[3,4] Sequence and RNA analysis identified a short transcript convergently transcribed and partially complementary to RNA I,[4] designated RNA II. It was later demonstrated that RNA II was capable of counteracting the toxic effects of RNA I both in cis and in trans, confirming its role as the antitoxin of the system.[3,5] Toxicity was shown to be due to a 33 amino acid open reading frame designated Fst for faecalis stabilizing toxin.[6] It was further demonstrated that the *par* locus, contained on a fragment of 457 nt, stabilized heterologous plasmids at the expense of host cell growth, confirming its role as a post-segregational killing (PSK) system.[3,7,8] More recently, multiple *par* homologs, sharing both toxin homology and similarity in genetic organization, have been identified on the plasmids and chromosomes of many Gram-positive bacteria.[9-11] It is presumed that the plasmid-encoded *par* homologs perform a function similar to that of pAD1 *par*, but the function of the chromosomal homologs is as yet unknown. For consistency in nomenclature, we have recommended using either the name of the mobile element or the chromosomal locus designation in subscript with the *par* component[11] and will use that convention here.

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The Genetic Organization of par_{pAD1} and the Interaction of Its RNAs

The genetic organization of par_{pAD1} and the structure of its transcripts are shown in **Figure 1 and 2**, respectively. The *par* RNAs are convergently transcribed and share a bidirectional intrinsic terminator. The terminator loop provides one region of complementarity at which the two RNAs interact. The RNAs are also transcribed across a pair of direct repeats, DRa and DRb, in opposite directions which provide a second region of complementarity between RNA I_{pAD1} and RNA II_{pAD1}. Interaction at both the terminator loop and the direct repeats is essential for proper regulation of Fst_{pAD1} translation, but the function of these interactions differs. The interaction between the par_{pAD1} RNAs is initiated at a U-turn motif, originally described for the *hok/sok* Type I TA system,[12] in the terminator loop of RNA I_{pAD1}.[13] Mutations in the terminator loop reduce the rate of interaction of the RNAs in vitro[13] and abrogate RNA II_{pAD1}-mediated protection in vivo,[5] suggesting that the rate of interaction is important for translational suppression. Following the initial reversible interaction between the terminator loops, binding is rapidly extended to the DRa and DRb repeats, sequestering the initiation codon, interfering with ribosome binding, and inhibiting translation of the toxic peptide, Fst_{pAD1}, as determined by ribosomal toeprinting and in vitro translation.[6,13]

The par_{pAD1} locus, therefore, has features of both cis- and trans-encoded systems.[14] As in cis-encoded systems, the genes

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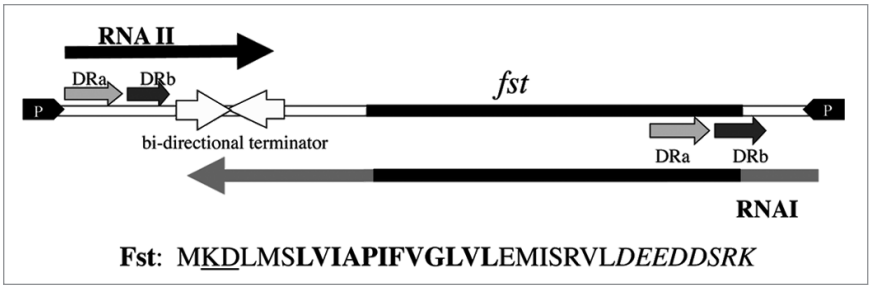

Figure 1. Organization of the pAD1 *par* locus and the Fst toxin. Converging promoters (black arrowheads labeled P) transcribe the toxin-encoding RNA I (red shaded arrow below line) and the antitoxin RNA II (dark green arrow above line) toward a bi-directional intrinsic transcriptional terminator (converging green arrows). The RNAs are transcribed across direct repeats DRa (pink arrows) and DRb (gray arrows) at which interaction occurs, suppressing translation of the Fst_{pAD1} coding sequence (dark red box on DNA and RNA). The amino acid sequence of the Fst toxin is shown using standard single letter amino acid designations. The essential, conserved hydrophobic domain is shown in bold red print. This forms part of a transmembrane domain in the recently published structure of Fst.[22] The two blue underlined amino acids at the N-terminus must be charged to retain toxin function. The non-essential C-terminal tail is shown in green italics.

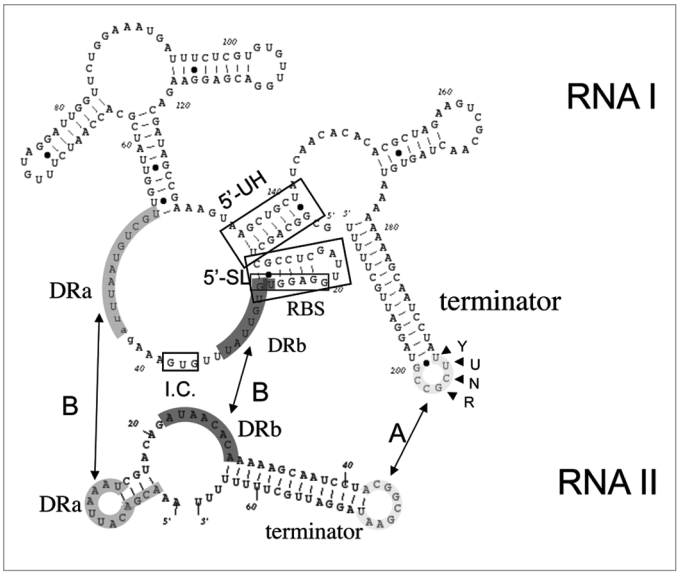

Figure 2. Secondary structures of RNA I_{pAD1} and RNA II_{pAD1}. The specific regions of interaction between the RNAs are shaded different colors to coordinate with **Figure 1** and labeled accordingly. Interaction is initiated at the U-turn motif (labeled YUNR) present in the loop of the terminator of RNA I (green shaded). This interaction is indicated by the arrow labeled A. The interaction then extends to the direct repeat sequences DRa (pink shaded) and DRb (blue shaded). This interaction is indicated by arrows labeled B and is responsible for preventing translation of Fst_{pAD1}, since the initiation codon (I.C.) and the ribosome binding site (SD) are sequestered by the interacting RNAs. The two structures, 5'-SL (blue box) and 5'-UH (red box) are responsible for preventing premature translation of Fst_{pAD1} and RNA I_{pAD1} stability, respectively.

for RNA I_{pAD1} and RNA II_{pAD1} overlap providing critical regions of complementarity required for interaction. However, overlap occurs at the 3' ends of the genes rather than the 5' ends as in most cis-encoded systems. Like trans-encoded systems, interaction between the par_{pAD1} RNAs occurs at dispersed regions of complementarity in which different interaction sites play different roles.

Critical Roles of RNA I_{pAD1} Intramolecular Structures in par_{pAD1} Regulation

In addition to intermolecular interactions with RNA II_{pAD1}, intramolecular structures of RNA I_{pAD1} affect ribosome access to the Shine-Dalgarno (SD) sequence and RNA stability. Two RNA I intramolecular structures, 5'-SL and 5'-UH (boxed and labeled in **Fig. 2**), impact par_{pAD1} function. 5'-SL is a stem-loop that sequesters the Fst_{pAD1} SD, suppressing translation and ribosome binding.[6,15] Translational suppression is not complete since low levels of translation can be observed in vitro and wild-type RNA I_{pAD1} is toxic in vivo in the absence of RNA II_{pAD1}. However, mutations destabilizing the 5'-SL cannot be established in cells expressing RNA II_{pAD1}[15] in spite of the fact that it is capable of binding to and suppressing translation from such mutants in vitro.[6] This discrepancy between in vivo and in vitro results may relate to the timing of RNA I_{pAD1}'s interaction with its two competing partners, ribosomes and RNA II_{pAD1}. Because the interaction between the RNAs is initiated at the terminator loop, the ribosome binding site of RNA I_{pAD1} is transcribed and available for ribosome binding before RNA II_{pAD1} can initiate binding. The 5'-SL is postulated to temporarily inhibit ribosome binding until the terminator loop can be transcribed. It is also possible that RNA I_{pAD1} is processed to remove the 5'-SL in vivo before it can be translated, but no such processing product has been observed in spite of multiple attempts.

The 5'-UH is an "upstream helix" composed of the extreme 5' end of the RNA I_{pAD1} transcript and a complementary sequence further downstream that folds back to interact with it. This helix sequesters the 5' nucleotides from digestion by cellular RNases and is at least partially responsible for the greater stability of RNA I_{pAD1} relative to RNA II_{pAD1}.[16] Mutations in the 5'-UH result in a > 4-fold drop in RNA I_{pAD1} half-life from > 40 min to around 9 min; the half-life of free RNA II_{pAD1} is approximately 4 min. Mutation of the 5'-UH makes RNA I_{pAD1} more susceptible to RNases J1 and J2, which have 5'-3'-exonuclease activity.[17] Whether these are the primary RNases responsible for degradation of RNA I_{pAD1} is not clear. It is also possible that other features of RNA I_{pAD1}, e.g., its relatively inaccessible 3' end and its compact structure, may contribute to its stability.

Interaction of par_{pAD1} RNAs Facilitates the Accumulation of a Stable Complex

In order for Type I TA systems to function as plasmid stabilizing PSK systems, a sufficient pool of the toxin message must accumulate to allow translation after the plasmid has been lost. In the

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prototypical *hok/sok* system, this is accomplished by the formation of alternate conformations of the *hok* mRNA (see x in this issue). In contrast, RNA I_{pAD1} does not appear to adopt alternate structures that control RNA interaction, degradation, and translation. Rather, interaction of the par_{pAD1} RNAs leads to stabilization of the RNAs and accumulation of the RNA I_{pAD1}-RNA II_{pAD1} complex. In the presence of RNA I_{pAD1}, RNA II_{pAD1} basal levels increase more than 2-fold and half-life increases from 4 to 16 min.[18] Similarly, the basal level and stability of the RNA I_{pAD1} destabilizing 5'-UH mutant (see above) was increased more than 2-fold in the presence of RNA II_{pAD1}.[16] These results suggest that formation of the RNA I_{pAD1}-RNA II_{pAD1} complex protects both RNAs from degradation by cellular RNases. While most regulatory RNAs appear to destabilize their targets, target stabilization is not without precedent.[19] These results led to the following model for regulation of par_{pAD1} function. Following transcription of RNA I_{pAD1}, the 5'-SL prevents ribosome binding until interaction with RNA II_{pAD1} can occur. The translationally inactive complex then accumulates as a pool in the cells with RNA I_{pAD1} to RNA II_{pAD1} ratios maintained at approximately 1:1.1.[18] It is possible that the discontinuous nature of the interacting sites in the complex prevents efficient degradation by RNase III which requires at least two helical turns of double stranded RNA for maximal binding and activity.[20] The lower stability of RNA II_{pAD1} suggests that it is preferentially removed from the complex and degraded by means that have yet to be described. This removal must be active, since in vitro results suggest that the complex does not spontaneously dissociate[18] and could involve RNA helicase and/or targeted RNase action. If plasmid remains in the cell, sufficient RNA II_{pAD1} is produced to replace that removed from the complex. If the plasmid is lost, degraded RNA II_{pAD1} cannot be replaced, the Fst_{pAD1} ribosome binding site becomes accessible, either through the processing of the 5'-SL or by the utilization of a ribosomal standby site[21] (perhaps within the adjacent large unstructured loop), and sufficient toxin is produced to kill the cell.

The Fst_{pAD1} Toxin Is a Small, Probably Membrane Localized, Peptide

The Fst_{pAD1} toxin is a 33 amino acid peptide with a charged N-terminus, a predicted central transmembrane domain, and a highly charged C-terminal tail (**Fig. 1**). Alanine scanning mutagenesis supplemented with select conservative and non-conservative amino acid changes revealed that the putative transmembrane domain was most important for toxin function.[11] Alanine substitutions in many of the amino acids in this region lost toxicity, while substitutions with bulky hydrophobic amino acids leucine and valine were tolerated, consistent with a role in membrane transit. The centrally located P11 residue appears especially important for function since substitution with four different amino acids, including alanine and acidic and basic amino acids, eliminated toxicity. At the N-terminus, substitutions of the two charged amino acids, K2 and D3, with either acidic or basic amino acids retained toxicity. However, alanine substitutions were non-toxic suggesting that polarity at the N-terminus is important for function. Substitution of L4 with either alanine or a charged amino acid eliminated toxicity. In contrast, the charged C-terminal tail appears to contribute little to toxin function. In most cases, alanine substitutions and substitutions reversing amino acid charge had no effect. Indeed, a nonsense mutation at D25 resulting in truncation of the C-terminus was still toxic, indicating that the last eight amino acids are not required for toxicity. A nonsense mutation in the adjacent L24 was non-toxic. It is important to note that the mechanism of testing toxicity could not distinguish degrees of toxicity, so it was not possible to test if toxic mutations might have had reduced toxicity.

An atomic resolution structure of Fst_{pAD1} has been determined in the membrane mimetic dodecylphosphocholine by NMR spectroscopy.[22] These results indicated that Fst forms a transmembrane α-helix with the first two and the last seven amino acids protruding. The charged C-terminal seven amino acids are disordered and were predicted to extend from the cytoplasmic side of the membrane. These authors suggested that the primary function of membrane insertion was to facilitate interactions with a specific target rather than being directed against the membrane itself. They also predicted that the disordered C-terminus might become structured upon recognition of the target, but this conclusion conflicts with mutagenic studies indicating that the last eight amino acids are not essential for toxicity.[11]

Overexpression of Fst_{pAD1} Affects Nucleoid Structure, Segregation and Cell Division

Fst_{pAD1} is toxic to *E. faecalis*,[23,24] *S. aureus*[11] and *B. subtilis*[23] when overexpressed from the native RNA I transcript. Toxicity can also be observed in *E. coli* if the 5'-SL structure is disrupted.[15] In all four species, the primary effect of toxin overexpression is condensation of the nucleoid. In *E. coli* and *B. subtilis* this results in elongation of cells, perhaps because the collapsed nucleoid interferes with formation of the division septum at the cell center. In *S. aureus*, the division septum forms and invaginates but the nucleoid is frequently trapped at the convergence point and completion of cell division is inhibited. *E. faecalis* cells initially elongate, then produce misplaced division septae and finally missegregate the nucleoid producing cells containing little or no DNA. The different effects of Fst may reflect differences in the control of cell division in the different species. In *E. coli* and *B. subtilis* nucleoid occlusion systems[25] apparently prevent the formation of division septae over the condensed chromosome. In both *S. aureus* and *E. faecalis* nucleoid occlusion appears to be ineffective in stopping invagination of the cell wall or Fst abrogates its function. In *S. aureus*, new cell wall growth occurs only at the septum,[26] so the presence of a condensed nucleoid effectively blocks both division and growth. In the chaining ovococci, however, cell wall growth occurs both longitudinally and septally,[27] allowing elongation of Fst-exposed cells with the nucleoid trapped at the division site. In at least some cells, the partition apparatus mobilizes the condensed chromosome, but only into one of the daughter cells.

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Fst_{pAD1} is Active at the Membrane But Its Specific Target is Unknown

The putative transmembrane domain of Fst_{pAD1} and its importance to toxin function suggest that the peptide is membrane localized. However, exposure to Fst_{pAD1}, unlike Hok,[28] does not result in the leakage of cell contents and the formation of "ghost cells." An increase in cell permeability is observed following Fst_{pAD1} overexpression but only after the appearance of cell growth and division anomalies, suggesting that membrane defects may be a secondary effect.[23,24] Nisin and Fst have synergistic effects suggesting that they have different but complementary targets.[24] Nisin is a pore forming lantibiotic that docks on lipid II and also affects peptidoglycan synthesis.[29] Unlike nisin but like Hok, synthetic Fst_{pAD1} has no effect on cell growth when added externally,[24] suggesting either that it is modified in some way within the cell or targets a component present only on the inner surface of the membrane or in the cytoplasm. Recent microarray data indicates that overexpression of Fst_{pAD1} results in induction of a variety of energy-dependent membrane transporters; interference with this induction by RNA polymerase mutation or interference of ABC transporter activity with reserpine leads to Fst resistance (Brinkman and Weaver, unpublished). It is possible that hyperactivity of energy-utilizing membrane transporters depletes the cells of energy thereby leading to the observed toxic effect.

par_{pAD1} Homologs Are Widespread in Gram-Positive Organisms

Work by several groups has revealed that Fst_{pAD1} belongs to a large family of RNA-regulated peptide toxins.[9-11,30] These peptides are smaller than 60 amino acids, hydrophobic, and predicted to contain an α-helical transmembrane domain. Indeed, many of the smaller peptides may consist solely of the transmembrane helix. Most are toxic when overexpressed in their native host.[30] An exhaustive bioinformatic search across 774 bacterial genomes identified hundreds of these peptides in the γ-proteobacteria and Firmicutes that were divided into eight families.[9] Fst_{pAD1} is the founding member of the Fst/Ldr family of peptide toxins, which in this analysis consisted of 161 members. In addition, Kwong et al. reported the identification of more than 200 Fst-related peptides in a diversity of Gram-positive bacteria.[10] While there is likely significant overlap between these two lists, it seems apparent that Fst peptides are ubiquitous in Gram-positive bacteria and the related Ldr peptides are prevalent in the γ-proteobacteria. In the Gram-positive bacteria, examination of the DNA sequences surrounding the Fst peptides revealed the existence of all of the elements originally defined in the par_{pAD1} locus, including the convergent promoters for RNA I and RNA II transcripts, a bi-directional intrinsic terminator, the DRa and DRb interacting sequences, and sequences providing the 5'-SL and 5'-UH of RNA I, suggesting that they may be regulated in a similar manner to par_{pAD1}.[10,11] U-turn motifs were not always present in the terminator loop, however, suggesting that some features of the interaction pathway might differ in individual systems. In addition, while the general features of the Fst-encoding *par* loci are conserved, their sequences are not, particularly in the DRa and DRb regions predicted to be involved in RNA-RNA interaction. This feature would allow related *par* systems present on different plasmids to operate in the same cell without interfering with one another. Furthermore, a number of *par*-homologs are chromosomally-encoded. For example, Fst_{EF0409} is present in all sequenced *E. faecalis* but not *E. faecium* strains.[31] Recent work in our laboratory indicates that it neither interferes with nor is essential for pAD1 *par* function (Weaver, unpublished results).

Many of the newly identified *par* homologs are present on plasmids, where they presumably perform the same PSK function as par_{pAD1}.[10] Indeed, a *par* homolog on the well-studied *S. aureus* plasmid pSK41 has been demonstrated to stabilize a heterologous plasmid and Fst_{pSK41} has been shown to be toxic when overexpressed in *E. coli* (Kwong and Firth, personal communication). Another abundant class of *par* homologs is associated with chromosomally-integrated mobile genetic elements including one within the SaPIbov2 staphylococcal pathogenicity island and one phage from *Lactobacillus gasseri*.[10] However, some chromosomally-encoded *par* homologs appear not to be linked to recognizable mobile genetic elements (MGE). The function of these chromosomally-located *par* loci, like most other chromosomal TA systems, is unknown. Interestingly, five of the *par* homologs not associated with MGE are intimately linked to genes involved in carbohydrate metabolism.[10,11] This includes par_{EF0409} located between genes for mannitol phosphotransferase components in *E. faecalis*, $par_{SSP0870}$ located between genes for 6-phosphoglucono-lactonase and an aldehyde dehydrogenase in *Staphylococcus saprophyticus*, $par_{LSEI2682}$ situated between genes for mannose-6-P isomerase and a two-component signal transduction system in *Lactobacillus caseii*, a locus in *S. aureus* MRSA252 located between genes encoding a putative ABC transporter and glycerate kinase, and a *Listeria monocytogenes* locus downstream of a gene encoding a glycosyl hydrolase. In *Streptococcus pneumoniae*, a pair of tandemly-encoded *par* homologs are located between genes for a regulator of a fucose operon and an ABC transporter.[9] The locations of these *par* homologs along with the association of Fst effects on ABC transporters are suggestive of a role in fine-tuning carbohydrate metabolism. This possibility is under active investigation.

Finally, a phylogenetic link between the Fst toxin and the Ldr toxin encoded in the *ldr/rdl* type I TA system present in the LDR (long direct repeat) sequences in the *E. coli* K12 genome was recently identified[9,32] (see also x in this issue for more detail on the *ldr/rdl* system). A superfamily signature consisting of a transmembrane helix followed by a highly conserved tryptophan with a C-terminal tail of charged amino acids was suggested, though the Fst_{pAD1} prototype lacks the conserved tryptophan. The possible relationship between these peptides is further strengthened by their strikingly similar effects upon overexpression in *E. coli*. How these apparently related peptides came to reside in such disparate hosts is a mystery. Although Fst homologs are frequently present on mobile genetic elements, phylogenetic analysis showing coherence between the phylogeny of Fst/Ldr peptides and their hosts of origin suggests that their distribution is not due

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to recent horizontal gene transfer. Interestingly, Ldr expression appears to be regulated by a mechanism similar to that of *hok/sok* rather than by a *par*-like mechanism.[33] It is interesting to speculate on the evolutionary path that fused an Fst-like peptide to a *hok/sok* like TA locus.

Conclusion

To date, the *hok/sok* and *par* systems remain the best studied Type I TA systems and a comparison of the two systems is instructive. While detailed analysis has identified many similarities between the two systems, there are also significant differences. For example, while both systems utilize regulatory RNAs to control the translation of their respective toxins and U-turn motifs are critical for interaction timing, the Sok RNA is a classic cis-acting antisense RNA while RNA II_{pAD1} interacts with its targets via dispersed regions of complementarity in a manner more similar to trans-acting RNA regulators. Both the *hok* RNA and RNA I_{pAD1} adopt a compact secondary structure that stabilizes the RNA and suppresses premature translation initiation. However, while the *hok* RNA structure prevents Sok binding in order to allow a stable pool of the toxin message to accumulate, RNA I_{pAD1} interaction with RNA II_{pAD1} is apparently immediate and it is the complex that accumulates. Both Hok and Fst_{pAD1} are membrane active peptide toxins that must be produced internally to exert their effects. But while Hok expression leads to the production of ghost cells, Fst_{pAD1} causes nucleoid condensation and division inhibition. It seems likely that *hok/sok* and *par* evolved independently and found partially convergent means to solve similar problems. The surprising hybrid nature of the *ldr/rdl* system certainly deserves more attention from an evolutionary perspective.

Significant features of par_{pAD1} function and regulation remain to be determined. The most pressing issue regarding RNA-mediated regulation is the mechanism of release of RNA II_{pAD1}-mediated repression of RNA I_{pAD1} translation. What enzymes and what processes are required to remove RNA II_{pAD1} from the RNA complex? Is the 5'-SL of RNA I_{pAD1} removed or in some other way altered prior to translation? The mechanism of action of the Fst toxins remains unclear. What are the targets on the cytoplasmic membrane to which these peptides bind? How do they insert into the membrane? How does Fst_{pAD1} affect nucleoid structure, partition and division? Why does Fst_{pAD1} have different effects on cell division in different hosts? Examination of this last question may provide insights into the regulation of cell division and chromosomal partition in cocci. The existence of large numbers of *par* homologs on the chromosomes of Gram-positive bacteria, many unassociated with mobile genetic elements, adds another layer of mystery to these apparently ubiquitous elements. Their association with genes involved in carbohydrate metabolism suggests that they may be responsive to growth conditions. Preliminary work with par_{EF0409} suggests that both RNAs of this system are regulated in response to growth phase (Weaver, unpublished results). If so, what is their role in regulating bacterial metabolism? Does the RNA II component regulate Fst expression in the same manner in plasmid and chromosomal systems? Do the chromosomal Fst's have the same target as the plasmid versions? Are the chromosomal versions as lethal as the plasmid versions? Answers to these questions concerning par_{pAD1} and its chromosomal homologs will go a long way toward determining the evolutionary relationship between them.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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