New perspectives into bacterial DNA transfer to human cells

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Abstract

The type IV secretion system (T4SS) VirB/D4 of the facultative intracellular pathogen *Bartonella henselae* is known to translocate bacterial effector proteins into human cells. Two recent reports on DNA transfer into human cells have demonstrated the versatility of this bacterial secretion system for macromolecular substrate transfer. Moreover, these findings have opened the possibility for developing new tools for DNA delivery into specific human cell types. DNA can be introduced in these cells covalently attached to a site-specific integrase with potential target sequences in the human genome. This novel DNA delivery system is discussed in the context of existing methods for genetic modification of human cells.

Keywords:

Type IV secretion / bacterial conjugation / *Bartonella* / R388 / gene therapy / DNA delivery

Genetic modification of human cells: pending issues

DNA-based therapeutic strategies such as DNA vaccination and gene therapy aim to alter the genetic content of human cells by the introduction and expression of exogenous DNA. Although it is possible to perform genetic modifications of human cells *ex vivo* followed by reintroduction of the modified cells, the *in vivo* delivery of DNA has multiple advantages, since it avoids the process of extraction and reintroduction of cells, thus saving time and minimizing intervention on the patient. Most significantly, in vivo modification could be addressed in theory to any cellular type, while many cell types, such as neurons, cannot be isolated, manipulated in vitro and subsequently reimplanted to their original location. To accomplish in vivo genetic modification, one of the biggest challenges is to access specifically certain cell types which requires the use of vectors with defined cellular tropism. Currently, a panoply of methods for the introduction of DNA into mammalian cells are available **(Figure 1)**, among which viral vectors are the most effective [1]. However, induction of unintended immune responses, the inherent risks associated with random DNA insertion and the limited size of the DNA that can be cloned into viral vectors represent critical issues for their safe use and limit their clinical application. Gene delivery methods based on synthetic vectors bear less risks, although they are also less effective due to the transient nature of transgene expression [2, 3].

Bacteria-mediated transfer of plasmid DNA into mammalian cells has been assayed for more than a decade [4-6]. Delivery of genetic material is achieved through entry of the entire bacterium into target cells. Once inside, bacteria lyse and the DNA liberated to the cytoplasm can transfer to the nucleus, probably during open mitosis, facilitating its expression. However, the complex processes underlying this gene

delivery process known as ‘bactofection’ remain elusive in many aspects. The efficiency of bactofection may vary depending on the ability of the bacterial strain to replicate inside host cells, or its location in intracellular vacuoles and the capacity to exit into the cytoplasm. Bacterial DNA delivery to human cells by bactofection has been accomplished mainly with invasive enterobacteria such as the intra-vacuolar pathogen *Salmonella enterica* serovar Typhimurium [7]. However, the intra-cytoplasmic pathogen *Listeria monocytogenes* has also been employed to specifically target DNA into tumor cells [8]. Once inside host cells, *Listeria* escapes from the vacuole into the cytoplasm, and has the potential for cell-to-cell spread. An alternative approach for bactofection with bacterial pathogens has been the use of lactic acid bacteria, which as commensal bacteria pose less safety issues, but also need further engineering to become invasive and deliver their cargo inside target cells [9]. Pre-clinical studies have already demonstrated the potential of bactofection for vaccination against infectious diseases. In oncology, bactofection has potential in immunotherapy and tumor targeting; and in gastroenterology, where this gene delivery method can be employed for the topical synthesis of immunomodulatory cytokines [10]. A key advantage of bacteria as DNA delivery vectors is their ability to transfer large DNA molecules. One of the problems encountered in transfection of mammalian cells is the possibility of mechanical breakage of these large molecules during the purification process. Bactofection via invasive bacterial vectors allows transfer of intact bacterial artificial chromosomes (BACs) containing therapeutic genes [11]. Another approach is the construction of ‘bacterial ghosts’ [12]: empty cell envelopes of Gram-negative bacteria, loaded with protein or DNA content, which retain the intact surface and are easily recognized by professional antigen presenting cells.

To accomplish stable expression, DNA delivery should be followed by integration of incoming DNA. Integrative viruses can randomly integrate into the genome, however, random integration bears the inherent risk of activating oncogenes or respectively inactivating tumor suppressor genes, as it has been reported previously [13]. The preferable alternative is site-specific integration. Gene targeting, intended to integrate foreign DNA in place of the endogenous counterpart, has advanced steadily in recent years mainly due to the use of zinc-finger nucleases [14], but these are hard to build, and may not be targeted to any desired DNA sequence [15] . Application of site specific recombinases, which direct integration of the foreign DNA into a specific site in the genome is limited in that target sites are rare or must be previously engineered [16]. Also, toxicity of recombinases of viral origin has been reported [17]. Novel approaches are required to overcome the limitations of the existing methodologies for DNA delivery and site-specific integration in the human genome.

DNA delivery through bacterial type IV secretion systems (T4SSs)

Some of the described problems for conventional gene delivery methods may be overcome by a recently reported means to deliver DNA into human cells that exploits a T4SS of the facultative intracellular human pathogen *Bartonella henselae* [18, 19]. Bacterial T4SS show a remarkable plasticity in terms of the nature of the substrate to be secreted, since both protein and DNA molecules are transferred. The final destination for that cargo is also quite variable since Type IV substrates can be targeted to the extracellular milieu, into other bacteria or into eukaryotic cells (either of plant and animal origin) [20]. This versatility allows them to be involved in a variety of biological processes such as DNA transfer among bacteria, known as bacterial

conjugation [21], or effector protein translocation into human cells [22]. *Bartonella* spp. infect vascular endothelial cells and erythrocytes with the help of two distinct T4SSs, VirB/D4 and Trw, respectively. During the invasion and subsequent intracellular colonization of vascular endothelial cells the VirB/D4 T4SS translocates bacterial effector proteins into the host cell cytoplasm and these proteins play a role in subverting cellular functions to the benefit of the pathogen [23, 24]. In bacterial conjugation, a plasmid containing a recognition sequence (origin of transfer, *oriT*) is transferred in the form of a linear single-stranded DNA (ssDNA) from a donor to a recipient bacterium. This allows DNA of large size (up to several megabases) and varied sequence to be transferred *in vivo*. A current model of the transfer mechanism postulates that a single strand of DNA is piloted out of the bacterium through the T4SS by a conjugative protein known as relaxase. In the recipient bacterial cell the relaxase recircularizes the transferred DNA strand [25].

In the recent reports on T4SS-mediated DNA transfer into human cells, the authors observed translocation of the substrate of a bacterial conjugation system (ssDNA covalently linked to the relaxase) mediated by the VirB/D4 T4SS of *B. henselae*. Efficient DNA transfer into vascular endothelial cells was detected by expression of an eGFP cassette encoded on a bacterial plasmid. The plasmid further contained the *oriT* and conjugative proteins from a cryptic *Bartonella* plasmid [19] or from the broad-host range conjugative plasmid R388 [18], respectively. In both cases, the conjugative relaxase was essential for DNA transfer; moreover, DNA helicase activity from the relaxase TrwC was also required for transfer of R388 [18], suggesting that the relaxase-DNA complex is translocated by a conjugation-like mechanism. DNA molecules of different

lengths were transferred with equal efficiency, as expected for a processive mechanism such as conjugative DNA transfer.

No manipulation of the T4SS or conjugative proteins was required to accomplish DNA transfer. Thus, T4SS involved in bacterial virulence are able to mediate transfer of heterologous substrates, including DNA molecules; a result that underscores the versatility of these bacterial secretion systems. In conjugation, it is believed that a specific protein, called coupling protein (CP), pumps out the DNA molecule, using energy derived from its ATPase activity. A CP, named VirD4, belongs to the VirB/D4 T4SS of *B. henselae* and was shown to be essential for DNA transfer [18]. Since DNA transfer, in contrast to protein transfer, has so far not been reported to play a role in the T4SS-dependent host cell subversion by *B. henselae*, it was unexpected that VirD4 may pump out DNA. It remains possible that DNA is naturally transferred through the *B. henselae* VirB/D4 T4SS during infection. Transfer of bacterial DNA would allow pathogens to genetically modify the infected cell to their benefit, similar to *Agrobacterium tumefaciens*-mediated plant transformation, which is accomplished by secretion of transfer DNA (T-DNA) through its T4SS. A bioinformatic search for eukaryotic regulation signals, which could reflect genes which are expressed in the eukaryotic host, could give clues of possible transferred bacterial genes. These findings also imply that plasmids which can naturally exist in *Bartonella* may be naturally transferred into human cells, contributing to possible trans-kingdom DNA transfer events.

The ubiquity and versatility of T4SSs provide multiple possibilities

In the report by Schröder *et al.* [19], addition of the defined VirB/D4 translocation domain (BID domain plus charged tail sequence, BID^+) to the conjugative relaxase MobA led to an increase in DNA transfer efficiency by two orders of magnitude (100-fold), confirming the role of the translocation signal and opening the way for translocation of other proteins or DNA-transfer complexes by addition of this signal. In contrast, in the report by Fernández-González *et al.* [18] addition of BID^+ to the relaxase TrwC did not improve DNA transfer efficiency, which was already as efficient as MobA-BID^+ mediated transfer. The additional requirement for the R388 CP TrwB for efficient DNA transfer argues for a TrwB-mediated recruitment of TrwC which may overcome the requirement for a VirB-specific translocation signal. Future experiments addressing requirements for substrate recruitment by T4SS will render valuable information in order to address heterologous DNA transfer by T4SS of other human pathogens. As a DNA delivery tool, the interest of this pathway would increase if the T4SS found in many human pathogens could be used in a similar way. Since each pathogen targets different cellular types, tropism could be acquired by selecting the appropriate T4SS-encoding bacterium depending on the tissue to be targeted – e.g. *B. henselae* to cure vascular deficiencies, *Helicobacter pylori* to combat gastric tumors, or *Burkholderia cenocepacia* to treat cystic fibrosis.

A key feature of T4SS-mediated DNA delivery for genomic modification purposes is the fact that DNA is introduced as a protein-ssDNA complex. We speculate that ssDNA may increase integration rates; ssDNA with a free 3´end may induce generation of nucleoprotein-filaments initiating homologous recombination, thus promoting gene targeting. Complementary strand synthesis is assumed to be carried out by replication factors of the host cell without requirement of hairpin structures or

special recruitment factors [26, 27]. Single stranded DNA binding proteins (SSB), a primase and a polymerase should be involved to form a primosome and to convert the ssDNA into dsDNA. Resulting dsDNA will be hemimethylated and thus more resistant to DNA endonucleases recognizing foreign methylation patterns.

The possibility of introducing DNA covalently linked to a protein raises other interesting possibilities. Schröder *et al*. [19] analyzed the fate of the transferred DNA upon selection of stable transformants, and found that incoming DNA integrated randomly in the human genome. In one out of eight investigated transformants, the 5´end of the transferred DNA was preserved, presumably by covalent attachment of the relaxase, as it happens in the case of *A. tumefaciens* T-DNA transfer to plant cells. So, incoming DNA will integrate randomly as reported for other DNA-delivery systems, but in this case, one of the ends of the DNA molecule is more likely to maintain its integrity. Other catalytic domains could be added to the transferred relaxase, providing for instance zinc-finger nuclease or transposase activity. There is an additional advantage for the R388 DNA transfer system employed by Fernández-González *et al.* [18]. In bacterial conjugation, the relaxase TrwC of R388 can catalyze site-specific integration of the transferred DNA strand covalently attached to the protein into the recipient genome [28]. TrwC has been shown to catalyze integration into DNA sequences present in the human genome, which are highly homologous to its natural target [29]. In addition, a TrwC domain with integrase activity is able to target the protein to the nucleus of eukaryotic cells [30]. Future research is required to test if TrwC can integrate the transferred DNA into the human genome with site-specificity. If this is the case, TrwC mutants could be selected which recognize different target sequences, as already reported [31], in order to address integration to selected sequences of the

genome; in this way, the number of possible natural targets for integration present in the human genome could be increased.

In addition to the covalently linked relaxase or fusion protein, T4SS delivery allows for co-delivery of other proteins, provided they carry the T4SS secretion signal. Co-transfer of DNA together with other effector proteins could have multiple applications, e.g. to assist nuclear targeting of the transferred DNA and/or to protect it from degradation, to concomitantly knock-out a gene at a different chromosomal locus (mediated by zinc-finger nucleases), or to induce cellular pathways. We speculate that zinc-finger nuclease delivery by T4SS with concomitant ssDNA delivery may represent a particularly effective tool for gene targeting [32, 33]. Delivery of heterologous proteins, or relaxase fusion proteins, will require a case-by-case study to determine if the substrate can be delivered and is active in the recipient cell; although many relaxases and heterologous proteins have been shown to be active upon T4SS secretion (e.g. relaxase TrwC, or the recombinase Cre fused to a variety of effectors; Draper et al, 2005; Schulein et al, 2005), it cannot be guaranteed that other substrates will be properly folded upon translocation.

Concluding remarks and future directions

In summary, the finding that DNA can be introduced into human cells through the T4SS of bacterial pathogens constitutes an additional example of the surprising versatility of these secretion systems, and it provides the foundation for new DNA delivery tools with exciting possibilities. In the long term, different T4SS-containing pathogens could be used to secrete a nucleoprotein complex accompanied by helper proteins, consisting of the DNA of choice covalently linked to a protein with integrase activity, directly into the cytoplasm of their target human cells. The helper proteins will then direct the DNA into the nucleus and catalyze its integration into specific target sequences already present in the human host genome (Figure 1).

Before reaching this point, further research is needed to characterize this DNA transfer proccess and its possibilities **(Box 1)**. Right now, it is not known if *Bartonella* delivers the DNA from the surface upon contact with the eukaryotic host cell, or if it does so from an intracellular compartment. If DNA is delivered from the surface, non-invasive *Bartonella* mutants could be used as delivery vectors and then removed with antibiotics, without any further perturbation of the eukaryotic cell. The use of pathogens with tropisms for specific cell types may allow cell and tissue-specific gene therapies, but this awaits proof that DNA delivery can also occur through other virulence-associated T4SSs. A possible role of ssDNA in promoting gene targeting would also have to be confirmed. Co-transfer of effector proteins or relaxases with fused active domains opens many possibilities, but this requires specifically designed assays to determine not only efficient protein transport, but measurement of the desired activity in human cells. Finally, the introduction of a site-specific integrase covalently attached to the transferred DNA strand may prove to be a decisive

advantage for genetic modification purposes, but the activity of TrwC on its possible target human sequences remains to be tested in human cells. Analysis of human integration sites in stable TrwC-mediated DNA transfer events from *Bartonella* will allow the frequency of site-specific insertions to be determined.

In spite of the work ahead, the potential advantages of DNA delivery by bacterial T4SSs as a tool for human genetic modification are outstanding. Conjugative DNA transfer allows the transfer of hundreds or even thousands of kilobases, allowing transfer of native genes with their regulatory sequences into specific cellular types. DNA could be transferred with a variety of proteins contributing to efficient chromosomal integration. The fact that assisting proteins can be transferred with the DNA from the bacteria, in place of expressing them in the recipient cell, minimizes toxicity problems. Future work will determine how far T4SS-mediated DNA delivery can get.

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Figures and Boxes:

Figure 1. Schematic representation of main DNA delivery routes into mammalian cells. Not shown are mechanical DNA delivery methods such as microinjection, particle bombardment, pressure-mediated injection, and electroporation [34]. **1.** Viral delivery involves the steps of: (a) binding to receptors, (b) endocytosis where the virus particle ends up contained in intracellular endosomes, (c) liberation and intracellular trafficking of the virus particle, (d) entry of the viral DNA (and proteins, e.g. a viral integrase) into the nucleus (before or after uncoating of the virus particle) (see [1]). The scenarios for integration of the DNA are described further below. **2.** Synthetic vectors, such as liposomes or nanoplexes containing DNA, or polymers (e.g. polylethyleneimine) or polypeptides complexed with DNA, follow the route: (a) receptor-mediated endocytosis, (b) liberation of the vector, (c) vector disassembly and (d) entry into the nucleus (see [3, 34]). **3.** Bactofection: (a, b) bacteria enter the cell via receptor binding and endocytosis, (c) the endosomal compartment is lysed, e.g. mediated by listeriolysin O of *Listeria monocytogenes*, thereby liberating the bacteria, (d) bacteria (optionally after replication) liberate DNA upon lysis (e.g. self-lysis induced in the cytosol), (e) DNA enters the nucleus (see [5, 10]). **4.** Bacterial type IV secretion (T4SS): (a) bacteria bind to receptors, (b) formation of the 'invasome' structure, where replicating bacteria are engulfed by the cell membrane, and secretion via the T4SS of a plasmid-derived single stranded DNA covalently bound to a relaxase or integrase protein (red spheres) and protein substrates (green and blue spheres) into the cytosol, (c) entry of the bacterial DNA and proteins into the nucleus. Solid black arrows designate active nuclear targeting (1d and 4c); dashed black arrows designate passive nuclear entry of DNA (2d, 3e). Once inside the nucleus, delivered DNA (in red colour) can be processed

in different ways: **(i)** transcription into mRNA (in green colour), export of mRNA into the cytosol, and translation into a target protein; **(ii)** site-specific integration of DNA mediated by a site-specific recombinase, integrase, nuclease or transposase (red arrow); **(iii)** random integration (e.g. unspecific recombination) (blue arrow). **(iv)** Zinc-finger nucleases (ZFN, green spheres), either co-transferred with the vector or expressed from delivered DNA, may be involved in site-specific DNA integration (see (ii)) or, alternatively may serve to knock out genes (green arrow) (see [29, 30]).

Box 1. Outstanding questions

- Does DNA delivery through the T4SS occur from bacteria residing outside of the human cell or from a vacuolar intracellular compartment?
- Is DNA a natural substrate for VirB/D4 T4SS? Is there DNA naturally transferred during *Bartonella* infection?
- Is DNA transfer to human cells a unique capability of the VirB/D4 T4SS of *B. henselae*, or could virulence-associated T4SS of other bacterial pathogens also be used to deliver DNA to specific cell types according to the specific cellular tropisms of the respective pathogens?
- Is TrwC a naturally occurring VirB/D4 substrate, and are other conjugative systems also used by virulence-associated T4SSs for DNA export?
- Does TrwC catalyze site-specific integration of transferred DNA in human cells?
- Will it be possible to broaden the number of possible natural targets for integration present in the genomes that we want to modify, through mutagenesis or engineering of conjugative relaxases?
- Will co-transfer of proteins with the T4SS secretion signal of VirB/D4 T4SS provide activities which will help integration of the incoming DNA?
- Does ssDNA delivery increase the rate of random integration compared to dsDNA, and/or does it promote targeted integration?

Figure

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