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Silencing quorum sensing and ICE mobility through antiactivation and ribosomal frameshifting

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Abbreviations: +1 PRF, A PRF event that results in a shift of the ribosome forward one nucleotide into next adjacent reading-frame on the mRNA; 3-oxo-C6-HSL, N-(3-oxohexanoyl)-L-homoserine lactone; antiactivation, A form of transcriptional repression that is dependent on protein-level inhibition of a transcriptional activator; C proteins, control proteins; ICEs, integrative and conjugative elements; ICE*Ml*Sym^{R7A}, the integrative and conjugative symbiosis island of *Mesorhizobium loti* strain R7A; MGE, mobile genetic elements; ORF, open reading frame; PRF, programmed ribosomal frameshift; QS, quorom sensing; RM, restriction modification

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Mobile genetic elements run an evolutionary gauntlet to maintain their mobility in the face of selection against their selfish dissemination but, paradoxically, they can accelerate the adaptability of bacteria through the gene-transfer events that they facilitate. These temporally conflicting evolutionary forces have shaped exquisite regulation systems that silence mobility and maximize the competitive fitness of the host bacterium, but maintain the ability of the element to deliver itself to a new host should the opportunity arise. Here we review the excision regulation system of the *Mesorhizobium loti* symbiosis island ICE*Ml*Sym^{R7A}, a 502-kb integrative and conjugative element (ICE) capable of converting non-symbiotic mesorhizobia into plant symbionts. ICE*Ml*Sym^{R7A} excision is activated by quorum sensing, however, both quorum sensing and excision are strongly repressed in the vast majority of cells by dual-target antiactivation and programmed ribosomal-frameshifting mechanisms. We examine these recently discovered regulatory features under the light of natural selection and discuss common themes that can be drawn from recent developments in ICE biology.

Mobile genetic elements (MGE) frequently harbor genes that contribute to the evolutionary success of their host organisms. The genetic cargo carried by MGE can increase metabolic capacity, confer resistance to antimicrobials or arm bacteria with weapons with which to attack other organisms.[1,2] In some cases MGE encode entire suites of genes that facilitate adaptation to a new habitat or lifestyle. The symbiosis island ICE-*Ml*Sym^{R7A} of *Mesorhizobium loti* is a 502-kb integrative and conjugative element (ICE) that carries a diverse array of genes involved in nitrogen fixation, plant signaling, effector protein secretion and metabolism.[3-6] Naturally-occurring non-symbiotic mesorhizobia that receive ICE-*Ml*Sym^{R7A} via conjugation gain the ability to become endosymbionts of the pasture legume *Lotus corniculatus*.[1] Converted mesorhizobia are able to communicate interactively with plants and form an intracellular infection within plant root cells, where they differentiate into 'bacteroids' that fix atmospheric nitrogen into ammonia.[7] Thus in this example, horizontal gene transfer results in the single-step evolution of an entire developmental and metabolic reprogramming of an organism.

Horizontal transfer of ICE*Ml*Sym^{R7A} was first identified *in situ* following investigation of rhizobial populations that arose under a *L. corniculatus* stand following inoculation with a single *M. loti* strain in New Zealand. The field-site soil was devoid of indigenous rhizobia able to nodulate the host. A diverse variety of native mesorhizobial recipients of ICE*Ml*Sym^{R7A} were identified and over time the exconjugants supplanted the original inoculant strain in nodules, directly demonstrating that these hybrid offspring produced from horizontal gene transfer were more

competitive in this environment than their parents.[1,8,9] A similar scenario was subsequently documented in Australian soils but, interestingly, the exconjugants were frequently inefficient nitrogen-fixers despite also being competitive in soil and nodule environments.[10] While the factors leading to the competitive success of these hybrid mesorhizobia are probably multifactorial, it is clear that ICE*Ml*Sym^{R7A} and related ICEs have expedited evolution by facilitating genetic reassortment of the pan-genome.

MGE can directly benefit their hosts in the short term and indirectly increase collective bacterial evolvability in the longer term but, because MGE ultimately promote their dissemination at the expense of the host, the fitness costs associated with mobility may present a direct fitness burden to the donor bacterium.[11,12] Conjugation systems, as well as occupying DNA real-estate, impart significant energy demands on hosts during conjugation.[13] These demands undoubtedly place active donors at a disadvantage compared to hosts lacking ICEs or those carrying ICEs that have lost mobility. Since there is no guarantee that horizontal transfer will result in the evolution of superior offspring, fitness deficits at the level of the host bacterium are also detrimental to the long-term survival of the ICE through vertical descent within its current host. Additionally, the unilateral transfer of DNA to a potentially non-isogenic competitor seemingly has no benefit to the bacterial donor and can (and does for mesorhizobial ICEs[8-10]) result in the original ICE host losing its niche to its partially-related offspring. Evolutionary models of plasmid persistence predict that plasmid mobility should eventually be lost regardless of the beneficial cargo the plasmid may carry, unless the rate of conjugation and generation of successful offspring overcome the selection for loss of mobility.[14] This prediction likely also applies to ICEs and other MGE and, assuming current presumptions of the selective forces shaping MGE evolution are correct, it would seem that successful extant MGE must have evolved mechanisms to mitigate these risks and avoid selection for loss of their mobility.[11,12,15]

Unlike plasmids, ICEs exhibit a clear partition between their 'stay-at-home' and 'mobile' lifestyles through their precise and regulated recombination with the host chromosome.[16] The integration of ICEs within the host genome affords ICEs the stability of replication and segregation provided by the host chromosome and, furthermore, likely reduces the fitness cost of DNA maintenance compared to an extrachromosomal existence. However, ICEs must excise from the host chromosome through site-specific recombination prior to conjugative transfer to form a circularized ICE capable of rolling-circle replication and conjugation. For most documented ICEs, excision and transfer is a rare event.[17-20] In wild-type laboratory populations of *M. loti* strain R7A, ICE*Ml*Sym^{R7A} is only observed in the excised state in 0.06–6% of cells and only ~3 in 10,000,000 cells act as donors. Nevertheless, genetic augmentation of the ICE*Ml*Sym^{R7A} transfer regulation system can induce excision and stable replication of ICE*Ml*Sym^{R7A} in 100% of cells, with a resulting 1000-fold increase in conjugative transfer.[17,21,22] These increased excision and transfer frequencies are achieved through derepression of the ICE*Ml*Sym^{R7A}-encoded quorum-sensing (QS) system (**Fig. 1**), which when activated establishes a positive autoinduction circuit leading to expression of the excisionase protein RdfS (**Fig. 2**).[23]

The original concept of an 'integrative and conjugative element' presumed that ICEs excised only transiently prior to transfer, before returning to an integrated state in the same cell.[16] It is now clear that several ICEs are able to replicate extrachromosomally and remain stable in this state through cell division.[24] Maintenance of ICE*Ml*Sym^{R7A} as an extrachromosomal

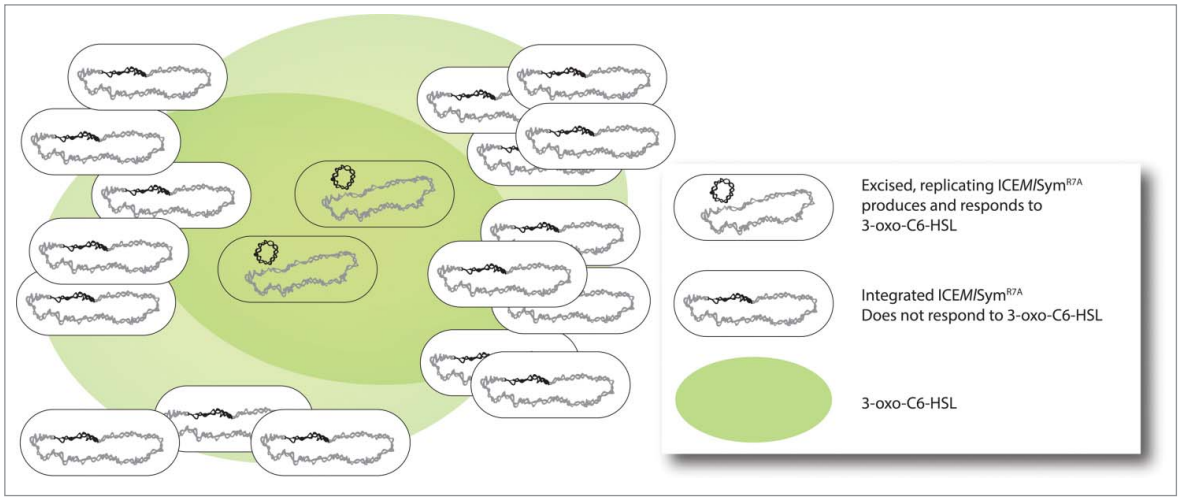

Figure 1. Population-level model of ICE*Ml*Sym^{R7A} excision and quorum sensing. In the vast majority of cells, ICE*Ml*Sym^{R7A} remains stably integrated within the host chromosome. In this state, cells produce negligible 3-oxo-C6-HSL and are unresponsive to large amounts of exogenous 3-oxo-C6-HSL.[22] However in a minority of cells in the population, ICE*Ml*Sym^{R7A} is excised and able to replicate extrachromosomally in a relaxase-dependent manner.[17] In this state, the QS autoinduction circuit is activated by minute amounts of 3-oxo-C6-HSL and is stimulated to produce 1000X more 3-oxo-C6-HSL than when in the integrated QS-off state.[22] The model implies that QS/excision-ON cells and QS/excision-OFF cells behave as 2 discrete populations.[21,23]

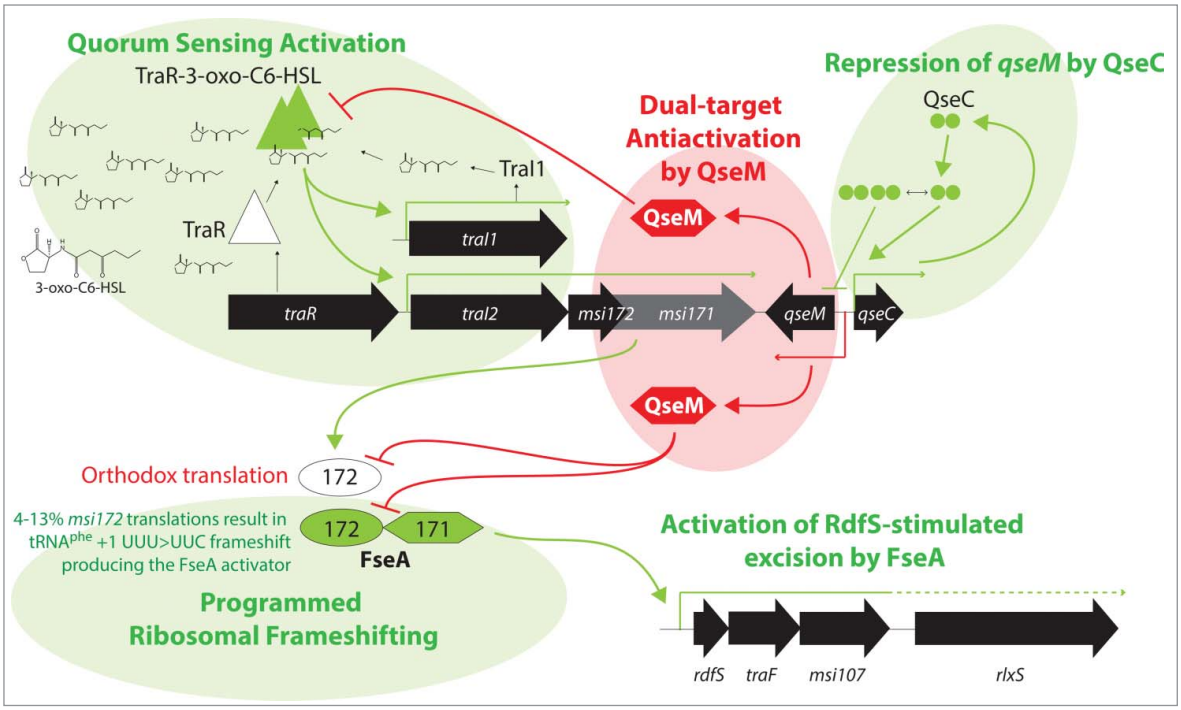

Figure 2. Molecular model of quorum sensing and ICE*Ml*Sym^{R7A} excision. The diagram illustrates the genetic and molecular factors involved in activation (in green) and repression (in red) of QS and excision of ICE*Ml*Sym^{R7A}. TraI1 synthesizes *N*-(3-oxohexanoyl)-L-homoserine lactone (3-oxo-C6-HSL) which activates TraR.[22] TraR activates transcription from the *traI1* and *traI2-msi172-msi171* promoters, further inducing 3-oxo-C6-HSL production.[22] In a minority of translation events of *msi172* (4–13%), an in-frame stop codon in *msi172* is bypassed through ribosomal frameshifting and the *msi172-msi171* open reading frames (ORFs) are translated into a single polypeptide, producing the transcriptional activator FseA.[23] FseA activates transcription of *rdfS*, which encodes the ICE*Ml*Sym^{R7A} recombination directionality factor (excisionase), which stimulates excision of ICE*Ml*Sym^{R7A}.[17] In most cells this activation pathway is repressed by the antiactivator QseM. QseM is able to bind both TraR-3-oxo-C6-HSL and the *msi172*-encoded N-terminal portion of FseA and inhibit the transcriptional activation functions of both proteins.[21,23] The transcription of *qseM* is controlled by the DNA-binding protein QseC, which positively autoregulates its own expression and represses expression of *qseM* through differential binding of 2 operator sites located between *qseM* and *qseC*.[21]

element is dependent on the conjugative relaxase gene *rlxS*, indicating that ICE*Ml*Sym^{R7A} replicates via relaxase-dependent rolling-circle replication in this state.[17] The phenomenon of relaxase-dependent replication of ICEs has subsequently been documented for ICEs in *Bacillus subtilis* (ICE*Bs1*)[25] and *Providencia rettgeri* (ICE R391).[26] ICE*Bs1* utilizes several host- and ICE-encoded proteins for extrachromosomal replication, including the conjugative relaxase NicK.[27] ICE R391, as well as exhibiting relaxase-dependent replication, is also stabilized through cell division by partitioning proteins when it is in the excised state.[26] Thus the consensus from these studies is that while ICEs spend most of their existence stably integrated within the host chromosome, they have also evolved the ability to exist as extrachromosomal elements that have an increased propensity for conjugative transfer. The concept of bacterial differentiation into "mating bodies" that carry excised ICE capable of conjugative transfer has been proposed for ICE*clc* of *Pseudomonas knackmussii* B13.[28] A low percentage (3–5%) of B13 cells in laboratory populations enter a slow-growing state in which ICE*clc* is excised. Mutations that inhibit this differentiation also reduce ICE*clc* transfer. This concept of differentiation of ICE-carrying bacteria into cell subpopulations that are either switched on or off for excision and conjugative transfer is consistent with the inducible and stable extrachromosomal replication exhibited by ICE*Ml*Sym^{R7A}, ICE*Bs1* and ICE R391.

Since ICEs are able to exist stably as extrachromosomal elements, it seems unlikely that ICE excision occurs transiently in all cells. Instead the vast majority of cells in ICE-carrying populations never participate in excision or conjugative transfer, while a minority of cells in the population carry stably excised ICEs and are the donors in horizontal transfer events. This population heterogeneity likely allows ICEs to ameliorate the fitness costs associated with their mobility, as the vast majority of ICE-carrying cells in a population likely never experience any costs other than those of maintaining the ICE DNA itself.[12] Similar phenotypic bet-hedging phenomena are observed for the induction of competence and sporulation in *Bacillus subtilis* in response to changing metabolic conditions.[29] Bet-hedging behavior is often underpinned at the molecular level by autoinduction circuits and layered antagonistic repression systems that together facilitate stochastic establishment of transcriptional and phenotypic bistability.[30]

The excision regulation system of ICE*Ml*Sym^{R7A} involves a QS autoinduction system that is able to induce excision in all *M. loti* cells.[17,22] Until recently however, the extent of the negative regulation that

prevents this activation in the majority of cells in the bacterial population was not fully appreciated.[21,23] ICE*Ml*Sym^{R7A} encodes a cluster of regulatory genes that control both the induction of excision and the expression of an N-acyl-homoserine-lactone-dependent QS system. TraR is a top-level regulator homologous to TraR of the *Agrobacterium tumefaciens* Ti plasmid.[31] TraR is activated in the presence of the diffusible signaling molecule N-(3-oxo-hexanoyl)-L-homoserine lactone (3-oxo-C6-HSL).[22] TraR activates transcription from 2 promoters, one of which initiates transcription upstream of the 3-oxo-C6-HSL synthase gene *traI1*, thus completing the paradigmal LuxRI-family QS-autoinduction loop.[22,32] The second TraR-regulated promoter activates an operon containing genes *msi172* and *msi171*. Expression of *msi172* and *msi171* is essential for expression of *rdfS*, the product of which stimulates the integrase IntS to catalyze excision of ICE*Ml*Sym^{R7A}.[22,23] As mentioned previously, despite this positive autoinduction circuit, excision is only observed in a minority of cells[17] and 3-oxo-C6-HSL production is almost undetectable in wild-type populations.[22] Repression of the autoinduction loop can be relieved through ectopic expression of *traR*, which induces excision in 100% of cells and copious AHL production,[22] but not by the addition of exogenous 3-oxo-C6-HSL.[22] This indicates that there is a fine balance between positive and negative regulatory components of this excision-regulating switch, but once the balance is tipped by increased TraR, cells are firmly set to the 'on' position.

Several QS circuits have evolved negative regulatory components that inhibit QS activation and prevent spontaneous autoinduction triggered by biological noise in transcription and signal-molecule production.[31,33-36] Like the QS system of the *Agrobacterium* Ti plasmid, ICE*Ml*Sym^{R7A} encodes an 'antiactivator' protein, QseM, which binds and inhibits activity of the QS transcriptional activator TraR. QseM shows no primary amino-acid sequence similarity to previously characterized antiactivator proteins, consistent with the observation that several unrelated QS antiactivators have independently evolved in association with LuxRI-type systems.[31,35,36] Using bacterial 2-hybrid assays, AHL bioassays and transcriptional fusion assays, we demonstrated that QseM binds ICE*Ml*Sym^{R7A}-encoded TraR in a 3-oxo-C6-HSL-dependent manner and prevents activation of QS and expression of *msi172* and *msi171*.[21] Strains deleted for *qseM* exhibit a phenotype identical to strains ectopically expressing *traR*, that is, 3-oxo-C6-HSL production is massively upregulated, and ICE*Ml*Sym^{R7A} is excised in 60–100% of cells, and transfer frequency is increased ~1000-fold.[21] Through binding and inactivating TraR, QseM likely dampens the effects of biological noise that might spuriously stimulate activation of QS; more importantly though, it appears to totally prevent the activation of QS in the vast majority of cells in the bacterial population.

Expression of QS and ICE*Ml*Sym^{R7A} excision require either that TraR expression is increased or that QseM expression is repressed; the balance between these 2 components is at the heart of the switch between the integrated and excised state. The expression of QseM is controlled by the 'quorum-sensing and excision control' protein QseC, a DNA-binding protein that autoinduces *qseC* expression and represses *qseM* expression through differential binding to 2 operator sites located between the divergently oriented *qseM* and *qseC* promoters.[21] Mutation of *qseC* prevents the activation of QS, even in the presence of ectopically expressed *traR*, due to derepression of *qseM* transcription. QseC and its operator sites strongly resemble the control (C) proteins and operator sites of type II restriction modification (RM) systems. Additionally, homologues of *qseC* are found adjacent to *traM* genes on several *Agrobacterium* and rhizobial plasmids.[21] This suggests that these C proteins provide a mode of regulatory control that is well-suited for regulation of restriction modification, quorum sensing and plasmid and ICE mobility. The C proteins of RM cassettes are critical for the lag between methylase and endonuclease expression following entry of the RM cassette into a naïve host, and for delicately adjusting the differential expression of these proteins during replication. C proteins, through a mix of positive and negative autoregulation, enable RM systems to adjust gene expression in response to changing C-protein concentration and operator copy number.[37] We suspect that, analogous to methylase gene expression on RM cassettes, *qseM* is strongly expressed following arrival in a new host and in actively replicating cells, but is stochastically repressed in a sub-population of slow-growing or non-dividing cells. In this repressed state, providing that 3-oxo-C6-HSL concentration is able to accumulate to enable activation of TraR (which may be as little as a few molecules per cell[22]), the QS autoinduction circuit is established, *msi172* and *msi171* are expressed and excision is activated. Once the switch has flipped, autoinduction of *qseC* expression largely prevents further *qseM* expression and *qseM* repression may be further augmented through transcriptional interference from the convergently transcribed *traI2-msi172-msi171* operon.

In a recent investigation, we found that the functional protein product of the *msi172* and *msi171* genes is a transcriptional activator FseA, which is translated as a single polypeptide through a programmed ribosomal frameshift (PRF).[23] FseA directly and strongly activates the *rdfS* promoter, connecting QS with the activation of excision. A PRF site exists near the 3′ end of the *msi172* gene, and encodes a slippery sequence UUUC that facilitates a +1 shift of the tRNA^{phe} from the UUU codon to the UUC codon, placing the ribosome in the *msi171* reading frame and joining the polypeptide sequences encoded by the *msi172* and *msi171* open reading frames (ORFs). Despite FseA having no structurally defined relatives, FseA homologues (DUF2283) are widespread in the proteobacteria and appear to be encoded on numerous putative ICEs, both as single-ORF and PRF-containing 2-ORF variants.[21,23] The PRF site of *msi172* contains several features common to other +1 PRF sites, such as the slippery codon sequence and a conserved upstream guanine-rich region. The PRF event appears to occur at a rate of between 4 and 13% of translation events, estimated from the detection of frameshifted protein products in *Escherichia coli* and through fusion of the PRF site to the β-galactosidase gene, expressed in *M. loti*. Activation of the *rdfS* promoter by *msi172-msi171* is only 0.8% of that induced by expression of a fused *fseA*

gene. Thus the PRF site of *msi172* markedly reduces the potential activation of the *rdfS* promoter by QS, but induced *rdfS* expression remains high enough that when TraR is active, excision is stimulated in 100% of cells. Overexpression of *rdfS* causes growth inhibition in the presence or absence of ICE*Ml*Sym^{R7A} and thus the PRF site may have evolved to reduce the deleterious effects of *rdfS* overexpression.[17] The inhibition observed when *rdfS* is strongly expressed suggests that, as for *P. knackmussii* ICE*clc*,[28] donors of ICE*Ml*Sym^{R7A} may differentiate into a slow-growing transfer competent state. However, given that *M. loti* cells active for QS and excision do not appear to exhibit growth inhibition,[17,21] the inhibition may be a side-effect of *rdfS* expression above levels that occur naturally and not a conserved feature of mating-body differentiation. The 4–13% translation of FseA may be adequate to provide the sustained expression of *rdfS* required for maintenance of ICE*Ml*Sym^{R7A} in the extrachromosomal state when QS is activated, but not high enough to induce growth inhibition or loss of ICE*Ml*Sym^{R7A}.[17]

In addition to reducing the deleterious effect of *rdfS* overexpression, the *msi172* PRF site may have evolved to dampen biological noise stemming from spurious *msi172-msi171* transcription.[23] At a molecular level, observed population-level ranges in gene expression are not analog, but rather the average of a series of discrete events. Promoter strength or promoter activation largely reflects the frequency at which transcription is initiated. Biological noise at the level of transcription can result in stochastic expression from even a weak or unactivated promoter, and once this transcript is produced, only post-transcriptional regulation can abate the translation of proteins encoded by it.[38] Quorum-sensing circuits are particularly sensitive to noise and stochastic autoinduction.[39,40] It has been proposed that QS circuits may not be able to avoid random autoinduction without the presence of negative regulatory components,[34] which is consistent with the unbridled QS activation in *M. loti* in the absence of *qseM*. Spurious expression of a transcript encoding genetically fused *msi172* and *msi171* genes (no PRF site) would likely result in sporadic expression of RdfS and excision of ICE*Ml*Sym^{R7A}. Untimely and unregulated excision of ICE*Ml*Sym^{R7A} could place both the ICE and the host bacterium at a competitive disadvantage that could lead to selection for loss of ICE*Ml*Sym^{R7A}, or at least loss of ICE*Ml*Sym^{R7A} mobility. We propose that the PRF site and FseA have together evolved to provide an appropriate level of expression of *rdfS* when the QS switch is activated, but negligible production of FseA in the absence of QS.

As we recently reported,[23] QseM is additionally able to bind the *msi172*-encoded N-terminal portion of FseA and directly prevent transcriptional activation of the *rdfS* promoter by FseA. To our knowledge, QseM is the first identified dual-target antiactivator. The additional inhibition of FseA by QseM illustrates that ICE*Ml*Sym^{R7A} has evolved extraordinarily robust safeguards to prevent expression of *rdfS*, and activation of excision, in the face of biological noise. It is not yet clear how QseM achieves binding and inhibition of two distinct transcriptional activator proteins. QseM shows weak amino-acid similarity to the *msi171*-encoded DUF2283 domain of FseA, suggesting their genes may have a common ancestor. Numerous ICEs that carry homologues of FseA lack QseM homologues, suggesting that DUF2283-domain proteins may have primarily evolved as transcriptional regulators rather than antiactivators. One possibility is that QseM arose from a duplication of an ancestral FseA gene that then lost its Msi172-like region and evolved antagonistic activity against its FseA-like paralogue. Interestingly, QseM and FseA are present on numerous ICEs that lack recognizable QS genes, further supporting the notion that the antagonistic relationship between QseM and FseA evolved prior to the ability of QseM to bind and inhibit TraR. These findings, considered together with the identification of several other independently-evolved QS antiactivators, suggest that antagonistic factors that repress QS have evolved frequently during evolution.[31,33-36]

Together the components of the ICE*Ml*Sym^{R7A} excision regulation system likely shield most cells from the potentially deleterious expression of its mobility genes and govern the proportion of cells in a population that enter into the transfer-competent state. It is likely that factors that favor the differentiation of ICE-carrying cells to become transfer-competent are the same factors that favor the evolutionary outcomes of horizontal gene-transfer events. Further understanding of the mechanistic factors that stimulate ICEs to enter into the excised, transfer-competent state, will undoubtedly shed light on the environmental factors that have favored horizontal gene transfer *in situ*. Horizontal gene-transfer events are of critical concern for the spread of antimicrobial resistance and virulence determinants and yet our understandings of the circumstances that lead to these low-frequency events are still relatively rudimentary.[41] Furthermore, tools could be derived from the exquisite repression systems inbuilt within MGEs for use in the creation of synthetic circuitry. The emerging field of synthetic biology has brought with it the promise of creating living organisms that might replace everything from electronic circuitry to molecular sensors and even biochemists![42] These switches often utilize quorum-sensing components and simple DNA-binding regulators similar to those described here. However a major stumbling block in the creation of synthetic biological circuits is their inherent variability and sensitivity to biological noise. In commentary on factors inhibiting the progress of the synthetic biology field, 3 of the "Five hard truths for synthetic biology" are directly related to the unwieldy effects of biological noise.[43] We argue that MGE have faced similar issues with noise throughout their lengthy evolutionary history and that elements like ICE*Ml*Sym^{R7A} have evolved very elegant solutions that await exploitation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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