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Review
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Regulation of bacterial conjugation: balancing opportunity with adversity

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Conjugative plasmids are involved in the dissemination of important traits such as antibiotic resistance, virulence determinants and metabolic pathways involved in adapting to environmental niches, a process termed horizontal or lateral gene transfer. Conjugation is the process of transferring DNA from a donor to a recipient cell with the establishment of the incoming DNA and its cargo of genetic traits within the transconjugant. Conjugation is mediated by self-transmissible plasmids as well as phage-like sequences that have been integrated into the bacterial chromosome, such as integrative and conjugative elements (ICEs) that now include conjugative transposons. Both conjugative plasmids and ICEs can mediate the transfer of mobilizable elements by sharing their conjugative machinery. Conjugation can either be induced, usually by small molecules or peptides or by excision of the ICE from the host chromosome, or it can be tightly regulated by plasmid- and host-encoded factors. The transfer potential of these transfer regions depends on the integration of many signals in response to environmental and physiological cues. This review will focus on the mechanisms that influence transfer potential in these systems, particularly those of the IncF incompatibility group.

Bacterial conjugation, transduction and transformation/competence are the major mechanisms for lateral gene transfer (LGT)/horizontal gene transfer (HGT) in the microbial world. Whereas transduction and transformation depend on the recipient to participate in the process via homologous recombination, conjugation is a function of the donor cell, which identifies a suitable recipient cell and transports the DNA into it in an active, donor-dependent manner. A wide range of recipients is possible, including those of other genera or kingdoms [1,2]. A single donor cell can convert a population of recipient cells to donor cell status via the process of epidemic spread, which is a feature of conjugation alone. The effectiveness of these mechanisms, particularly conjugation, in disseminating traits such as antibiotic resistance is remarkable, with resistance to newly available antibiotics appearing within months of their introduction [3].

In general, conjugative elements, either plasmidic or integrative in nature, accumulate antibiotic and heavy-metal resistance as well as many other traits on smaller mobile elements such as integrons, insertion sequences, integrative phage-based elements and transposons (reviewed in [4]). These elements, which are also capable of intracellular movement, are then introduced into a new host via conjugative transfer. It is now recognized that maintenance of the conjugative element in the new host/recipient cell is not essential, and that the cargo of genes can be incorporated into the recipient cell genome by homologous recombination, transposition or integration. Thus, while we can easily observe the evidence for lateral gene transfer, it has been more difficult to monitor it happening in nature.

A key feature of conjugative elements is the expression of the genes for transfer. Transfer systems utilize type IV secretion systems, which form the transferosome, to transport the DNA between cells (Figure 1) [5]. A nucleoprotein complex called the relaxosome consists of the relaxase, which makes a single-stranded break ('nicks') the DNA at *nic* within the origin of transfer (*oriT*), as well as auxiliary proteins bound to adjacent sites. The relaxosome is then linked to the transferosome via the coupling protein. Mobilizable elements encode an *oriT* region and the components of the relaxosome but use the transferosome and coupling protein (usually) to effect their transfer [6]. Plasmids can be defined as broad or narrow host range depending on their replication systems with the transfer and replication regions coevolving, suggesting that they are coregulated. The sequencing of many bacterial genomes has revealed the presence of mobile elements that are related to prophages in that they are capable of

Keywords
■ alternate σ factors ■ conjugation ■ extracytoplasmic stress ■ horizontal gene transfer ■ lateral gene transfer ■ plasmid ■ silencing ■ transfer region

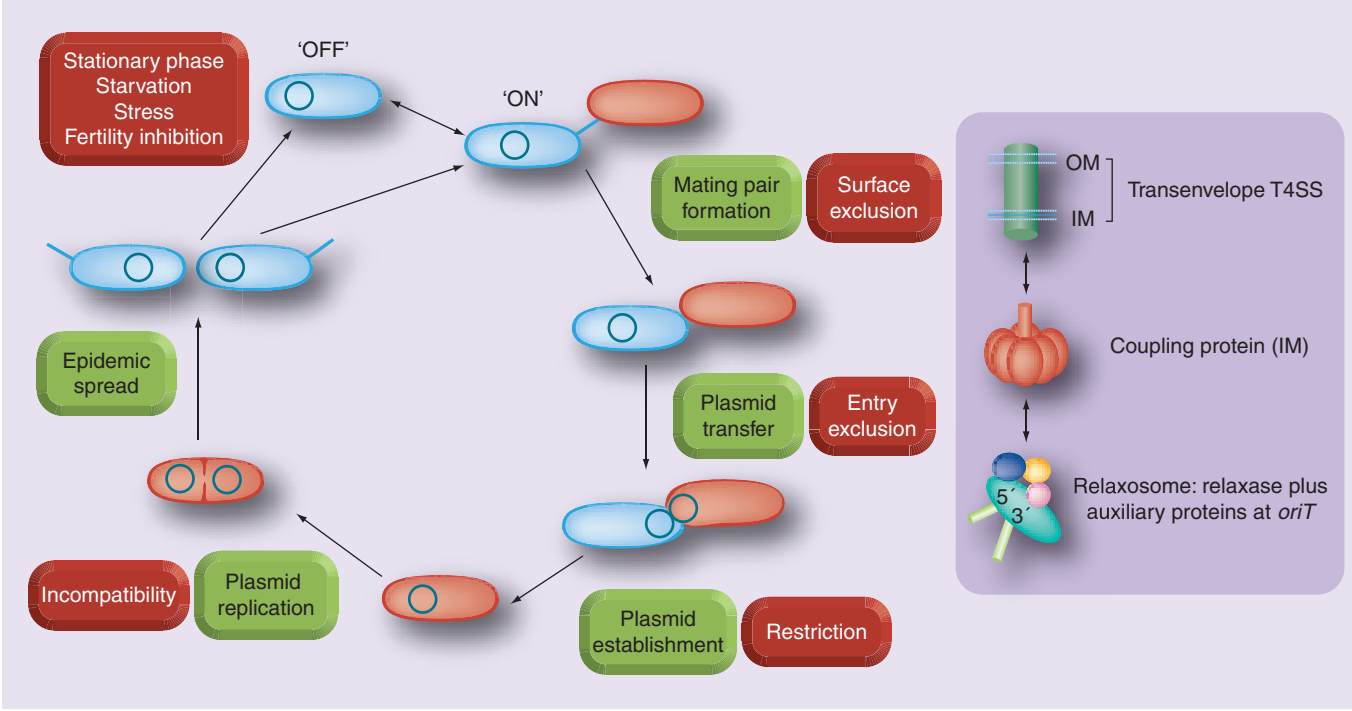

Figure 1. Diagrams of the main steps and components in the conjugation process in Gram-negative bacteria. The inset shows the main components of the transfer apparatus. All conjugative systems require a coupling protein, an ATPase that energizes transfer. Most Gram-negative and -positive conjugative systems require a T4SS, which forms the conjugative pore, and a relaxosome composed of the relaxase, which nicks at *oriT* and other auxiliary proteins [6]. Gram-negative systems, but not Gram-positive ones, produce an extracellular appendage called a pilus, which identifies a suitable recipient cell. Some systems, such as in *Streptomyces*, require only a coupling protein for DNA transfer. Main diagram: the donor cell in blue binds the recipient cell in red via the pilus, which retracts and brings the cells together. A signal is transmitted through the T4SS to the coupling protein that then attracts the relaxosome to the conjugative pore. A single strand of DNA, covalently linked to the relaxase, is transported into the recipient cell, followed by the establishment and replication of the plasmid, or in the case of an integrating conjugative element, incorporation into the recipient chromosome. Recipient cells that are newly converted to donor cell status exhibit high frequency of transfer, which can lead to epidemic spread. Many control elements alter the level of transfer gene expression in the donor cell or interfere with the transfer and establishment of the plasmid in the recipient. The major negative influences are indicated in red boxes whereas the steps in conjugation and establishment are indicated in green boxes.
IM: Inner membrane; OM: Outer membrane.

excision and integration, but, unlike prophages, they are not thought to be capable of autonomous replication (discussed later). Many of these elements, called integrating conjugative elements (ICEs), encode transfer regions that enable them to conjugate into a wide variety of recipients. ICEs now include the previously designated conjugative transposons [7].

One would expect that conjugative elements should maximize transfer gene expression, only in the presence of recipient cells and that this should be downregulated once a recipient cell population has been converted to donor status. Based on the detailed examination of a few paradigms for conjugation, this appears to be essentially correct. Transfer gene expression is either induced in response to specific signals produced by recipient cells or it is tightly controlled in a complex manner by factors encoded by the host or mobile element. In the latter case, transfer gene expression does not depend on the presence of recipient cells or signaling molecules produced by them, resulting in a varying fraction of the donor cell population that express the transfer genes.

Induced systems are exemplified by the well-studied systems in Gram-positive *Enterococcus*, which use a pheromone detection system to trigger expression of the transfer genes prior to conjugative DNA transfer with the pheromone being released by the recipient cell [8,9]. In the Ti plasmid of *Agrobacterium tumefaciens*, signaling compounds trigger expression of the virulence genes for T-DNA transfer into a wounded plant cell during crown gall tumor formation [10] whereas a quorum sensing mechanism is used to upregulate gene expression of the transfer region that promotes Ti plasmid transfer or RSF1010 plasmid mobilization between bacterial cells [11]. Quorum sensing is also important in the regulation of conjugation in *Yersinia* [12].

Integrative and conjugative elements regulate their transfer using mechanisms that resemble cI repression for maintaining the lysogenic state in lambdoid prophages. Transfer potential is increased when environmental and physiological signals promote excision and recircularization of the ICE with the release from cI-like repression and immunity that is characteristic of these prophages [13,14]. Recircularization allows expression of the transfer genes, which leads to nicking in a strand- and site-specific manner, as is characteristic of plasmidic transfer systems [6]. In *Bacillus subtilis*, ICEBs1 is capable of autonomous replication and depends on the relaxase NicK and *oriT* to excise the ICEBs1 element and initiate replication [15]. Other systems, such as the conjugative transposon CTnDot – an ICE – upregulate transfer gene expression in response to the presence of antibiotics such as tetracycline rather than the presence of recipient cells [16]. In the *Actinomycetes*, transfer ability is linked to the hyphal stage of growth. A single essential transfer protein, similar to a coupling protein and homologous to SpoIIIE and FtsK, contributes to plasmid partitioning and maintenance as well as spread throughout the hyphae prior to sporulation [17].

Plasmids of the well-studied Gram-negative systems in the incompatibility groups IncF, -H, -I, -P and -W limit their transfer potential by employing both transcriptional and post-transcriptional mechanisms to ensure that transfer gene expression is not an undue burden on the host cell. These systems are in 'standby mode' and their transfer potential is the readout of the sum of the responses to environmental and physiological signals as well as the presence of recipient cells. Whereas detailed analyses of many transfer systems are unavailable, the regulatory networks controlling transfer gene expression in the IncF and IncP-1 groups are approaching the level of understanding needed for the predictive methods used in system biology. This review will concentrate on these systems and the exceptionally diverse array of mechanisms used to control their expression.

IncF plasmids: integrating plasmid- & host-encoded control mechanisms

The transfer regions encoded by IncF plasmids have been the subject of much study both in terms of their complex T4SS, their mechanism of DNA processing and transfer, as well as the regulation of their transfer (*tra*) operons. The F plasmid itself has a derepressed transfer region and is capable of very efficient mating (nearly 100%), whereas other F-like plasmids isolated from natural environments have a repressed phenotype whereby mating efficiency is reduced 10–1000-fold. Usually, derepressed mutants of these plasmids, which mate with efficiencies comparable to F, can be isolated relatively easily. This has greatly facilitated the study of these systems and has allowed us to build a relatively complex picture of the network of positive and negative signals that result in the observed mating ability (Figure 2).

Organization of the F transfer control region

IncF plasmids, including F, R1, R100, pRK100 and pSLT, encode more than 30 kb transfer regions, with two monocistronic operons encoding *traM* and *traJ* followed by the long *tra* operon *traY–X* . The *traM* gene is immediately adjacent to the origin of transfer (*oriT*) containing the *nic* site [6]. TraM is essential for DNA processing in preparation for transfer and also autoregulates its own promoters, P_m1 and P_m2, collectively known as P_m. This is followed by *traJ*, which encodes the main activator of *tra* gene expression and is expressed from its own promoter P_j (Figure 2). The multicistronic *tra* operon (*traY–traX*) encodes over 30 genes for DNA metabolism, T4SS and regulation of transfer potential. The first gene product in the *tra* operon is TraY, which is important for processing the DNA prior to transfer and for autoregulating the main P_y promoter. Secondary promoters have been proposed at sites within the *tra* operon but these have never been studied further (reviewed in [18]). Aside from the many regulatory elements brought to bear on *traJ* and its gene product (discussed later), the only other proteins known to affect transfer ability are SfrA and IHF, which stimulate the P_y promoter [19]. SfrA was later identified to be ArcA, a response regulator [20], but no corresponding sensor kinase has been implicated in the regulation of F *tra* gene expression. A regulatory loop exists among the three main regulators, TraJ, TraM and TraY [21], which has also been termed a 'latch relay' mechanism [22]. TraJ activates P_y, which gives rise to TraY. TraY has a stimulatory effect on P_m whereas transcripts from P_m readthrough into *traJ,* stimulating further expression. Eventually, TraM and TraY levels rise and their respective promoters, P_m and P_y, are repressed. One puzzling aspect of this loop was that these promoters, when studied in isolation, often failed to demonstrate the same pattern of control seen *in vivo*. For instance, P_m

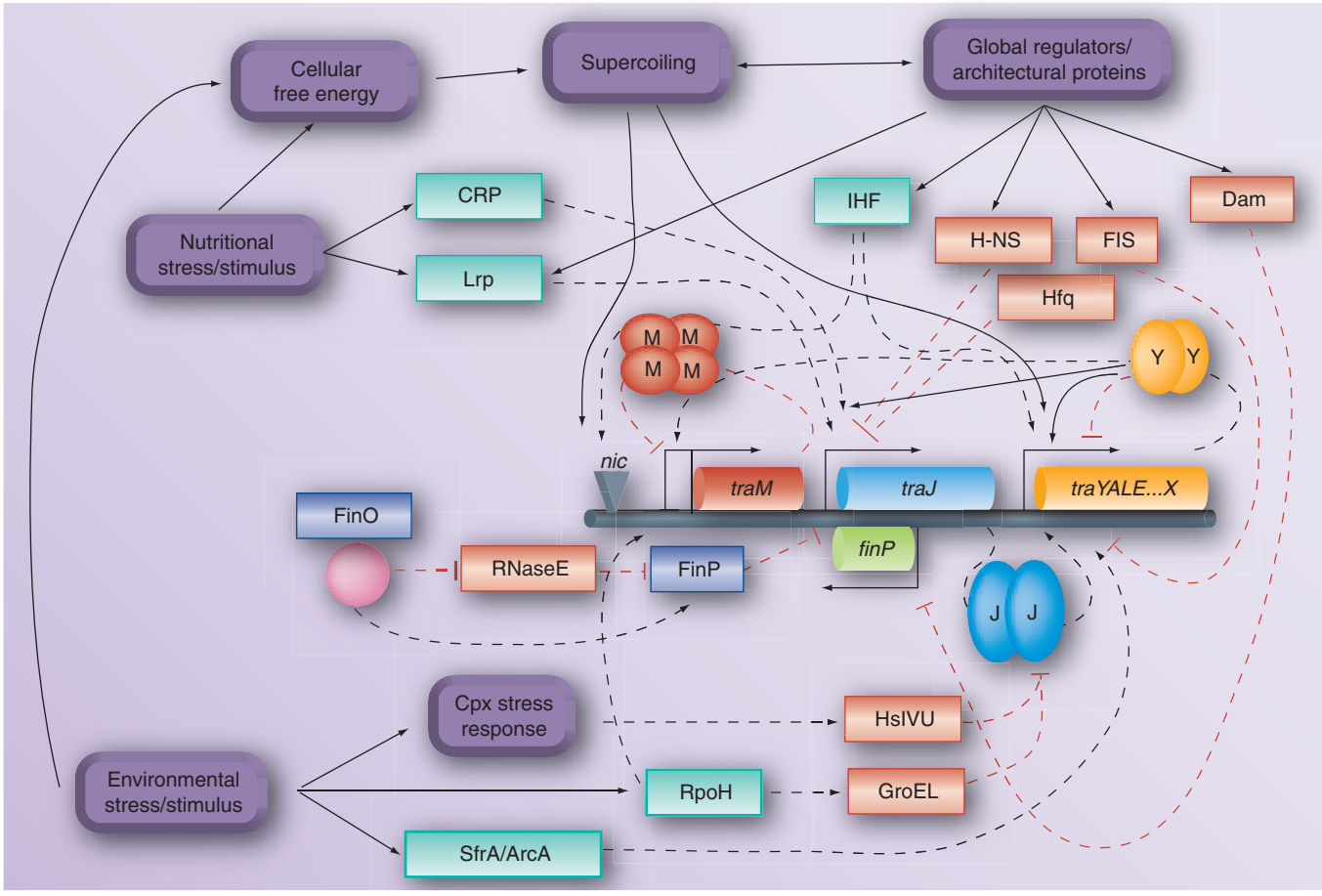

Figure 2. Summary of the mechanisms acting on the regulatory region of the IncF plasmids. The transcripts arising from the three promoters (P_m, P_j, P_y) at the head of the transfer region for monocistronic traM and traJ, the 33 kb mRNA from traY to traX, and the finP antisense RNA, which is encoded on the strand complementary to the traJ untranslated leader region, are shown as green arrows (*traM* has two promoters). F transfer regulatory proteins are represented as a tetramer (TraM), dimer (TraJ, -Y) or monomer (FinO). The outer ring of rectangles represents physiological and environmental factors that influence transfer gene expression. Inner rectangles refer to plasmid- (blue lettering) and host-encoded regulators (green and red lettering for positive and negative effects on transfer potential, respectively). Red dashed lines ending in bars indicate repressive or inhibitory effects whereas black dashed lines ending in arrowheads indicate activation. Solid lines ending in arrows indicate generalized effects.

cloned onto a promoter assessment vector failed to respond to the presence of TraY supplied *in trans* [Penfold SS, Frost LS, Unpublished Data]. This suggested that the control of transfer gene expression was more subtle than could be discerned using promoter assessment vectors.

Fertility inhibition

An interesting aspect of IncF transfer region expression is the downregulation of *traJ* mRNA translation by an antisense RNA, FinP, through a process termed fertility inhibition. Fertility inhibition was discovered when certain 'R factors', which were later determined to be IncFII plasmids carrying multiple antibiotic resistance determinants, were introduced into F^+ cells. These plasmids supplied a protein named FinO, which reduced F transfer ability by 10–2000-fold by increasing the intracellular levels of FinP [23]. F itself is naturally 'derepressed' because there is an IS3 element inserted into the *finO* gene and has almost undetectable levels of FinP [18].

FinP forms two stem loops, SLI and SLII, whereas the untranslated leader region of *traJ* mRNA contains two stem loops complementary to those in FinP, SLIc and SLIIc, and a third stem loop, SLIII, near the *traJ* transcriptional start site. FinO binds FinP and *traJ* mRNA and aids in duplex formation, which triggers its degradation by RNase III. FinP is a target for RNase E that cleaves the linker between SLI and SLII [24]. FinO binds and protects FinP from RNase E cleavage, thereby allowing intracellular levels of FinP to rise [25]. X-ray crystallographic analysis of a fragment of FinO revealed an extended structure that resembles a pointing hand [26] with two RNA-binding sites near the

N- and C-termini [27]. FinP recognizes the *traJ* mRNA via loop–loop interactions in SLI that require 5′ and 3′ tails on the FinP RNA [23,28]. SLII is a 14 bp perfect duplex that is partially unwound by FinO in an ATP-independent manner, thereby facilitating full duplex formation [29]. Thus, FinO can be considered to be an RNA chaperone that binds and protects its substrate and helps catalyze duplex formation.

An interesting phenotype exhibited by FinOP-containing IncF plasmids is that of high frequency transfer (HFT) whereby new transconjugants mate at high frequency to ensure complete conversion of a recipient cell population to plasmid-bearing status. This has also been termed 'epidemic spread' and is important in the dissemination of cargo genes (antibiotic and heavy metal resistance, virulence determinants, and so on) by these plasmids during LGT (reviewed in [30]). HFT is thought to be curtailed by rising levels of FinP and FinO in potential donors. Thus, fertility inhibition results in only a few cells in a bacterial population being transfer-competent (e.g., in the case of R1, 0.1%) with single cells either in the off (default) or the on state as a consequence of this positive feedback loop.

Other regulatory elements in F-like plasmids

Other factors can influence the stability of *traJ* mRNA, both in donor cells and new transconjugants. The *traJ* mRNA, but not FinP, is a substrate for Hfq, a global regulator known to modulate gene expression by mediating repression by small RNAs (reviewed in [31]). Hfq binds to the linker region between SLIII and SLIIc in the *traJ* mRNA leader region and promotes its degradation, thereby limiting TraJ levels and transfer potential [32]. A small RNA encoded outside the transfer region of F, called UtpR, has homology to the *traJ* mRNA leader region. Hfq can bind UtpR, suggesting that this small RNA is involved in *traJ* mRNA degradation [Majdalani N, Will WR, Frost LS, Unpublished Data].

Many IncF plasmids contain 5′–GATC–3′ sequences, the sites for Dam methylase recognition and methylation of the A residue, within their *traJ–finP* promoter regions [33]. *finP* transcription appears to be sensitive to Dam-methylation in F, R100 (IncFII) and pSLT (virulence plasmid of *Salmonella enterica* serovar Typhimurium) since FinP levels decrease and transfer ability increases in a *dam* host [34]. Paradoxically, Dam-methylation was also found to reduce *traJ* transcription in pSLT and R100; however, this turned out to be due to the ability of Lrp to respond to the methylation state of the *traJ* promoter region [35]. Lrp activates P_J in these transfer systems by binding to two sites, Lrp-1 and Lrp-2, in the *traJ* promoter region. Lrp binds Lrp-2 when it is hemimethylated, which occurs during plasmid replication. Full methylation inhibits its binding, thereby reducing *traJ* expression. Lrp has also been found to bind preferentially to hemimethylated DNA when the methyl group is on the template (noncoding) strand. This would have consequences during entry of the methylated template strand into a recipient cell. Replacement strand synthesis would result in transient hemimethylation that would stimulate *traJ* transcription and promote transfer operon expression, a property that would increase HFT [36]. Interestingly, F-transfer ability is not affected by Lrp nor is it affected by growth in rich media, which represses pSLT and R100 transfer [35].

ArcA (SfrA) and ArcB have been found to influence transfer gene expression in pSLT depending on oxygen levels. Conjugation is repressed in an *arcA* mutant under aerobic and microaerobic conditions whereas ArcB, the sensor kinase, is only required for pSLT transfer under microaerobic conditions. Phosphorylated ArcA (ArcA-P) has been shown to bind the P_y promoter in plasmid R1, suggesting that binding of ArcA-P is a prerequisite of P_y promoter activation [37]. Succinate dehydrogenase (*SdhABCD*) has a general repressive effect on pSLT transfer under aerobic, but not microaerobic, conditions, probably by regulating *traJ* transcription. This is complicated by the repressive effect of ArcAB on *sdhABCD* under microaerobic conditions [38]. Interestingly ArcB does not have a role in regulating F under aerobic conditions. Instead, SfrB, originally thought to be ArcB or another sensor kinase, was shown to be identical to RfaH, which acts as an antitermination factor for long transcripts containing rho-dependent termination sites [18].

Silencing & desilencing the F transfer region

Earlier studies on the mechanism of F-like plasmid transfer were carried out using cells grown to mid-exponential phase at optimum temperatures and in rich media. Further insight into the underlying mechanism came from studies on cells in stationary phase, or undergoing changes in temperature or nutritional status as well as being subjected to extracytoplasmic stress. For instance, F is repressed in stationary phase and

gives 'F-phenocopies' whereby levels of key transfer proteins decrease to undetectable levels and F^+ cells can act as recipients [39]. F-encoded proteins were not involved, suggesting that the host was repressing the transfer region. A similar phenomenon was observed in plasmid R1–16, which is a derepressed variant of R1, whereby TraM protein and *traA* (pilin) mRNA levels as well as transfer frequencies decreased in stationary phase [40]. Interestingly, pSLT does not undergo this loss of transfer ability in stationary phase, perhaps because of its naturally low transfer potential [34].

Repression of virulence genes in *Shigella flexneri* by nucleoid-associated proteins (NAPs) such as H-NS, its homolog StpA, Fis, HU and IHF [41], suggest that newly acquired genes might be selectively repressed by these proteins and that the problem for foreign DNA was finding mechanisms to reverse this repression and allow gene expression in an appropriate manner. In 2006, two groups reported that genes acquired by HGT or LGT in *Salmonella* or *Shigella* had GC content that varied from that of the chromosome and that AT-rich sequences were present in key promoter regions for virulence and other acquired traits [42,43]. These genes were overexpressed in an *hns* mutant. Since H-NS does not bind in a sequence-specific manner but does bind to intrinsic bends in the DNA formed by runs of As or Ts, it was concluded that these genes were repressed by H-NS and that genes acquired by LGT, at least in enteric bacteria, would be subject to H-NS repression, a process termed 'xenogeneic silencing'. H-NS was named the 'genome sentinel' for its role in modulating foreign gene expression [44]. This concept, along with the mechanism for desilencing, has been the subject of several recent reviews [45,46].

When the derepressed F plasmid was introduced into an *hns* mutant, it exhibited multipiliation and increased transfer ability in stationary phase [47]. DNA footprinting with purified H-NS revealed that it bound to predicted intrinsic bends within the main regulatory region encompassing P_m, P_j and P_y. Northern blotting revealed that all three of these promoters were greatly repressed in wild-type compared with an *hns* mutant and that readthrough transcripts from P_m extended into *traJ* as well as the *tra* operon beyond P_y. Transfer-region expression was found to be derepressed in a double *traJ hns* mutant suggesting that TraJ functioned in desilencing the *tra* region rather than activating the P_y promoter in a classical sense [32].

In cells carrying R1, which has a functioning FinOP fertility inhibition system, *traK* mRNA levels were approximately tenfold higher in an *hns* mutant (compared with wild-type) and a similar increase in transfer ability was detected [Schiffer D, Wagner MA, Koraimann G, Unpublished Data]. Thus, the absence of H-NS did not transform R1 into a fully derepressed plasmid, suggesting that desilencing of the PY promoter might be different in F and R1.

An examination of many large self-transmissible plasmids in enteric bacteria, especially pathogens, reveals the presence of F-like transfer regions containing orthologs of TraJ (**Figure 3**). Remarkably, while some orthologs are virtually identical to one another (e.g., F and APEC-E3 TraJ), there is only a low level of identity between most TraJ orthologs (e.g., R1 and F TraJ are only 38% similar). However, all contain a putative helix–turn–helix) DNA-binding motif, which has been shown to be important for F TraJ function. This is in agreement with earlier results for R100 TraJ, which was shown to bind DNA containing an inverted repeat within the P_y region [48]. Mutations in F TraJ that introduced deletions at its C-terminus revealed that the last four amino acids are important for TraJ function but affected neither DNA binding nor dimerization [Rodriguez-Maillard JM, Frost LS, Unpublished Data]. This is reminiscent of SlyA and RovA, which are involved in desilencing H-NS-repressed promoters for virulence determinants in *Salmonella* and *Yersinia* species [49–51]. These proteins are members of a subgroup of MarR regulators that are activators rather than repressors and have been characterized as dimeric, winged helix–turn–helix proteins in which deletion of the last four or more amino acids blocks function. These proteins act in conjunction with two-component response regulators (e.g., SlyA and the response regulator PhoP), among other mechanisms, to counteract H-NS repression [52]. Considering that F TraJ shares homology with these proteins within their C-terminal regions and that the response regulator ArcA is involved in Py activation, the mechanisms of activation by TraJ and these proteins might be related. Furthermore, R1 TraJ has been shown to bind P_y DNA at a site adjacent to that for ArcA-P and both proteins can bind simultaneously to the P_y promoter [37] [Schiffer D, Wagner MA, Koraimann G, Unpublished Data].

Other mechanisms that are involved in desilencing include the use of alternate σ factors to stimulate transcription from H-NS-silenced promoters that are normally transcribed by σ70-RNA polymerase holoenzyme during growth. σS, the stationary σ factor, has been implicated

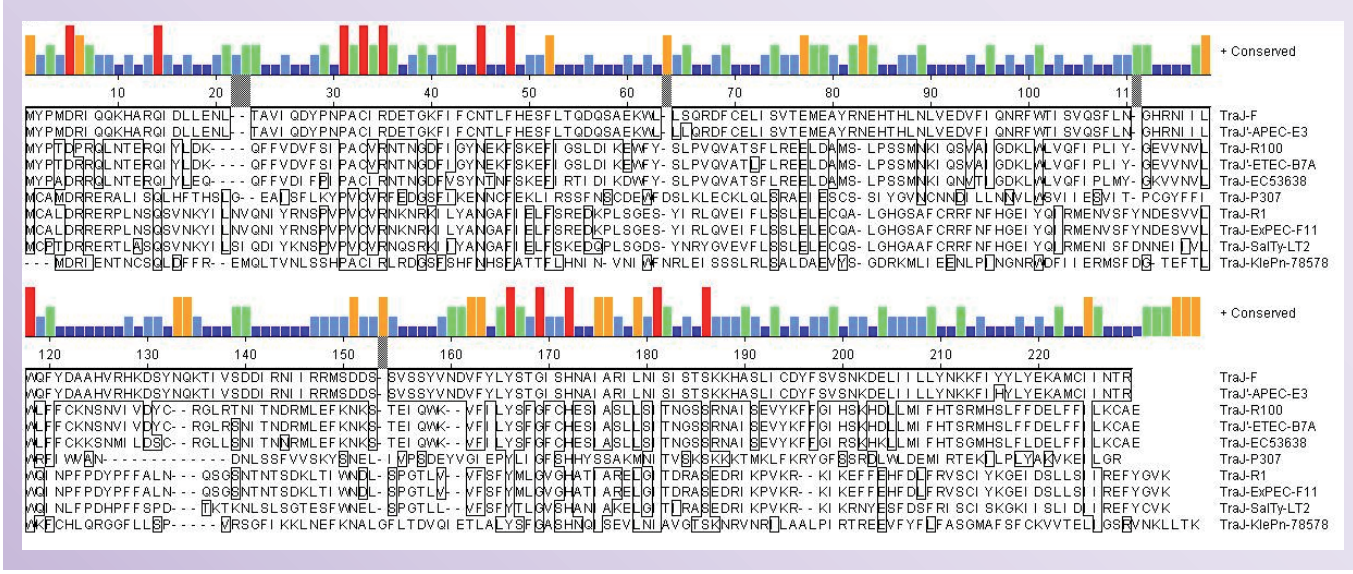

Figure 3. Orthologs of TraJ from the paradigmatic F-like plasmids (F, R100, P307, R1 and TraJ-KlePn-78578) as found in enteric pathogens. Sequence alignment of TraJ-F plasmid (gi 8918895) and nine homologs from different strains and plasmids TraJ-APEC-E3 from avian pathogenic *Escherichia coli* (gi 169546529), TraJ-R100 plasmid (gi 5103213), TraJ-ETEC-B7A from enteropathogenic *E. coli* (gi 191169437), TraJ-EC53638 from *E. coli* 53638 (gi 188574230), TraJ-P307 plasmid (gi 150466), TraJ-R1 plasmid (gi 223634723), TraJ-ExPEC-F11 from exopathogenic *E. coli* (gi 191173602), TraJ-SalTy-LT2 from *Salmonella enterica* serovar Typhimurium, plasmid LT2 (gi 17233441), and TraJ-KlePn-78578 from *Klebsiella pneumonia* (gi 152973560). Consensus residues are shown on top of the alignment as red boxes with lower homology shown with progressively shorter boxes; dark blue short boxes indicate low identity. The number of amino acids in F TraJ is shown between the colored boxes and sequences, with gray boxes indicating gaps in the sequences. Boxed residues are conserved amino acids compared with the F TraJ sequence.

in the desilencing of the *hdeAB* promoter when it is not accessible to the housekeeping σ factor, σ70. This suggests that σS recognizes the architecture of the H-NS-bound promoter rather than the sequence [53]. In another example, the F plasmid requires the heat shock σ factor, σ32 (RpoH), for both replication [54] and transfer. RpoH is required for *traJ* transcription in the presence of H-NS but not in an *hns* mutant, suggesting that it has a role in desilencing [Lau-Wong I, Frost LS, Unpublished Data]. Thus, much of the complicated regulation of F-transfer gene expression might center on balancing silencing and desilencing to achieve optimum levels of transfer potential.

Silencing & desilencing other transfer regions

Many large, self-transmissible plasmids carry copies of H-NS as well as members of the Hha/YmoA family that regulate virulence determinants in response to changes in environmental conditions, such as temperature [55]. These proteins mimic the oligomerization domain of H-NS and modulate its function by directing it to specific promoters for silencing [56]. The IncFII plasmid R100 carries an ortholog of Hha/YmoA, called RmoA [57], which represses transfer ability approximately five-fold. Similarly, orthologs of H-NS and Hha/YmoA/RmoA have been identified in the IncM plasmid R466 [58] and a Hha/YmoA/RmoA ortholog has been identified in the F-like plasmid pRK100 [59]. The R27 plasmid of *Salmonella enterica* serovar Typhi is an IncHI1 plasmid that exhibits thermosensitive transfer, with an optimum between 22 and 30°C, with transfer being undetectable at 37°C. R27 encodes two transfer regions that are expressed from six promoters, all of which are repressed at 37°C compared with 30°C [60]. R27 encodes orthologs of H-NS and Hha (open reading frames 164 and 182, respectively), which selectively repress a subset of chromosomal operons acquired by LGT, such as pathogenicity islands, as well as most R27 operons. They do not appear to interfere with the regulation of housekeeping genes by chromosomally encoded H-NS [61,62]. The almost identical plasmid pSf-R27 encodes an H-NS ortholog, Sfh, which has a 'stealth' function in that it aids in LGT without reducing bacterial fitness. This was determined by observing the surprisingly small effect on gene expression within the recipient cell during conjugation [63]. Thus, cells containing R27 or pSf-R27 contain chromosomally encoded H-NS and its paralog, StpA, plasmid-encoded H-NS or Sfh, and Hha. The interplay of these

four repressors should reveal important aspects about the regulation of global networks that are divided into housekeeping and foreign genes.

Other NAPs & the regulation of transfer

Other nucleoid-associated proteins such as StpA, Dps, IHF, HU, FIS and Hfq have been tested for their effect on transfer. Mutations in StpA, Dps and HU do not affect F-plasmid transfer whereas mutations in FIS, IHF and Hfq do [64]. Since StpA and HU resemble H-NS and IHF, respectively, mutations in their corresponding genes might be suppressed by their paralogs. The effect of FIS on transfer ability is also difficult to assess because of its role in modulating H-NS repression since it also binds to AT-rich sequences and competes with H-NS for these sites. It is also sensitive to the superhelical density of DNA and has a role in altering it. Thus, FIS can be both a repressor and activator depending on whether it acts alone or in competition with H-NS [65].

IHF has a complex role in F-like plasmid transfer and is essential for F, but not R388, relaxosome formation [6]. It also affects transcription from the P_m and P_j promoters in R100 [66] and P_y in F [67]. Like H-NS, IHF also binds AT-rich sequences, making it necessary to separate its role as an architectural protein in relaxosome formation and as a modulator of H-NS repression [47]. Hfq has a role in *traJ* expression, which is discussed above.

Early experiments suggested that F plasmid transfer was sensitive to catabolite repression and that F transfer is upregulated dramatically in the presence of glucose [18]. More recent studies revealed that CRP might affect transfer in response to the carbon source, specifically the concentration of glucose. As well as being an antagonist of H-NS silencing, CRP, in complex with cAMP, is a positive regulator of *traJ* transcription in pRK100 [68]. The CRP binding site was mapped to a sequence within the *traM* transcription termination region upstream of the *traJ* promoter, P_j. Thus, CRP–cAMP might effect readthrough transcription from *traM,* during desilencing of the P_j promoter, in response to the glucose levels in the cell.

Transfer regulation & the response to stress

One of the interesting activities exhibited by Gram-negative bacteria is their ability to sense events external to the cytoplasm and translate them into an intracellular response. This usually involves two component regulatory systems that may be combined with alternate σ factors to effect expression of entire regulons. One of the best studied two component systems, CpxAR, controls the extracytoplasmic stress response that monitors events as disparate as cell envelope composition to a rise in misfolded proteins within the periplasm (reviewed in [69,70]). Cpx stands for 'conjugative plasmid expression' and refers to the early finding that certain mutations in *cpxA,* termed *cpxA*,* led to reduced levels of TraJ and reduced transfer ability. Thus, CpxA was considered to be required for *traJ* expression [71]. This role for CpxA was undermined by the observation that deletion of *cpxA* did not affect TraJ levels and that *cpxA** mutations affected TraJ levels post-transcriptionally (summarized in [72]). Newly synthesized TraJ is a substrate for HslVU, the protease–chaperone pair involved in the degradation of other cytoplasmic regulators such as SulA (reviewed in [73]). TraJ is cleaved by HslVU both *in vitro* and *in vivo*, with HslVU regulated by CpxAR as part of the extracytoplasmic stress response. Interestingly, TraJ accumulates within the cell in an inactive form that is resistant to degradation during the stationary phase. An otherwise cryptic gene in the F transfer region, *traR*, stabilizes TraJ, preventing its cleavage by HslVU, via mechanisms that are not understood [74]. This has consequences for desilencing the transfer region. If the F^+ cell is under stress when the opportunity for growth resumes (e.g., dilution into fresh media), and the CpxAR regulon is upregulated, newly synthesized TraJ will be degraded and be unable to counteract H-NS repression.

One of the best illustrations that 'sex is stress' is the transient upregulation of the CpxAR regulon in response to stimulation of F-like R1–16 transfer gene expression upon entering the early exponential phase of growth. The transient burst of transcription needed to overcome H-NS repression leads to the production of the many conjugative T4SS proteins that are inserted into the cell envelope. The transport of these gene products to their correct destination in the outer and inner membranes as well as the periplasm must require monitoring by the cell, with the CpxAR regulon being an obvious candidate for this process. Interestingly, *traJ* transcription requires σ32 (RpoH) to overcome H-NS silencing (see previous sections), whereas the activation of the *rpoH* P1 promoter by CpxR is required, at least in part, for the extracytoplasmic stress response [40]. Thus, desilencing cannot be accomplished unless the

Cpx regulon is activated and is ready to deal with the possible overexpression of the transfer gene products. The involvement of the CpxAR stress response in the resumption of cell growth seems paradoxical. On one hand, the presence of extracytoplasmic stress when growth resumes triggers degradation of TraJ by HslVU, resulting in little to no *tra* gene expression. On the other hand, early expression of the transfer genes triggers the extracytoplasmic stress response and TraJ is presumably degraded or rendered inactive, thereby downregulating *tra* gene expression. The system seems to sense the levels of CpxR-P when growth resumes and balances desilencing of P_J via the σ32 pathway with degradation of TraJ by HslVU, where both the *rpoH* and the *hslVU* promoters are under CpxR-P regulation.

The sensitivity of mating ability to growth temperature is well known; however, the mechanisms are not well understood. F-like plasmids have an optimum temperature for mating ability of approximately 37°C, and is almost undetectable at high (50°C) or room temperatures [75]. In plasmid R1–16, transfer gene expression has been measured and is undetectable at or below 22°C [76]. The R27 plasmid, with its unusual requirement for lower temperatures for optimal transfer gene expression, appears to be regulated by H-NS, as are many virulence determinants [60,61,62]. In R1–16, mating ability has also been shown to be downregulated upon an upshift in temperature to 43°C. This is overcome in a *groEL* mutant, which was linked to decreased degradation of TraJ. It appears that the chaperonin GroEL, which binds TraJ, targets it for degradation by an unidentified protease [76].

Post-translational regulation of IncF conjugation

Progress has been made in understanding mechanisms for controlling transfer potential at the protein level, especially in F-like transfer systems. The targeted degradation of TraJ, discussed above, belongs in this category. However, other mechanisms that do not affect transcriptional regulators are also at play. Some fertility inhibition mechanisms use protein–protein interaction to achieve repression of conjugation. One of the best described is the inhibition of RP4 (IncP-1α) transfer by F-encoded PifC [77], which appears to involve physically blocking the activity of the RP4 coupling protein, TraG [Wong, Frost LS, Unpublished Data]. Similarly, the Osa protein encoded by IncW plasmids as well as the mobilizable plasmid RSF1010 inhibit T-strand and VirE2 (single-stranded DNA binding protein) transport during plant tumorigenesis by the Ti plasmid [78].

Other mechanisms involve the relaxosome proteins, which also have a role in modulating transfer potential. F TraI (180 kDa) contains both a relaxase and helicase linked by a central domain that inhibits helicase activity, presumably to prevent inappropriate DNA unwinding when DNA transfer is not taking place [79]. Beyond its many regulatory duties, F TraM has also been shown to change conformation in response to changes in temperature or pH and release the coupling protein, TraD, thereby providing a simple and fast response to changing environmental conditions [80].

An intriguing mechanism for blocking DNA transfer between two related donor cells is entry exclusion (Eex) with many conjugative systems encoding *eex* genes as well as associated genes for surface exclusion (Sfx), which block donor-to-donor cell contact [18]. In the F-like plasmid systems F and R100, as well as the ICEs SXT of *Vibrio cholera* and R391 of *Providencia rettgerii*, the entry exclusion proteins TraS and Eex, respectively, in one donor recognize the TraG protein of another donor and block conjugation. The specificity regions of both proteins have been mapped to small domains within TraG as well as Eex [81,82]. Remarkably, both TraG and TraS/Eex are inner membrane proteins, suggesting that TraG, previously identified as a mating pair stabilization protein, is translocated to the inner membrane of the recipient cell as part of the establishment of the mating pore [83]. If TraG contacts TraS/Eex, conjugation proceeds no further.

Regulation of conjugation in broad host range IncP plasmids

In contrast to narrow host range plasmids in the IncF and IncHI1 groups, broad host range plasmids such as those in the IncP-1 group (also known as IncP-1α and IncP-1β) have a very different mechanism for coordinating transfer gene expression (reviewed in [84]). These transfer systems depend on plasmid-encoded factors to modulate the level of transfer gene expression via repression rather than activation and appear to be independent of host-encoded NAP control. These plasmids coregulate the genes for replication, stable inheritance (partitioning) and transfer via four principle regulators KorA, -B, and -C, and TrbA. A fifth regulator, TrfA, which regulates replication,

binds to a site within the *trfAp* promoter that overlaps *trbAp*. This provides coordinated gene expression of the replication and transfer genes via binding sites for KorA, KorB and TrbA as well as TrfA (Figure 4). KorB, which also acts as a partitioning protein, binds to 12 sites on the IncP-1α plasmid RK2. It can be thought of as a substitute for H-NS in that it modulates total gene expression in response to environmental and physiological conditions as well as the different transcription levels in various hosts as befits a broad host range plasmid. With the exception of one operator, O_B9, which is near the promoter for *trbA* (*trbAp*), KorB binds at several locations within the transfer regions in keeping with its potential role as a silencing protein [85].

The transfer genes of RK2 are expressed from four promoters within two transfer regions. They are repressed by TrbA, the first gene product from transfer region 2 [86]. TrbA appears to prevent overexpression of the transfer genes, especially upon arrival of the plasmid in a recipient cell, whereas KorB provides a housekeeping function in vegetatively growing cells. KorB is capable of cooperating with TrbA to increase repression at transfer promoters. TrbA and KorA, as well as KlcB, have similar C-terminal domains that are involved in dimerization and interaction with KorB to amplify repression [87,88]. TrbA binding sites are imperfect palindromes that ensure a modicum of transfer gene expression by reducing the affinity of TrbA for these sites [89]. Thus, these systems are very 'robust' in that they maintain a highly repressed conjugative system that is a minimal burden to the host cell [90].

Constitutively regulated transfer systems in Gram-positive bacteria

Aside from IncF, IncHI1 and IncP plasmids in Gram-negative bacteria, relatively little is known about the regulation of transfer in other systems that do not require induction by signals from the recipient cell. This also is true for constitutive transfer systems in Gram-positive bacteria [91]. Many conjugative plasmids in these transfer systems have high efficiencies of transfer whereas others, for reasons that are not well understood, have low efficiencies, sometimes at a barely detectable level (reviewed in [92]). pCW3, a conjugative plasmid in *Clostridium*, which mates at high frequency, has not been studied extensively at the level of regulation [93]. Other plasmids, such as pSK41 in *Staphylococcus aureus*, which is closely related to pGO1 in *Lactococcus lactis,* encode repressors ArtA and TrsN, respectively, presumably resulting in their characteristically low transfer efficiency. The crystal structure of ArtA has been determined and was shown to be an unusual DNA-binding protein with a ribbon–helix–helix motif and an extended loop in its N-terminal region. Deletion of *artA* causes plasmid instability, presumably because ArtA is also important for replication and partitioning of the plasmid [94].

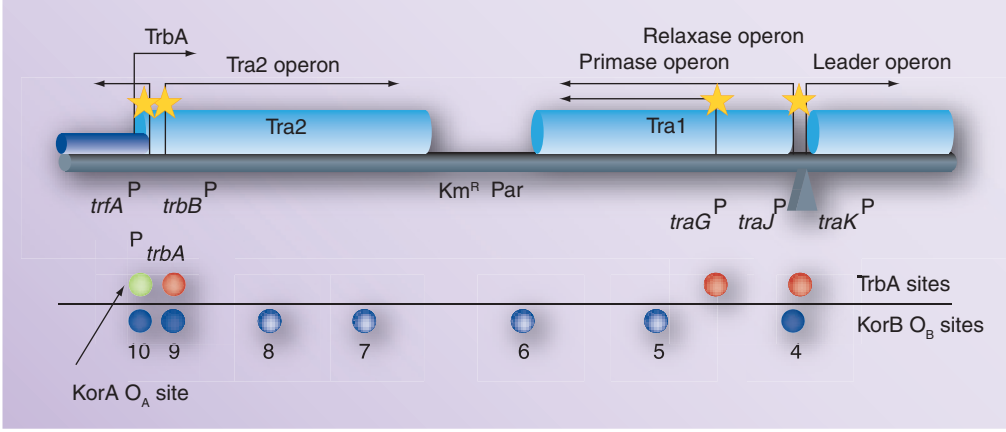

Figure 4. Regulation of IncP-1 plasmids by KorAB and TrbA. Blocks of transfer genes are represented by Tra1 (relaxase and primase overlapping operons) and Tra2 (Tra2 operon) whereas the transfer-related leader region is shown to the right and replication-related genes are shown on the left as a blue box. Promoters of interest are shown below the boxes whereas the direction of transcription is shown as angled arrows above the boxes. Green and red circles above the lower black line represent one KorA binding site (O_A) and TrbA binding sites, respectively, whereas blue circles represent KorB binding sites (O_B) 10 to 4 as discussed by Chiu *et al.* [85]. Dark blue circles represent strong KorB binding sites whereas light blue circles represent weaker binding sites. The stars represent sites for cooperative interaction between KorA and TrbA or TrfA, with TrfA being the connection between the regulation of replication and conjugation.

Whereas the pheromone-inducible conjugative systems of plasmids in *Enterococcus faecalis* are justly famous [8], other plasmids, such as pRE25, which has a broad host range, have a transfer system akin to that of pIP501, originally isolated from *Streptococcus agalactiae* [95]. pIP501 encodes its relaxase, TraA, at the head of the transfer operon, which simultaneously nicks the DNA at *oriT* and represses further transfer gene expression, thereby preventing deleterious overexpression of the transfer proteins [96,97]. A useful classification of Gram-positive plasmids based on replication mechanisms is given in [98] although the transfer and replication mechanisms are not as closely linked as in Gram-negative bacteria.

Interestingly, the expression of mobilization genes in mobilizable plasmids appears to be constitutive and is linked to the plasmids' replication machinery. These plasmids are usually relatively small and have a higher copy number than the self-transmissible plasmids on which they rely to supply a suitable T4SS and coupling protein. Therefore, their replication and, by extension, mobilization genes, are constitutively expressed in growing cells. A useful way to categorize these plasmids is on the basis of their relaxase proteins [99].

Expression of transfer genes in the real world

Although the effect of physiological and environmental parameters on the transfer ability of very few transfer systems have been studied in the laboratory, increased effort is being made to track conjugative events in the natural world and to discover how the regulation of transfer genes affects this process and contributes to LGT. The effects of carbon source, temperature, pH and oxygen levels, among others, have been studied over the past few decades for self-transmissible plasmids in the IncF, IncHI1 and IncP [92]. For instance, conjugation has been monitored in exotic locations that include the low gravity conditions of space [100]. In mice, conjugative transfer of the *Salmonella enterica* virulence plasmid pSLT was found to occur in the distal portion of the small intestine. It was inhibited by sodium deoxycholate (bile salts) and feces [101], which confirmed observations by Bidlack and Silverman [102] and Brinton [103]. Conjugation was responsible for the acquisition but not the dissemination of tetracycline resistance by pIP501 from *E. faecalis* to *E. italicus,* which is important for the dairy industry [104]. Conjugation is thought to aid in biofilm formation [105] and indeed biofilms are interesting systems for the study of conjugation because of the possibility for inter- and intraspecies transfer. An extension of this work is the study of the kinetics of transfer in natural settings, which is beyond the purview of this review.

Conclusion

This brief review of the recent literature on the regulation of transfer genes in Gram-negative systems reveals that, whereas there are differences in how plasmids use the tools at their disposal, there appear to be only a few types of regulatory mechanisms. These include the inducible systems that respond to plant phenolics, pheromones, or quorum sensing molecules of the homoserine lactone family. Among constitutive systems, F and other narrow host range plasmids take advantage of many host-encoded regulatory factors that act at the DNA level (activators and repressors, architectural proteins, excision and recircularization effectors, and Dam methylation); the RNA level (RNase susceptibility of antisense RNA and its target); and protein level (inhibitor binding, proteolysis and conformational change). Other systems, exemplified by the IncP-1 plasmids and perhaps characteristic of all broad host range plasmids, use a complex set of plasmid-encoded repressors to fine tune transfer gene expression. These systems appear to be unencumbered by host-encoded regulatory mechanisms in accordance with their promiscuous nature. As more transfer systems are identified, especially by *in silico* analysis of the numerous microbial sequences appearing in the databases, researchers will be able to predict the type of transfer gene control a given plasmid might have. Hopefully, this will allow them to optimize transfer gene expression for further study as in the serendipitous use of a derepressed mutant of F by Tatum and Lederberg over 60 years ago [106].

Future perspective

Although expression of the transfer genes is crucial to understanding the factors that affect the rate of gene transfer in nature, very few systems have been studied in detail at the mechanistic level. Whereas some systems have been studied extensively *in vitro,* there is only scant information on how physiological and environmental conditions affect gene expression in them. Other self-transmissible and mobilizable elements have been studied at the level of mating ability under various conditions but mechanistic detail is lacking. In yet other systems, regulators have been predicted or identified by genetic analysis, but again mechanistic detail is not available.

Nevertheless, current knowledge is sufficient to allow future studies on microbial populations both at the level of the individual cell as well as in natural communities such as in biofilms or aqueous environments involving mixed populations of microbes. There appears to be considerable variation in *tra* gene expression among individual cells that affect the transfer potential of an entire population. This is especially important in mixed populations, which is normal in natural environments. The goal will be to trace the path of LGT in these environments and identify reservoirs for efficient transfer as well as bottlenecks and impediments that affect the flow of genetic information. Except in a very few specific situations, LGT is probably impossible to control using conventional drug therapies [107]. As Perez-Mendoza and de la Cruz noted, transfer systems appear to be able to drill a hole through any recipient cell envelope and effect DNA transfer in a recipient-independent manner [108]. Instead, effective public health measures based on a knowledge of what triggers efficient gene transfer will be more effective in curbing threats such as total antibiotic resistance.

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Executive summary

Conjugation: a definition

- Bacterial conjugation involves the transfer of ssDNA (or in some cases, dsDNA) between donor and recipient cells that are normally in close contact.
- Conjugation requires a coupling protein that links the transferosome (a type IV secretion system in Gram-negative bacteria) to the relaxosome, a nucleoprotein complex at the origin of transfer (*oriT*).

Conjugative elements

- Transfer (*tra*) regions can be encoded by plasmids and integrating conjugative elements (ICEs), which include conjugative transposons, which resemble integrative phages.
- Mobilizable plasmids encode a *mob* region containing the origin of transfer and a relaxosome, and borrow the transferosome/coupling protein of coresident self-transmissible plasmids or ICEs to effect their transfer.

Mating potential versus mating ability

- Mating potential is qualitative and refers to transfer gene expression in a donor cell or in a donor population of cells.
- Mating ability is quantitative and is measured as a percentage of a positive control set at 100% and can be expressed as the number of transconjugants/100 donor cells (%).

Lateral/horizontal gene transfer

- Lateral or horizontal gene transfer refers to the spread of genetic information via conjugation, transduction or transformation.
- Evidence for lateral gene transfer is the appearance of new traits in an organism and does not require the survival of the agent (e.g., plasmid ICE) in the new host.

Types of conjugative gene expression

- Conjugative elements can be narrow or broad host range, depending on their ability to be established and maintained in the new host.
- Transfer gene expression can be continuous and modulated by repressors and activators; induced via small molecules or peptides; or in response to excision from the host genome (ICEs).

Silencing and desilencing

- The repression of newly acquired genes within the transconjugant is known as 'xenogeneic silencing'. This involves nucleoid-associated proteins (NAPs) such as H-NS, the genome sentinel, as well as StpA, Fis, HU, IHF, Lrp and CRP, among others.
- Desilencing refers to the activation of NAP-repressed promoters by host- and plasmid-(ICE) encoded mechanisms such as TraJ from IncF plasmids.

Mechanisms for controlling *tra* gene expression

- Transfer gene activators such as IncF TraJ are subject to post-translational and post-transcriptional regulation including proteolytic degradation and fertility (Fin) inhibition, which is the interference in the expression or assembly of the conjugative pore.
- Many IncF plasmids encode a protein chaperone-antisense RNA system, FinOP, which downregulates *traJ* mRNA translation.
- The IncP-1 plasmids encode repressors (KorA, -B, TrbA), which modulate *tra* gene expression independently of host-encoded functions. Transfer potential is defined by the level of repression.

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