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Mini-review

Bacterial conjugation: a potential tool for genomic engineering

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Abstract

Bacterial conjugation is a mechanism for horizontal DNA transfer with potential for universal DNA delivery. The conjugal machinery can be separated into three functional modules: the relaxosome, the coupling protein, and a type IV protein secretion system. Module interchangeability among different conjugative systems opens up the possibility of "à la carte" engineering of DNA delivery into virtually any cell type.

Keywords: Bacterial conjugation; Coupling proteins; Type IV secretion; DNA relaxases; Genomic manipulation

1. Introduction

To achieve an adequate level of genetic diversity, bacterial cells overcome their lack of sexuality by horizontal DNA transfer. Thus, an extended pool of genetic information is made available to the whole population. Any bacteria, in spite of carrying a limited amount of genetic information in its gene-packed chromosome(s), can access the extended DNA pool. Any specific gene in the extended gene pool is maintained in the population by just a few cells, thus consuming little energy from the population as a whole, but is ready to spread upon selective pressure. The most dramatic example of the power of this process has been illustrated by the spread of antibiotic resistance genes among prokaryotes in just a few decades, since antibiotics started to be widely used in hospitals [26].

Horizontal DNA propagation is mediated by several mechanisms; bacterial conjugation is the most widespread and the one that contributes most to the horizontal gene pool in the prokaryotic world [7]. The promiscuity of bacterial conjugation goes beyond prokaryotes. The ultimate example of this versatility is the DNA transfer system of *Agrobacterium tumefaciens* [38], mediated by a transfer machinery highly homologous to a Gram-negative conjugative apparatus [17], that efficiently transfers DNA into the nucleus of plant cells. In addition, but only under laboratory conditions, conjugative DNA transfer has been observed from bacteria into yeast, plant and animal cells [3,14,36].

2. Modular elements in the transfer machinery

The molecular mechanism of conjugative DNA transfer has been studied extensively, especially in Proteobacteria. The DNA molecule to be transferred (usually a plasmid, that encodes its own transfer apparatus) must carry an origin of transfer (*oriT*), a short DNA sequence where the process starts and ends. The rest of the conjugal machinery consists of no less than 15 proteins that carry out several functions, including the DNA processing reactions and the active transport into the recipient cell. The conjugal machinery can be thought as consisting of three functional modules:

- The substrate *selector* consists of a nucleoprotein complex (also called relaxosome) formed by an *oriT*, a relaxase and one or more accessory nicking proteins. The

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relaxase protein specifically cleaves *oriT* in the DNA strand to be transferred, remains covalently bound to the cleaved strand, and presumably religates it at the end of the transfer process. Conjugative relaxases are related to rolling-circle replication initiator proteins, and their target *oriT* sequences are related to rolling-circle replication *oriV* sequences [37]. The selector is so called because it is very specific for each plasmid system. Relaxases act specifically on their cognate *oriT*, and not on those of related plasmids.

- The transmembranal *conduit* is a multiprotein complex, formed by about 10 different proteins, that spans both the inner and outer membrane. Its components belong to a family of protein transporters known as type IV secretion systems (T4SS) [6,23]. Many T4SS family members are constituents of mammalian pathogens that use them to inject virulence factors into target host cells [8,31].
- The *coupling protein* (CP) brings together the selector and the conduit, thus approaching both parts of the transfer machinery. In addition, based on its atomic structure and similarity to other DNA transporters, it is proposed that it actively pumps the T-DNA strand out of the donor and into the recipient cell [20].

3. Molecular architecture of the conjugal machinery

Our research team has extensively studied the conjugative system of the IncW plasmid R388, a 34-kb self-transmissible plasmid that devotes half of its genetic information to conjugative functions. Still, it represents one of the smallest known transfer regions in Gram-negative bacteria. Its simplicity and promiscuity make it an ideal model system to study the transfer process. The R388 transfer region (TRAw) is encoded in a contiguous 15-kb DNA segment that consists of the *oriT* at one end and 15 *trw* genes (*trwA* to *trwN*) that code for the corresponding Trw proteins (Fig. 1a). Our knowledge of the individual components is outlined below. Other well-characterized bacterial conjugation systems, such as RP4 or F, contain the same minimal set of molecular components plus a number of additional components.

The *oriT* is a DNA segment of 330 bp that includes the *nic* site (the site recognized and cleaved by the relaxase) and a number of structural features, namely inverted and direct repeats for protein binding [18]. R388 proteins TrwA and TrwC, and the host protein IHF, bind *oriT*. Together, this nucleoprotein complex is called the relaxosome. The architecture and assembly of the R388 relaxosome have been studied in detail [29,30]. TrwA and IHF are involved in al-

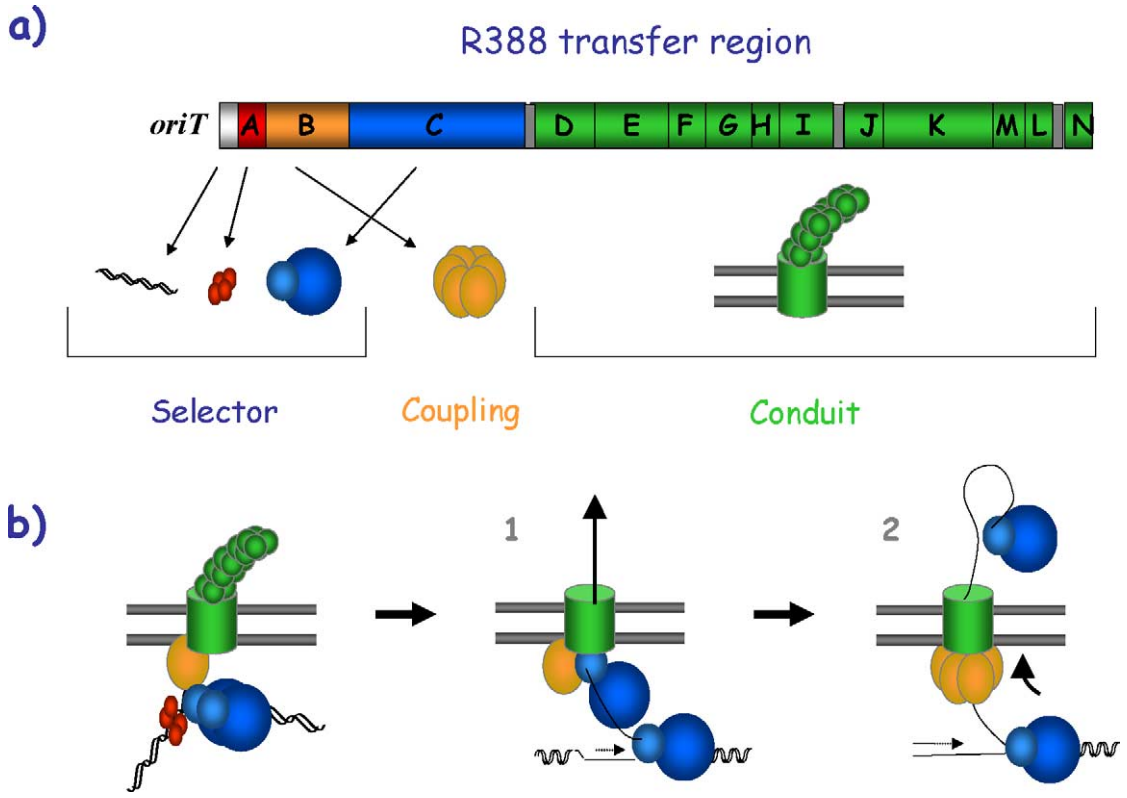

Fig. 1. Structure and function of a conjugative DNA transfer system. (a) Genetic organization of the transfer region of plasmid R388 and the resulting protein products. The *trw* gene prefix has been omitted for clarity. Proteins are arranged in the indicated functional modules. (b) Scheme of the shoot-and-pump model for conjugal DNA transfer. Step 1: the relaxase is secreted through the T4SS, with the trailing covalently bound DNA strand. Step 2: the remaining DNA is pumped out via the CP.

tering the topology of the DNA around *nic* so that a single strand is exposed to TrwC at the right site for cleavage.

TrwC is a bifunctional enzyme that can be dissected into an N-terminal domain that contains the DNA strand transferase activity and a C-terminal domain that displays a DNA helicase activity [22]. The N-terminal domain can introduce a specific cleavage at the *nic* site on supercoiled or single-stranded substrates [21]. Upon cleavage of one strand of the *oriT*, TrwC remains covalently bound to the 5′-end of the nick through a tyrosyl residue, and a second catalytic tyrosine is responsible for the strand-transfer reaction that presumably ends the transfer process [12]. The N-terminal domain can also catalyze an *oriT*-specific recombination in the absence of conjugation [19]. Recently we have determined the 3D structure of this domain [13], which allowed us to present a detailed biochemical model of the strand transfer reactions held at *oriT* by TrwC.

Little is known about the molecular architecture of the transmembranal conduit in conjugative systems. Our knowledge is mostly inferred from other members of the T4SS family: the prototypical T4SS of the *A. tumefaciens* DNA transfer system and the T4SS of mammalian pathogens such as *Helicobacter pylori*. Although 3D structures of two of the individual components have become recently available, most structural information is inferred from protein–protein interaction studies (for a recent update, see [23]). The protein components are named VirB1 to VirB11 in the *A. tumefaciens* T4SS (TrwN to TrwD in R388, see Fig. 1a). Evidence is accumulating that there is a conserved T4SS core made up of proteins VirB6 to VirB10; these proteins interact with each other and probably assemble a complex that spans both membranes. In addition, two traffic ATPases are associated with the core complex at the cytoplasmic side: VirB4 and VirB11. The pilus appendage, probably involved in interaction with the recipient cell, is composed of subunits of VirB2 and VirB5 as major and minor components. Finally, a lytic transglycosilase (VirB1) probably helps assembly of the conduit by degrading the peptidoglycan in the periplasm.

Coupling proteins (CPs) were postulated as such based on primordial work carried out with R388 protein TrwB [4]. Genetic evidence showed that CPs interact both with relaxosome components and with the conduit. A key question was to decipher the specific protein–protein interactions between the CP and the two other functional modules. TrwB has been shown to interact in vitro with the relaxosomal components TrwA and TrwC, and in vivo with the T4SS component TrwE [24], a conserved component of the core complex that belongs to the VirB10 family. Interestingly, recent data suggest that the VirB10 component could be an energy transducer and thus a strong candidate for transmitting the incoming mating signal to the CP:relaxosome complex [23].

The 3D structure of the cytoplasmic domain of TrwB is known [10,11]. Biochemical work, first with this soluble domain and more recently with the whole protein [16,28], showed that TrwB is a non-specific DNA binding protein that also binds NTPs. The ability to bind DNA and the presence of Walker boxes are also features of the CPs of plasmids F and RP4 [33], suggesting an active role in DNA transport.

4. The functional model

Once the modules of the machinery are assembled, DNA transfer is believed to happen in two steps (the "shoot and pump" model; [20]) as shown in Fig. 1b:

(1) The shooting step, or the active transport of the relaxase by the T4SS, with the DNA strand passively attached to it.
(2) The pumping step, consisting of an active movement of the threading DNA across the conduit, motored by the CP.

Although neither the physical exit of the relaxase nor the ability of the CP to track on DNA have been shown experimentally, the model is based on the following facts:

(i) The T4SS substrate. T4SSs are protein transporters, as shown by the fact that many are used solely to secrete proteins (namely T4SSs involved in injecting virulence factors into mammalian host cells). It is reasonable to assume that DNA transport is a consequence of its covalent attachment to the real T4SS substrate, the relaxase protein.
(ii) The relaxase as a pilot protein. In the related *A. tumefaciens* DNA transfer system, the relaxase VirD2 was shown to play a functional role in the plant cell [32]. Conjugative relaxase MobA from plasmid RSF1010 seems to get into the recipient cell too, as judged from indirect assays [25]. Finally we have preliminary results that show that TrwC enters the recipient cell in a functional form (our unpublished data).
(iii) The CP as a DNA pump. In addition to their DNA and NTP binding abilities, CPs show sequence similarities to a family of proteins involved in DNA transport across membranes [9], including proteins FtsK and SpoIIIE, for which a DNA-tracking activity has been demonstrated [1,34]. Another piece of indirect evidence is the fact that CPs are associated with T4SS involved in DNA transport (conjugative and *A. tumefaciens* T4SS), while those of intracellular pathogens, such as *Brucella* or *Bartonella*, that are only known to deliver virulence proteins into the host cell, lack a CP. The only exception is the T4SS of *H. pylori*, which has a CP, and no DNA transfer has been shown to date. However, it is noteworthy that this CP also binds DNA [33], and that *H. pylori* contains two genes with sequence similarity and motifs conserved in relaxases [2].
(iv) The exit pathway of the DNA. Using a variant of the chromatin immunoprecipitation technology, evidence for the *A. tumefaciens* T-DNA exit pathway has been

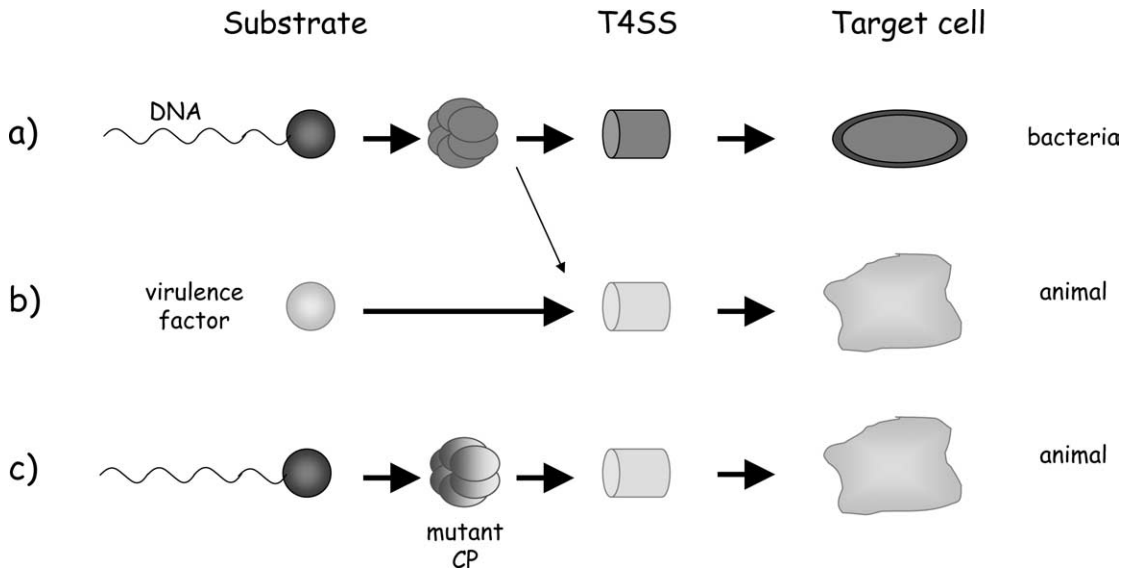

Fig. 2. Engineering DNA injectors. Design of TrwB-based CPs specifically adapted to a variety of conduits will allow DNA transport to specific cell types. (a) A conjugative T4SS recruits the relaxase + DNA through the CP and delivers it into a recipient bacterial cell. CPs can use heterologous T4SS through interaction with different VirB10-like proteins. (b) A pathogen T4SS is used to deliver virulence factors into a mammalian host cell. (c) Mutant CPs could be selected with stronger heterologous VirB10 interactions, and used to couple the DNA substrate to the pathogen T4SS. The result would be the efficient transfer of the DNA into a specific type of eukaryotic cell.

presented [5]. The T-DNA strand (presumably bound to the pilot protein, VirD2) comes in contact first with the CP, then with the cytoplasmic ATPase VirB11, then with different components of the T4SS core complex: first those in contact with the inner membrane (VirB6 and B8), then those anchored to the outer membrane (VirB9) and the pilin subunit (VirB2). The positioning of the CP at the beginning of the DNA secretion process is in agreement with our proposed "shoot and pump" model.

5. Engineering universal DNA injectors

The biotechnological potential of the conjugal machinery as a DNA injector has long been appreciated. Bacterial conjugation was shown to transport DNA from bacteria into all types of eukaryotic cells, albeit at low frequencies. This "natural" potential might be improved by applying our growing knowledge of the molecular mechanism.

The interactions of the CP with the selector module are highly specific. However, the CP–T4SS interactions are more flexible. It was shown that CPs can interact with heterologous VirB10-like components, and that the strength of this interaction correlates with the ability of the transporters to serve as conduits for the heterologous substrates [24]. In other words, the selector can be driven to different transporters, and this will depend, at least in part, on the ability of the CP to interact with the VirB10 element of each transporter.

The discovery of the interactions that direct the capacity of a CP to switch its cognate substrate into a given selector raises the possibility of its manipulation by mutagenesis in order to improve it at wish. This is especially significant considering that T4SS transporters include several systems that intracellular pathogens use to deliver their substrates (virulence factors in general) into the host cytoplasm. Provided the same CP:T4SS rules apply to these systems, we can expect to engineer a hybrid DNA injector that would transfer any given DNA into the host cytoplasm selected by the pathogen harboring the T4SS (Fig. 2). Thus, such systems have the potential for DNA delivery into several specific mammal cell types. For instance, *Brucella* infects macrophages, *Bartonella* infects epithelial cells and erythrocytes, *H. pylori* infects gastric cells, and so on. It is important to keep in mind that this would be accomplished by bacteria that can infect whole organisms and target specific tissues. Our preliminary data show indeed that conjugative CPs interact physically with VirB10-like components of the pathogens' T4SS (unpublished results).

In addition to its potential as an in vivo DNA delivery tool, another advantage of bacterial conjugation is that the DNA enters bound to a functional relaxase. The strand transfer abilities of those enzymes are probably used to recircularize the transferred DNA strand. A more powerful biotechnological tool would be obtained if this strand-transferase activity could be used to catalyze site-specific integration of the transferred DNA. In *A. tumefaciens*, VirD2 plays a role in the chromosomal integration of the T-DNA [32], although the integration is not site-specific. How could we use the conjugative mechanism to target the transferred DNA into specific sites of the host chromosome?

In R388, protein TrwC is able to catalyze a site-specific recombination reaction between two *oriT* copies both intra-

and intermolecularly [19]. This specific ability of TrwC has not been shown so far for other conjugative relaxases. The TrwC relaxase domain shows structural homology to the N-terminal domain of the Rep protein of the AAD adeno-asociated virus [13,15], that catalyzes integration of the viral genome—or a plasmid containing the terminal repeats—into a specific site of the chromosome [35]. A general feature of site-specific integration systems is the small size of the target DNA sequence. In the case of the AAV Rep protein, the target sequence required for integration is 28 bp [27]. We are working to show whether TrwC can catalyze the integration of an incoming *oriT*-containing DNA into a resident target sequence, and to define how small that target can be (work in progress in our lab). The future goal is to identify naturally occurring potential target sites in the growing list of sequenced eukaryotic genomes.

6. Concluding remarks

Bacterial conjugation is a promiscuous DNA transfer mechanism used by Nature for gene delivery into a variety of hosts. Our knowledge of its molecular mechanism can improve its versatility. We propose to exploit this knowledge to develop biotechnological tools for eukaryotic genome engineering. First, new conjugative coupling proteins can be engineered so that they adapt their cognate substrates (relaxase + DNA complex) to heterologous transmembrane transporters. Mutagenesis on protein TrwB is expected to result in coupling proteins that interact with transporters involved in protein secretion into specific animal cells. Second, conjugative relaxases enter recipient cells with intact DNA strand-transfer activities. Target sites can be selected within eukaryotic genomes to achieve site-specific integration of the transferred DNA by the appropriate relaxase.

As a result, controlled infections by attenuated bacteria carrying the DNA of interest could be used in vivo to deliver genes into specific cell types and to integrate those genes into specific chromosomal locations. Conjugal DNA transfer technology thus provides a potential new tool for genomic manipulation (including gene therapy) which could be an attractive alternative to existing methods for gene delivery.

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