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Integrative and conjugative elements (ICEs):
what they do and how they work

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Abstract

Horizontal gene transfer plays a major role in microbial evolution, allowing microbes to acquire new genes and phenotypes. Integrative and conjugative elements (ICEs, a.k.a. conjugative transposons) are modular mobile genetic elements that reside integrated in a host genome, and are passively propagated during chromosomal replication and cell division. Induction of ICE gene expression leads to excision, production of the conserved conjugation machinery (a type IV secretion system), and the potential to transfer DNA to appropriate recipients. ICEs typically contain "cargo" genes that are usually not related to the ICE life cycle and that confer phenotypes to host cells. We summarize the life cycle and discovery of ICEs, some of the regulatory mechanisms, and how the types of cargo have influenced our view of ICEs. We discuss how ICEs can acquire new cargo genes and describe challenges to the field and various perspectives on ICE biology.

INTRODUCTION

Microbes can acquire (and donate) new genes and phenotypes rapidly by horizontal gene transfer (HGT), the transfer of DNA from one organism to another. There are three well-studied types of horizontal gene transfer in microbes: 1) Transformation, the natural ability to take up exogenous DNA from the environment (reviewed in 83). 2) Transduction, the transfer of DNA from one cell to another by bacteriophage (139) and references therein. 3) Conjugation, the contact-dependent, unidirectional transfer of DNA from a donor to a recipient via a conjugation (or mating) apparatus expressed in the donor (first described by Lederberg 86). Both transduction and conjugation are mediated by mobile genetic elements, which can mediate their transfer from one cell to

another. Other types of mobile genetic elements are mobile within an organism, but not necessarily between organisms. A fourth type of horizontal gene transfer, fusion of two cells, and perhaps fusion of cells with DNA-containing vesicles, appears to be less common in prokaryotes than transformation, transduction and conjugation.

This review focuses on integrative and conjugative elements (ICEs), also called conjugative transposons, which make up a large family of mobile genetic elements. There are two defining features of ICEs: 1) they are found integrated in a host genome, and 2) they encode a functional conjugation system, a type IV secretion system (described below and reviewed in 2, 38, 143), that mediates their transfer to other cells. We limit our scope to ICEs that transfer linear single strand DNA (ssDNA) and will not cover the ICEs of actinomycetes (AICEs) that are transferred as double stranded DNA (dsDNA) by an FtsK-like ATPase (reviewed in 18, 61, 138).

There are many excellent reviews of ICEs (14, 122, 141, 156) and various functions associated with ICEs and other mobile elements (14, 141). Here, we briefly summarize some of what has been previously reviewed and describe some of the key features of the ICE life cycle. We focus on aspects of ICE biology that are emergent and have not been extensively summarized.

Integrative and conjugative elements

ICEs and conjugative plasmids are both mobile genetic elements that carry genes encoding the machinery necessary for conjugation. ICEs are typically found integrated in the host chromosome, and contain genes needed for integration and excision. They are propagated passively during chromosomal replication, segregation, and cell division. In contrast, conjugative plasmids exist as extra-chromosomal elements that

replicate separately from the host chromosome. However, several ICEs, and we postulate many more, are capable of autonomous plasmid-like replication (see below).

ICEs and conjugative plasmids contain genes and sites needed for processing their DNA for transfer. Most of these genes are not expressed when the ICE is integrated in the chromosome. However, upon activation, expression of ICE genes needed for excision and conjugation is induced, and the ICE excises from the host chromosome. Cells then have the ability to transfer the ICE (or other DNA) through the ICE-encoded conjugation machinery to an appropriate recipient.

ICEs are typically mosaic and modular, ranging from ~20 kb to >500 kb in size. They contain functional modules from different sources. Genes of similar function are typically grouped together on the element (79, 105, 110, 121, 122, 142, 155, 156).

Because of the mosaic and modular nature of ICEs, knowledge of other elements greatly informs our views of different aspects of the ICE life cycle. Regulatory mechanisms controlling ICE gene expression can be similar to those of phages, plasmids and host genes. Mechanisms of integration and excision from the host genome are similar to those of viruses and transposons. Processing of ICE DNA for conjugative transfer is similar to that of conjugative plasmids and analogous to rolling circle replication of some plasmids and viruses.

The conjugation machinery encoded by ICEs, a type IV secretion system, is homologous to that encoded by conjugative plasmids, and much of what we know about the mechanism of conjugation comes from many beautiful studies of conjugative plasmids, including the F plasmid from *E. coli*, R plasmids, and the Ti plasmid (reviewed in 2, 3, 80, 154). In some cases ICEs and plasmids use conjugation systems to mobilize non-conjugative elements to new hosts. As with some plasmids, conjugation can be regulated by cell-cell signaling. Like both plasmids and phages, many ICEs carry

cargo genes with functions unrelated to the ICE life cycle. These cargo genes are typically thought to provide some benefit to the host cells.

Historically, the study of cargo genes and their transfer led to the discovery of ICEs. Cargo genes that were typically studied encoded an easily selectable or identifiable phenotype that facilitated study of the ICE, including antibiotic resistance or the ability to metabolize a new carbon source. Recently, ICEs have been identified by sequence analysis, and in many cases it is not obvious what benefit these ICEs confer to their hosts. Therefore, our current understanding of ICE-encoded phenotypes is skewed by the selective studies of ICEs that confer specific phenotypes. We speculate that many of the recently identified ICEs confer beneficial phenotypes that are outside the range of those already associated with ICEs.

The biology of ICEs can be viewed from both mechanistic and evolutionary perspectives. We believe that the most interesting aspects of ICE biology are those that are likely conserved and not well understood for other elements. This includes understanding the mechanisms of transfer, the processing and fate of ICEs upon introduction into a transconjugant, and the identity and roles of host genes in ICE biology. In addition, the study of cargo genes and the phenotypes conferred will provide insight into the evolutionary aspects of ICEs, their interactions with hosts and other mobile genetic elements, and their roles in enabling organisms to grow in different niches.

The ICE life cycle

Under normal circumstances, most ICEs are integrated into the host chromosome and conjugation genes are not expressed (Fig. 1A). When ICE gene expression is induced, by specific cellular conditions, or perhaps stochastically, the ICE excises from

the chromosome and forms a circular, dsDNA molecule, essentially a plasmid (Fig. 1B). Several of the ICE gene products assemble into a mating pore that is capable of transferring the ICE DNA. Other host and ICE-encoded proteins recognize the origin of transfer (*oriT*) and process the ICE DNA to generate a linear ssDNA-protein complex, referred to as the transfer DNA (T-DNA) (Fig. 1C). The mating machinery pumps the T-DNA into the recipient (Fig. 1D), where the ICE likely recircularizes, becomes double stranded (Fig. 1E), and then recombines into the chromosome using an ICE-encoded recombinase (integrase) (Fig. 1F). Because the known recombinases require dsDNA as template for recombination, it is inferred that the ICE ssDNA must be converted to dsDNA. Many ICEs integrate into a specific chromosomal site, often a tRNA gene. Others are more promiscuous and can integrate into many locations. In all cases, if the ICE is to be maintained in the original donor, it must eventually integrate back into that chromosome (Fig. 1F).

CARGO GENES AND THE DISCOVERY OF ICEs

Identification of ICEs that confer antibiotic resistances

The discovery and earliest studies of ICEs resulted from interest in resistances to antibiotics and heavy metals, and how those resistances were spread between organisms. At the time, the spread of many of these types of resistances was known to be mediated by conjugative plasmids that harbored the resistance genes. Work with *Enterococcus faecalis* (52), *Bacteroides* species (98, 117), *Haemophilus influenzae* (124, 136), *Streptococcus pneumoniae* (128), *Proteus rettgeri* (108) and *Clostridium* species (94, 134) identified antibiotic and heavy metal resistance determinants that were transferred via conjugation. Importantly, the resistance genes were later found to be transiently or permanently located on the chromosome and not on a stable plasmid.

Conclusive evidence of a conjugative element that integrated into DNA came from studies of Tn916 in *E. faecalis* (then called *Streptococcus faecalis*). It was found that tetracycline resistance could be transferred between strains of *E. faecalis* via conjugation in the absence of plasmids, and that the recipients in these experiments were converted to donors that could then transfer the resistance to another recipient. This work demonstrated that Tn916 encoded genes necessary to mediate its own conjugative transfer and that it could integrate into various sites on a plasmid or the host chromosome (often in multiple copies) much like a transposon. Furthermore, Tn916 functioned in recombination-deficient strains, demonstrating that homologous recombination was not needed for integration of the element into other DNA (52, 58). Because of these properties, Tn916 was called a conjugative transposon.

Identification of ICEs that confer other phenotypes

ICEs were also identified based on their ability to enable cells to utilize an alternative carbon source. This trait, like antibiotic resistance, provided a selective phenotype that facilitated study of the ICE. The ability of *Pseudomonas knackmussii* B13 to degrade chlorocatechols and use them as a carbon source was found to be transferrable to other strains via conjugation of the chromosomal element ICE_{clc} (119). Genes allowing the fermentation of sucrose were determined to be on the ICE CTn_{Scr94} in *Salmonella* (71) and on the ICE Tn5276 in *Lactococcus lactis* (118). Genes allowing *Pseudomonas putida* to metabolize biphenyls and salicylate are located on the ICE *bph-sal* (107).

Some genomic, pathogenicity, and symbiosis islands are also ICEs. These are regions of bacterial genomes that are present or absent in otherwise closely related bacterial strains. For example, the opportunistic pathogen *Pseudomonas aeruginosa* has an

extremely plastic genome, in large part due to pathogenicity islands (67, 97). One of these islands, PAPI-1, is an ICE (32). The genes that allow *Mesorhizobium* (then *Rhizobium*) species to form nodules on *Lotus* species are on a symbiosis island ICEMISym^{R7A} (116).

The selective advantages and phenotypes conferred to the bacterial hosts by ICEs led directly to the identification of these mobile elements and have been a convenient and powerful means for identifying and tracking these ICEs. However, identification of ICEs based solely on the phenotypes conferred by the cargo genes provides a limited means for identifying ICEs. Other means for identifying ICEs provides a more complete view of these elements, their distribution in various organisms, and their potential contributions to microbial phenotypes.

Identification of ICEs based on conserved features

Many ICEs, or putative ICEs, have now been identified based on sequence similarities rather than the phenotypes conferred by cargo genes. Bioinformatic approaches have been used to: 1) Find new ICEs based on the presence of conserved conjugation and DNA processing genes. 2) Compare closely related ICEs to identify conserved features, and 3) Survey a wide variety of genomes for conserved features indicative of conjugative elements.

Functional ICEs have been identified in diverse organisms using bioinformatic approaches. For example, ICEA of *Mycoplasma agalactiae* (47, 96), ICEPmu1 of *Pasteurella multocida* (101) and ICE-βox (also LpPI-1) of *Legionella pneumophila* (21, 51) were found by identifying variable regions within the chromosomes of closely related bacteria that contain conjugation genes. ICEBs1 of *B. subtilis* was identified both bioinformatically (25) and by analysis of a peptide signaling system (*rapI-phrI*) that was found to regulate

conjugation (5).

Examination of genome sequences and sequencing of known ICEs has facilitated comparisons between closely related ICEs, such as those of the ICE*Hin1056* (79) and SXT/R391 families (8, 155). Such studies helped distinguish conserved genes that contribute to the ICE life cycle from cargo genes. In addition, they help to reveal the breadth of cargo genes associated with closely related ICEs and to highlight the fact that ICEs carry many of their cargo genes in defined regions (hot spots) that tolerate gene insertion without disrupting ICE function. An online tool, ICEberg, has been recently created that facilitates the identification and comparison of closely related ICEs (<http://db-mml.sjtu.edu.cn/ICEberg/>) (16).

Recently, a systematic approach has been applied to the broad-scale identification of candidate conjugative elements, including ICEs, in genomic sequence databases (66). Regions that contained conserved features of conjugative elements were identified in genomic sequences, including genes predicted to encode conjugative relaxases, type IV coupling proteins, and ATPases of type IV secretion systems. A search of over 1000 genomes revealed 335 putative ICEs and 180 putative conjugative plasmids, documenting that ICEs are present in most clades of bacteria and are likely more common than conjugative plasmids (66).

Approaches to identify cargo genes and benefits of ICEs to host cells

Many of the putative ICEs that have been identified bioinformatically are likely to have cargo genes with functions distinct from those already associated with most well characterized ICEs. Understanding the function of these cargo genes can reveal important information about the specific ICE, its host, and the environment in which the host normally resides.

A variety of approaches have been used to identify phenotypes conferred by ICEs. In some cases, a phenotype can be inferred from bioinformatic analyses based on homology of cargo gene to genes of known function, and testing for that function (51, 101). High throughput screening of multiple possible phenotypes (phenotype microarrays) has been used to identify phenotypes conferred by ICEs (51, 55). Many ICEs confer multiple, seemingly unrelated phenotypes to their hosts, so the identification of one phenotype conferred by an ICE does not preclude the existence of another.

It is possible that not all ICEs benefit their hosts. Some ICEs have a broad host range, and as such, an ICE that confers a benefit to one host may not confer a benefit to another. In some cases, particular cargo genes may have mutated and become non-functional. Genes associated with mobile genetic elements are more likely to be pseudogenes than are chromosomal genes not associated with mobile genetic elements (91).

Generation of diversity among ICEs

ICEs are largely modular. Genes responsible for related functions, including conjugation, recombination, regulation, and cargo genes, are often grouped together. Conjugation genes are usually the most highly conserved, but the specific gene order can vary between otherwise closely related ICEs due to insertion of other genes (8, 64, 79, 155). Several mechanisms have been described that generate this diversity (Fig. 2).

Tandem insertion and accretion at the cognate *att* site

Composite ICEs can be generated by tandem insertion of an ICE alongside an existing mobile genetic element, followed by transfer of both elements. This occurs when the *att* site normally targeted by an ICE is occupied by an element and the ICE

inserts in one of that element's flanking *att* sites (Fig. 2A). Cis mobilizable elements (CIMEs) are non-conjugative elements that occupy an *att* site used by an ICE (13, 23, 111). When the ICE inserts next to the CIME, the *att* site that the ICE inserts into is duplicated, forming a composite ICE with an internal and external *att* site at one end, either of which may be used during subsequent excision and conjugation events (13, 111).

An ICE can insert next to another ICE that is occupying its normal *att* site (Fig. 2B), forming a tandem array {e.g., ICE*clc* (119) and SXT (27, 70)}. These ICEs may be capable of moving together to a new host. However, if the tandem ICEs carry genes with high sequence similarity, then recombination between the ICEs can occur, removing the intervening sequence and generating a new ICE that inherits sequences from both parents. The SXT/R391 family of ICEs carry homologs of the lambda red genes *bet* and *exo* that enhance RecA-dependent recombination between tandem ICEs, promoting genetic exchange within this family of ICEs (57).

Recombination into an existing ICE

ICEs can accrue other mobile genetic elements within the boundaries of their attachment sites and thereby transfer these elements during conjugation (Fig. 2C). Several ICEs, including ICE*Hin1056* (79), SXT/R391 (17, 72), and ICE*Ec2* (125), carry insertion sequences and-or transposons. Other ICEs, including SXT^{ET} (72), ICE*Ec2* (125), and Tn5253 (6, 76) contain integrons (reviewed in 141). In some cases, a plasmid has integrated, apparently by single crossover, into an ICE, for example Tn5253 (6, 76).

Relaxase-mediated recombination

Some conjugative elements are able to insert into *oriT*-like sequences by a relaxase-mediated recombination event (Fig. 2D). The relaxase of the conjugative plasmid R388 is able to direct recombination of plasmid T-DNA into a cognate *oriT* site in a recipient

(1, 49). Although this activity is not conserved in all conjugative relaxases (1, 35, 49), we suggest that relaxases from some ICEs or plasmids could mediate this type of integration into a cognate *oriT*, either in another mobile element or in a host chromosome.

Imprecise excision

In addition to the mechanisms described above, we suspect that imprecise excision of an ICE could bring along flanking genes, analogous to the imprecise excisions that generate lambda transducing phage (reviewed in 30). Acquisition of flanking sequences could also occur if multiple sequences resembling an attachment site are present beyond the ends of an ICE, for example in the ICE in *Bacillus atrophaeus* (our unpublished observations and 60). Imprecise excision might be more common with ICEs that have promiscuous integration sites (e.g., Tn916 58) rather than a single preferred site. It might also be more prevalent with ICEs that use DDE recombinases (24) that usually have low sequence specificity (see below).

Summary of cargo genes and our view of ICE biology

ICEs were initially discovered because of interest in the genes they carry. Thus, it appeared that most ICEs (at least many of those that have been characterized) contained genes that provide obvious phenotypes and benefits to the host, including antibiotic resistances, the ability to metabolize various compounds, and the ability to colonize various hosts (symbiosis, pathogenesis). However, recent bioinformatic analyses have identified ICEs throughout the prokaryotic domain (66), and there are at least a few functional ICEs that provide no obvious (known) benefit to the host. We postulate that there will be many ICEs with cargo genes that confer previously uncharacterized phenotypes to the host cells, and that these phenotypes are likely advantageous under

conditions normally experienced by the host. These phenotypes might be difficult to identify initially, but could contribute to interactions between ICEs and the host or between ICEs and other mobile genetic elements of a cognate host. Understanding some of these phenotypes will likely provide further insight into the continuing evolution of ICEs and microbes and how cells inhabit particular niches.

MECHANISMS OF ICE FUNCTION

As described above, the two defining features of ICEs are that they integrate into the host genome and that they encode a functional conjugation system that mediates their intercellular transfer. To integrate into the host chromosome, ICEs employ integrases that are homologous to those encoded by phage and some genomic islands. To mediate conjugation, most ICEs encode a type IV secretion system and DNA processing proteins that are homologous to those used by conjugative plasmids. In addition, like certain plasmids, some ICEs undergo autonomous rolling circle replication. This is likely to be a critical property of many ICEs that facilitates maintenance of an ICE in a population of cells (88).

Integration and excision

ICEs integrate into and excise from DNA using an ICE-encoded recombinase. Though, referred to as an integrase, it is needed both for integration and excision. The recombinase is often homologous to phage integrases and, like temperate phage, ICEs frequently insert at a particular attachment site. The chromosomal *att* site for ICEs is often in a tRNA gene. Many ICE integrases are tyrosine recombinases, but some ICEs use serine recombinases or DDE recombinases (for example 24, 47, 65, 147). Tyrosine and serine recombinases catalyze slightly different biochemical reactions, but both have

the same functional consequence for the ICE. These enzymes mediate site-specific recombination between double-stranded DNA molecules at short stretches of similar sequences (reviewed in 62, 114). When these sequences, termed attachment (*att*) sites, are on a circular ICE and the chromosomal target, recombination results in insertion of the ICE flanked by two *att* sites (*attL* and *attR*, on the left and right sides respectively). When recombination is between two *att* sites flanking the ICE, the result is excision of the ICE and reestablishment of the unoccupied chromosomal *att* site. Frequently, in addition to the integrase, an ICE encoded recombination directionality factor, often designated *xis*, is required for excision. This factor influences the direction of recombination mediated by the integrase to favor excision (reviewed in 69).

There is a great deal of variation in the specificity that ICEs have for a particular attachment site. Many ICEs target a single *att* site in the host chromosome that is similar to the *att* site in the ICE. If the normal *att* site is unavailable, some ICEs target alternate sites at lower efficiency (26, 87, 100). Other ICEs have lower specificity for a specific *att* site. For example, CTnDOT inserts at multiple sites that contain a consensus sequence (11, 37). Tn916 insertion is not site specific in most organisms (references in 122).

Recently, ICEs have been identified that integrate into the host chromosome using DDE recombinases. These recombinases are typically associated with transposons, insertion sequences and phage. DDE recombinases employ a variety of recombination mechanisms and frequently do not target a specific site for integration (reviewed in 68). The ICEs TnGBS1, TnGBS2 and ICEA all encode DDE recombinases (24, 65). None of these ICEs integrate at a specific target site, however TnGBS2 inserts upstream of promoter sequences and has a preferential insertion site that it uses more frequently than others (24, 47).

Conjugation and the Type IV secretion system

Like conjugative plasmids, ICEs encode conjugation machinery, at type IV secretion system, for the transfer of DNA to another cell. The mechanism by which type IV secretion systems transfer DNA has been extensively studied in Gram negative bacteria (reviewed in 2, 29, 38). Homologous type IV secretion systems of conjugative elements have been identified in most bacterial phyla (66), indicating that the general mechanism of conjugation is likely to be widely conserved. Some of the details of the transfer mechanism likely vary as the number and composition of proteins within conjugation systems varies widely. There are also different challenges in transferring across the larger cell wall in Gram positive bacteria versus both the inner and outer membranes of Gram negative bacteria, though it is noteworthy that the “minimal” conjugation system of Tn916 successfully negotiates both. We briefly summarize the conserved aspects of type IV secretion systems, including the mating machinery and DNA processing. More extensive descriptions of these features have appeared elsewhere (reviewed in 15, 29, 38, 143, 146).

DNA processing

Plasmids and excised ICEs exist as covalently closed circles of dsDNA and must be processed prior to conjugation. A dsDNA circle is converted to linear ssDNA covalently bound to a relaxase protein, the substrate for conjugative type IV secretion systems. The proteins that perform these functions are homologous for ICEs and plasmids.

During conjugation an ICE-encoded relaxase recognizes its cognate *oriT*. The relaxase then nicks one strand of the ICE DNA and is covalently attached to the 5' terminus to form the T-DNA. Both host and ICE factors assist in unwinding the two DNA strands and an ICE-encoded type IV coupling protein (an ATPase) engages the T-

DNA to the type IV secretion system. The type IV secretion system then enables translocation of the T-DNA into the recipient cell where the DNA is recircularized by the relaxase. The ssDNA is presumably converted to dsDNA, which is eventually integrated into the host chromosome by site-specific recombination. This conversion of circular ssDNA to dsDNA is thought to be the same as second strand synthesis of rolling-circle replication employed by some plasmids and phage (reviewed in 82). Many conjugative plasmids use separate origins for replication and transfer and distinct proteins for processing each origin (77). In contrast, some ICEs use the same origin and DNA processing proteins both for conjugation and to replicate using a rolling-circle replication mechanism (see below).

Autonomous replication of some ICEs

Studies with ICEBs1 provide the most direct evidence that an ICE can replicate autonomously. When ICEBs1 is induced, it excises from the chromosome and the copy number of all ICE genes, but not adjacent chromosomal genes, increases (88). Replication is unidirectional, starts at *oriT* and requires the conjugative relaxase, NickK, the ICE encoded helicase processivity factor Help, the chromosomally encoded helicase PcrA, PolC, and the β -clamp (DnaN) (88, 140). Replacement synthesis of the unwound (leading) strand is presumably primed from the 3'-OH terminus created by the nicking event. The leading strand is presumed to be recircularized by NickK, by analogy to other systems (reviewed in 82). Synthesis of the complementary, or lagging strand is primed by a single strand origin (*ssO*) (158). The nicking and unwinding of DNA for rolling circle replication is very similar to that needed to transfer ICE ssDNA during conjugation (Fig. 3) (reviewed in 36).

ICEMISym^{R7A} of *Mesorhizobium loti* R7A also replicates using its conjugative relaxase. When ICEMISym^{R7A} is genetically induced, the element excises and is maintained in a host population at a copy number of 1.5. If the conjugative relaxase is deleted the element is lost from the population (116).

Some of the earliest evidence for autonomous replication of an ICE comes from *Haemophilus influenzae*. ICEHin1056, and related elements, integrate into a host tRNA^{Leu} gene (46). These elements were originally described as a family of antibiotic resistance vectors that were integrated in the chromosome of the donor, but could be recovered as plasmids from outgrown transconjugants (45, 136). This implies that these elements are maintained as replicative plasmids in the recipient for some generations before integrating into the chromosome.

There are additional elements in which replication can be inferred because the copy number of the circular form of the ICE is greater than the copy number of the chromosomal attachment site. The ICE RD2 of Group A *Streptococcus* exists as both an integrated and a plasmid form in stationary-phase cultures. Treating the host with the DNA damaging agent mitomycin C increases the copy number of the plasmid form (132). ICESt3 of *Streptococcus thermophilus* also excises from the host chromosome and exists as a multicopy plasmid following induction with mitomycin C (31). Additionally, SXT of *V. cholerae* may also be capable of replication under certain situations (26).

There are also ICEs that replicate via dedicated replication systems, independent from the conjugative relaxase. TnGBS1 and TnGBS2 of *Streptococcus agalactiae* encode a replication initiation protein and a conjugative relaxase. Loss of the replication initiation protein reduces copy number whereas loss of the conjugative relaxase does not (24, 64). Additionally, after conjugation, circular forms of TnGBS1 and TnGBS2 can be isolated from outgrown transconjugants. In the majority of cases, the attachment site in the ICE

matches that of the donor, and does not match a potential integration site in the recipient, indicating that the circular forms result from replication, rather than integration and subsequent excision in the recipient (64).

ICEs can mobilize non-conjugative elements

Non-conjugative mobile genetic elements can use the conjugation machinery encoded by an ICE or a conjugative plasmid to transfer to new hosts, a phenomenon known as mobilization. Mobilizable elements can exist as freely replicating plasmids, or as chromosomally integrated genomic islands (mobilizable genomic islands) that excise prior to mobilization.

ICEs can mobilize both plasmids and genomic islands. CTnDOT/ERL (127), Tn916 (106, 145), SXT (73), and ICEBs1 (90) have all been shown to mobilize plasmids. Additionally, the CTnDOT/ERL family of ICEs can mobilize genomic islands known as nonreplicating *Bacteroides* Units (NBUs) (129, 135) and the SXT/R391 family of ICEs has recently been shown to mobilize genomic islands of *Vibrio* species and related organisms (42-44). In both cases, these genomic islands encode their own integrase, but excision is regulated by the mobilizing ICE and transfer depends on the ICE conjugation machinery. Once in a new host these mobilizable genomic islands are capable of independently integrating into the chromosome.

Plasmid mobilization by ICEBs1 blurs the distinction between conjugative and replicative relaxases

Relaxases are frequently classified as conjugative or replicative, depending on whether they are required for conjugation or replication. Sequence analysis has revealed that these relaxases are related, but appear to be in distinct families (63, 77).

However, this distinction has become blurred as evidenced by the ability of some relaxases from plasmids and ICEs in Firmicutes to function in both replication and conjugation. For example, the replicative relaxase of the Staphylococcal plasmid pC194 and the conjugative relaxase of ICEBs1 are both bi-functional. That is, they can act as both replicative and conjugative relaxases (88, 89). Both ICEBs1 and Tn916 are capable of mobilizing pC194 (90, 106). In the case of ICEBs1 this requires the pC194 replication protein, which does not resemble a conjugative relaxase (90, 133). The sequences of the conjugative relaxases of ICEBs1 (Nick) and Tn916 (Orf20) more closely resemble replicative relaxases than other conjugative relaxases (56, 89). The conjugative relaxase of ICEBs1 supports replication of the extrachromosomal form of this ICE, as previously discussed (88). These examples blur the distinction between conjugative and replicative relaxases and raise the possibility that replicative relaxases may also serve as conjugative relaxases when recognized by the correct type IV secretion system. These findings also blur the distinction between ICEs and rolling circle replicating plasmids.

Lagging strand synthesis

During conjugation, ssDNA is transferred into the recipient. Once there, the ICE must either function as ssDNA or convert to dsDNA. ICEBs1 of *Bacillus subtilis* possesses a single-strand origin of replication (*ssO*), located on the transferred strand, enabling its conversion from ssDNA to dsDNA (158). *ssOs* are DNA sequences that form a particular secondary structure when single stranded. This structure mimics other DNA elements to recruit host factors that synthesize a short RNA to prime DNA synthesis. The *ssO* of ICEBs1 is homologous to the *ssOs* of plasmids of Gram positive bacteria that replicate by a rolling circle mechanism. These *ssOs* fold into a structure recognized as a promoter by RNA polymerase, which then synthesizes a leader RNA

that is used to prime DNA synthesis (reviewed in 82). Other *ssos* use the primase DnaG for synthesis of the RNA (84, 152). Once a primer is generated at the *ss0*, the host replication machinery is recruited to complete synthesis of the second strand.

Plasmids are known to prime complementary strand synthesis after conjugation. The RCR plasmid pMV158 is mobilized by conjugative elements and contains two *ssos* that function in different recipient species (92). In addition, several plasmids in Gram negative bacteria encode primase proteins, which directly polymerize RNA synthesis on the T-DNA, either through recognition of a cognate *oriV* (75) or via a general mechanism that functions on ssDNA (153).

Summary of ICE replication

We postulate that many (perhaps most or all) ICEs undergo autonomous rolling circle replication. This view is based on the conserved nature of ICE-encoded relaxases, the known roles of some of these relaxases in conjugation and replication, and the role of replication in the stability of ICEs in a population of cells. We suspect that it has been difficult to detect autonomous replication of most ICEs due to the low frequencies of induction and excision.

REGULATION OF ICE ACTIVATION

Normally, ICEs are maintained as quiescent elements in the host chromosome. The excision and conjugation genes are not expressed, usually due to active repression of transcription. Constitutive expression of conjugation genes has been found to be detrimental to the host and maintenance of the ICE, providing selective pressure for repression of these genes. Under certain conditions, the ICE can become induced, alleviating repression and permitting expression of ICE genes, leading to excision of the

ICE and the potential for conjugation. Even under inducing conditions, most ICEs appear to excise from the chromosome of a relatively small subpopulation of cells (95, 102). The signals that induce ICE gene expression and the mechanisms of repression are not universally conserved, but there are some common themes.

Pressures against ICE activation

Expression of conjugation genes is likely to be maladaptive under most circumstances for both the host and the ICE. In general, expression of the genes likely creates a metabolic burden on the host, diverting cellular resources away from essential processes. It has been shown that constitutive activation of SXT, either by deletion of the CI-like repressor, SetR, or overexpression of the transcriptional activators SetC and SetD is deleterious to the host (9, 10). This, however, may be due to loss of the element and subsequent activation of a toxin-antitoxin system carried by SXT (157). For its part, an excised ICE can no longer rely on chromosomal replication to ensure vertical inheritance. Even ICEs that replicate are lost from a population if they are constitutively activated (5, 116). In addition to these general considerations, ICE activation can be deleterious to a host for specific reasons. In the case of Gram-negative conjugation systems, expression of a mating pilus may make the host susceptible to male-specific phage. In other cases, induction of the ICE results in host death. Activation of ICE*clc* causes a portion of the cells with the element to differentiate into potential donors that have reduced growth kinetics and eventually lyse (120). In the absence of its preferred attachment site, ICE*Bs1* will integrate into secondary sites within the host chromosome. If the ICE cannot efficiently excise from these secondary sites, induction of the ICE kills the host in a manner dependent on activity of the relaxase (100).

ICEs are induced by a variety of signals

ICEs are induced in response to a wide variety of signals. The inducing signals vary for each ICE, but there are common stimuli known to activate multiple ICEs. These include: induction of the cellular SOS response to DNA damage; secreted signaling molecules from potential recipients; the growth phase of the host, and mechanisms tied to selective advantages conferred by the cargo genes in an ICE. Some ICEs are known to respond to more than one cue.

Some of the signals that induce ICEs activate other mobile genetic elements. The SOS response to DNA damage induces many lysogenic phages to enter the lytic cycle (41, 109). Cell-cell signaling regulates conjugation of some plasmids (50, 113).

Induction during the SOS response

DNA damaging agents cause induction of the *recA*-dependent SOS response in host cells and also induce several ICEs (5, 10, 12, 19, 132). During the SOS response, DNA damage generates ssDNA. This is bound by and activates RecA, which causes auto-cleavage of the LexA repressor, the phage lambda repressor CI, and related repressors (reviewed in 28, 41, 109).

SXT, ICEBs1, and ICESt3 all contain genes homologous to phage repressors and these ICEs are induced by the SOS response (4, 5, 10, 12, 26). This induction is known to be RecA-dependent for SXT and ICEBs1. Whereas canonical CI-like repressors (LexA, CI) mediate their own cleavage, the ICEBs1 repressor, ImmR, is cleaved by a separate protease, ImmA (19). *immR* and *immA* are linked in ICEBs1 and are immediately upstream of the gene for the site-specific recombinase *int*. In addition to ICEBs1, this gene arrangement and protease-mediated cleavage of a repressor appears to be a common property of many phages (19).

Induction of ICEs by the SOS response likely indicates that the host is facing a potentially lethal challenge and that the ICE must rely on horizontal, rather than vertical transmission in order to propagate. Alternately, induction via the SOS response may be a way of maintaining a low level of horizontal transmission without incurring an undue metabolic burden on the population as a whole. The SOS response is generally activated in a small subset of growing cells (81, 112), usually in response to replication fork stress (39, 112). Induction of ICE within these cells would ensure that a small portion of an ICE-bearing population is primed to act as donor cells at any given time.

Control by cell-cell signaling

Some ICEs respond to cell-cell signaling, becoming induced when the population density of potential recipients is high. The use of quorum-sensing pathways can allow ICEs to activate expression of conjugation genes when the density of related cells is high, indicating the presence of potential mating partners. In some cases, multiple signals are used to indicate the presence of potential recipients and whether or not the recipients already contain a copy of the cognate ICE, allowing an additional level of control such that conjugation is induced only when it is likely to result in horizontal transmission to a naïve recipient, rather than a cell with an established ICE.

The *Mesorhizobium loti* symbiosis island, ICE*Mlsym*^{R7A} is also induced by quorum signals. It specifies the production of at least one and possibly two acyl homoserine lactones (AHLs), as well as a transcription factor, TraR, that drives expression of genes needed for conjugation (115, 116). TraR is activated by several different AHLs, likely enabling this element to respond to a variety of potential recipients.

ICE*Bs1* of *B. subtilis* is controlled by cell-cell signaling in two ways (Fig. 4). First, secreted peptides produced and sensed by *B. subtilis* strains indicate the presence of

neighboring cells, likely as an indicator of the presence of potential mating partners. These peptides stimulate transcription of *rapI*, leading to activation of *ICEBs1*. RapI activates *ICEBs1* gene expression by stimulating the ImmA-mediated proteolysis of the *ICEBs1* repressor ImmR (4, 5, 19). Second, *ICEBs1*-containing cells produce an additional secreted peptide that inhibits activation of *ICEBs1* gene expression. This inhibition occurs when the peptide PhrI is imported and inhibits the activity of RapI. The *ICEBs1* encoded peptide PhrI signals that the surrounding cells already have a copy of *ICEBs1* and serves to limit activation and potential loss of the element. In addition to this peptide-mediated mechanism of limiting activation of *ICEBs1*, there are other mechanisms for limiting acquisition of *ICEBs1* by cells that already have a copy (e.g., 4).

Induction during stationary phase

Several ICEs are induced during stationary phase. In *ICE_{clc}* expression of genes that regulate conjugation is driven by the host-encoded stationary phase sigma factor RpoS (104). *ICEMISym^{R7A}* preferentially excises during stationary phase, likely in response to the growth phase of the host in a manner independent of cell-cell signaling. Deletion of *TraR*, the ICE-encoded transcription factor that responds to acyl-homoserine lactones has a negligible effect on ICE excision, although conjugation is reduced (115). *ICE_{St3}* is also activated during stationary phase as evidenced by an increase in excision from the chromosome and expression of conjugation genes (31). Induction of an ICE during stationary phase does not necessarily lead to conjugation during stationary phase, but could indicate that the ICE is primed to transfer once nutrients become available, as with *ICE_{clc}*. Stationary phase may also serve as an indicator that the host is poorly adapted to grow under current conditions. In this case, it might be beneficial for the ICE to move to a different host rather than relying on vertical inheritance in the starving stressed host.

Phenotype-dependent induction

In several instances, the induction of an ICE is tied to a selective advantage that the ICE provides to the host. Tn916 was originally identified because it confers tetracycline resistance to its hosts (52). Tetracycline induces conjugation of Tn916 and other related elements (48, 130). This induction is initially regulated at the level of transcriptional attenuation. Under non-inducing conditions, most of the transcripts that initiate at the promoter for *tetM* (Ptet) in Tn916 terminate at a factor-independent terminator upstream of *tetM* (the gene for tetracycline resistance) and other genes. Following exposure to tetracycline, transcription extends past this terminator, into *tetM* and downstream regulatory genes. Full expression of the conjugation genes occurs when Tn916 circularizes upon excision from the chromosome (Fig. 5 and 34, 137).

Tetracycline also induces conjugation of the CTnDOT/ERL family of ICEs from *Bacteroides* sp., which confer tetracycline resistance to their hosts. In this case, induction is regulated at the level of translation initiation, rather than the level of transcriptional attenuation. Transcription of a *tetQ* is constitutive, however, the ribosome binding site is inaccessible because of the secondary structure of the mRNA. Tetracycline causes ribosomes to stall on a leader peptide, also encoded on the *tetQ* mRNA. The paused ribosomes change the mRNA secondary structure so that the *tetQ* ribosome binding site is accessible, allowing translation of TetQ and the downstream regulators RteA and RteB (148, 149). This initiates a regulatory cascade leading to excision and conjugation of CTnDOT (reviewed in 150).

Other ICE-encoded phenotypes can also serve as activation signals. ICE*clc* enables *Pseudomonas* sp. to metabolize chlorocatechols, permitting some species to use 3-chlorobenzoate as their sole carbon source. Growth of ICE*clc* donors on 3-chlorobenzoate enhances expression of conjugation genes and conjugation of ICE*clc*

(102, 126). The advantage of linking conjugation to an adaptive phenotype conferred by the ICE is two-fold. Any bacterium that acquires the ICE and can benefit from the phenotype immediately gains a selective advantage over its peers, promoting vertical transmission of the ICE. Additionally, any metabolic burden on the donor due to the expression of conjugation genes may be offset by the ability to exploit a distinct niche made available by the ICE.

Induction upon entry into a new host

Some ICEs are active immediately upon entering a recipient cell. When *ICEBs1* is introduced into a new recipient, it is able to spread rapidly to other cells in a manner that requires the conjugation machinery (7). This activity does not require *rapI*, which is needed for activation of *ICEBs1* in response to cell density. Similarly, if *Tn916* is delivered to a cell on a conjugative plasmid, it frequently transposes to a new location before becoming quiescent (58, 59). The initial burst of activity seen in these ICEs is likely due to a lack of repression. When the ICE enters a recipient cell, the ICE-encoded regulators that repress gene activity are absent. It is only after the ICE has expressed these regulatory genes that repression of ICE functions is achieved.

CHALLENGES TO THE FIELD

Although there has been a tremendous increase in our knowledge of ICE biology since the description of *Tn916* (52), there is still a tremendous amount to learn. One area of intense interest is the nature of the mating machinery and how a protein attached to ssDNA is transferred out of the donor cell and into the recipient. Many components of the type IV secretion system encoded by ICEs and conjugative plasmids are conserved across the bacterial domain. Recent biochemical and structural advances (e.g. 33, 53, 54,

93) have improved our understanding of this macromolecular machine, but a thorough understanding of its structure and function remain elusive.

It is not known if there is a specific cue that signals the mating machinery to export the T-DNA. There is reason to believe that such a cue exists and that conjugation systems do not pump DNA into the environment when no recipient is present. Studies of conjugative plasmid R1 indicate that the mating channel is gated and that communication occurs between the inside and outside. Entry of the R17 phage via the conjugation channel requires that a T-DNA and type IV coupling protein be docked at the inner opening of the channel (85).

It is becoming evident that there is not a clear distinction between conjugative and replicative relaxases in Firmicutes. The distinction between conjugative plasmids and ICEs is also becoming blurred as several ICEs appear to undergo plasmid-like rolling circle replication. Further studies are needed to identify and characterize interactions between relaxases and conjugation systems of Gram positive bacteria. In particular, investigation into the role of type IV coupling proteins in determining the range of relaxases that can be recognized might prove fruitful.

Host genes, both essential and non-essential, are likely involved in every step of the ICE life cycle. Some of these genes, such as IHF (99) and components of the host replication machinery (88, 140) have been identified and their roles are understood to varying degrees. However, the contribution of other host genes remains to be elucidated (78) and it is likely that other, unidentified, genes are also involved in conjugation.

Similarly, the relationship between the host range of an ICE and the genetic content of the permissive (or non-permissive) hosts is not well understood. Some ICEs, such as Tn916, have a very broad host range, whereas others are more restricted. Host range

could be limited by incompatibility between a given type IV secretion (conjugation) system with a particular recipient's cell envelope. Host range could also be restricted by cytoplasmic factors, for example, restriction/modification or CRISPR systems, an inability of a specific ICE to replicate or integrate in some hosts, or incompatibility with other resident mobile genetic elements.

A mechanistic understanding of what happens to an ICE once it enters a recipient has not been thoroughly developed. It is generally assumed that the ICE must generate a complementary strand to the T-DNA prior to integrating into the chromosome. For ICEs that replicate prior to integration this is certainly the case, and this is also likely for those that encode a mechanism for generating a complementary strand. However, it is not clear that such a requirement exists for all ICEs. Some integrases of the tyrosine-recombinase family can insert single-stranded DNA elements into the chromosome, provided the DNA forms a double-stranded structure at the attachment site (20, 144). The integrase itself might be expressed from a single-stranded promoter on the T-DNA, or transferred from the donor to the recipient, as is thought to be the case for Tn916 (22). Alternately, the relaxase could directly mediate integration of the covalently bound T-DNA into the chromosome, though this would require that the chromosome already harbor a cognate *oriT* (49).

ICEs have tremendous potential to be developed as tools for genetic engineering. Conjugation can be used to deliver DNA to organisms from all domains of life, provided there is a match between the conjugation system and the recipient. ICEs have the added benefit, over plasmids, of being able to insert into a host chromosome, provided the integration system functions in the recipient. This avoids the need for replication of and selection for the element to ensure maintenance, and inherent variability in copy number of most plasmids. In the past, conjugative delivery of Tn916

has been used to mutagenize a variety of bacteria including Gram positives (151), Gram negatives (74) and the wall-less Mollicutes (123) and to mobilize other elements. In the future, ICEs could allow the delivery of specific genes or metabolic pathways to an incredibly diverse array of organisms.

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Sidebars:

BOX 1. Typeset near introduction

Types of horizontal gene transfer used by bacteria

- Transformation: The direct uptake of DNA from the environment and its incorporation into an organism's genome.
- Transduction: Phage-mediated transfer of DNA from one host to another. The DNA is generally genomic DNA from the original host of the phage that was packaged into the phage head instead of or in addition to phage DNA.
- Conjugation: Contact-dependent, unidirectional transfer of DNA from one host to another mediated by the mating pore of a conjugative element.
- Fusion: Acquisition of DNA via fusion with a DNA-containing outer membrane vesicle or with another cell (protoplast fusion)

BOX 2. Typeset near introduction

General types of mobile genetic elements that can move within or between cells.

Individual elements may have characteristics of more than one type of element.

- Transposable elements: Including transposons and insertion sequences with many different subtypes. Capable "moving" to different DNA sites with varying degrees of site-selectivity. Some excise from original site and insert into new site (cut and paste) whereas others use replicative mechanisms to create a copy at a new site (reviewed in 40, 131).
- Phages/ viruses: Mobile between cells. Viral nucleic acid contained in proteinaceous particles. Many kill the host during growth.
- Conjugative plasmids: Mobile between cells using conjugation machinery. Require cell-cell contact for transfer. Replicate independently of the host chromosome.

- ICEs: Mobile between cells using conjugation machinery. Able to integrate into DNA sites via site-specific recombination. Some are also mobile within cells.
- Many types of elements are not themselves mobile, but can be mobilized by one or another of the mobile elements.

BOX 3. Typeset near Generation of Diversity among ICEs

Diversity of ICEs.

- Size range: about 18 kb (Tn916) to over 500 kb (ICE*MISym*^{R7A})
- Some phenotypes conferred by ICEs: antibiotic resistance(s) (Tn916; SXT; CTnDOT and many others); heavy metal resistance (R391); carbon-source utilization (ICE*clc*, *bph-sal*, CTn*Scr94*, Tn5276); symbiosis (ICE*MISym*^{R7A}); pathogenesis (PAPI-1); restriction-modification (ICESt3), bacteriocin synthesis (Tn5276) and biofilm formation (PAPI-1).

Figure legends

Figure 1. The ICE life cycle. A model of ICE conjugation is shown and is described in the text. The bacterium bearing the ICE (donor) is shown in gray, the bacterium acquiring the ICE (recipient) is shown in green.

Figure 2. Mechanisms that generate diversity between ICEs. Specific mechanisms are described in the text. Double black lines represent the host chromosome whereas double red, orange and blue lines represent mobile genetic elements as indicated. Double green lines indicate regions of homology. Rectangles behind double lines indicate *att* sites or insertion sites. (A,B) An ICE (blue lines) can insert in tandem next to a heterologous mobile element (A) or an ICE (B) occupying the ICEs preferred *att* site (purple boxes). (C,D) Other mobile genetic elements can insert into an ICE. C. Elements can transposase from another chromosomal location (Ci) or extrachromosomal elements can recombine into an ICE (Cii) either by site-specific recombination or homologous recombination. D. T-DNA from an external element might recombine into a cognate *oriT* site on the ICE. Insertion is more efficient when the *oriT* is located on the lagging strand, likely indicating that the target is ssDNA, as shown. The relaxase is bound to the transferred DNA at one catalytic tyrosine residue. The relaxase nicks and binds the *oriT* of the resident element with a second catalytic tyrosine residue (both catalytic tyrosine residues are required for this activity (1)). The relaxase also joins the free 3'-OH end of the chromosomal nick to the 5' end of the T-DNA and the 3'-OH end of the T-DNA to the 5' end of the chromosomal nick. The insertion is then fixed by replication.

Figure 3. Conjugation resembles rolling-circle replication. Both processes require similar initial steps that generate a substrate that may be suitable to act as a T-DNA during conjugation. The ICE is shown as double blue lines. In both processes a relaxase recognizes and nicks a cognate *ori*, binding to the free 5' end. An additional helicase activity and single-strand binding protein is required to unwind the single-stranded DNA. Replacement synthesis of the unwound strand can occur, but is not required for unwinding. If replacement synthesis occurs, a second nicking event at the reconstituted *ori* is likely required in order to generate a free 3'-OH group for recircularization of the unwound strand.

Figure 4. Cell-cell signaling induces *ICEBs1*. The pathway by which cell-cell signaling regulates *ICEBs1* gene expression is shown. Arrows indicate positive regulatory effects. Lines with bars indicate negative regulatory effects. Proteins and peptides are shown with a brief explanation of their role or activity. The PhrI signaling peptide is likely either a pentapeptide (5) or a hexapeptide (103) with sequence (A)DRVGA.

Figure 5. Excision of *Tn916* allows expression of conjugation genes. Both linear and circular maps of *Tn916* are shown. Genes are shown as arrows on the map. Some known promoters are shown as bent arrows. While the element is integrated into the chromosome, low levels of transcription (red arrows) occur within the regulatory region (white arrows), but not within conjugation genes (black arrows), such as the relaxase encoded by *orf20*. Excision and circularization of *Tn916* makes the conjugation operon contiguous and codirectional with the regulatory region, allowing conjugation genes to be transcribed. The red arrows are meant to demonstrate this phenomenon and do not depict the known variety or relative abundance of transcripts produced by *Tn916*.

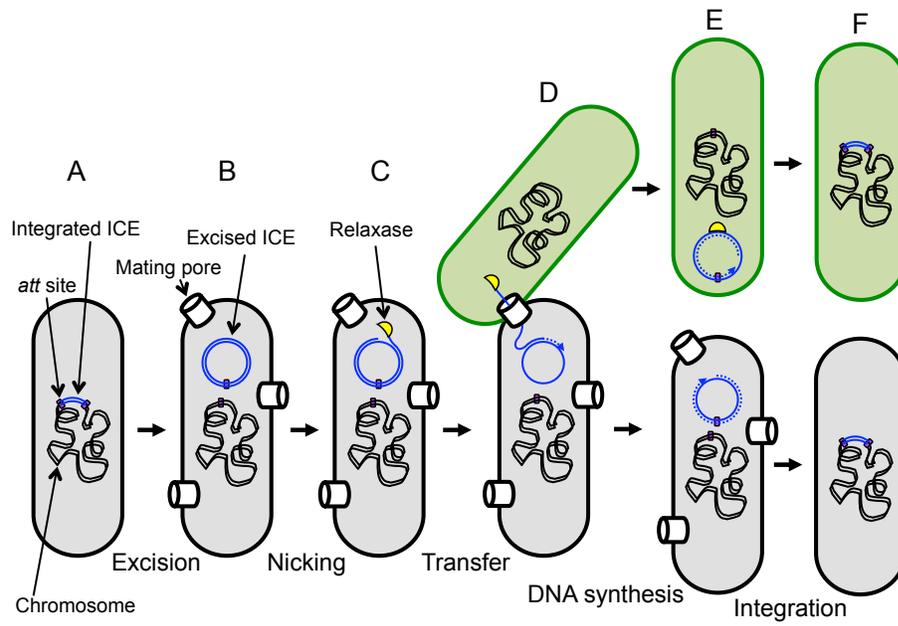


Figure 1

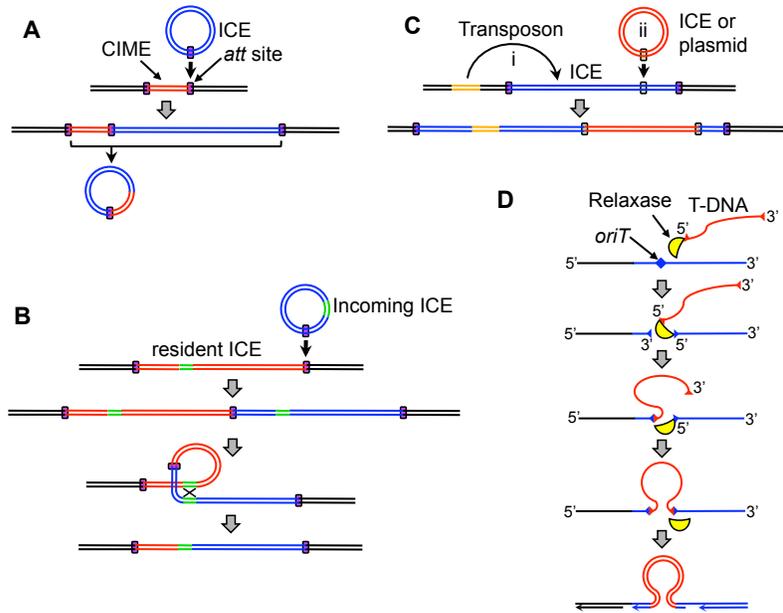


Figure 2

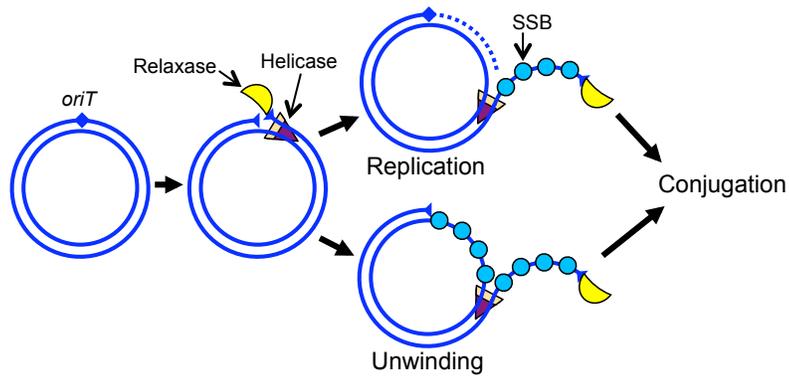


Figure 3

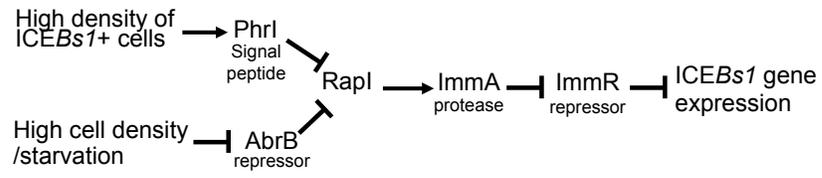


Figure 4

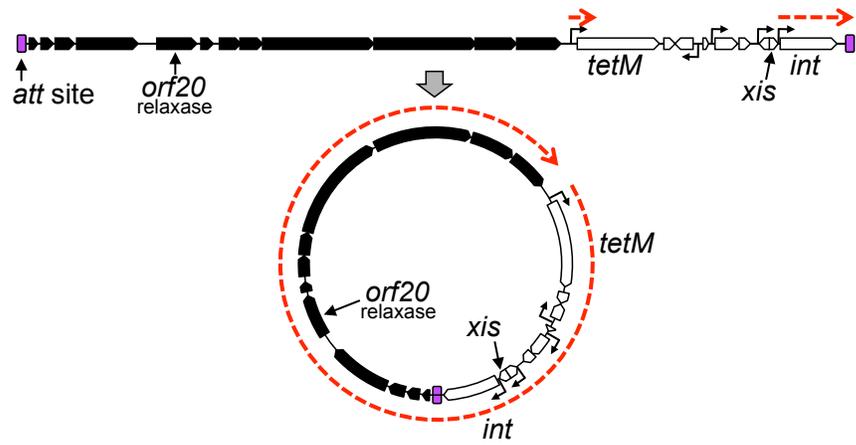


Figure 5