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Rationale redesign of type III secretion systems: towards the development of non-pathogenic *E. coli* for *in vivo* delivery of therapeutic payloads

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Abstract

Transkingdom secretion systems that bacteria use to inject proteins directly into the cytosol of mammalian host cells play an essential role in the virulence of many Gram-negative bacterial pathogens. Current efforts are underway to repurpose these machines as novel therapeutics; type III secretion systems as vectors for the delivery of proteins of therapeutic value including heterologous antigens for vaccine development and type IV secretion systems as vectors for DNA. While initial studies focused on the use of attenuated or replication incompetent pathogens, the recent development of non-pathogenic *Escherichia coli* that encode programmable type III secretion systems expands possibilities for the *in vivo* directed delivery of therapeutic payloads.

Graphical abstract

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Repurposing bacterial T3SSs as therapeutic vectors

Conflicts of interest: none

Introduction

Gram-negative bacterial pathogens utilize a variety of secretion systems to directly inject virulence proteins, often referred to as effectors, into the cytosol of host cells [1]. Currently, type III secretion systems (T3SSs) are arguably the best characterized of these complex transkingdom nanomachines [2]. T3SSs are complex machines composed of at least 25 different proteins that assemble to form supramolecular complexes, which act as conduits through which effectors traverse the inner and outer membranes of bacterial envelope and the host cell plasma membrane before accessing the host cell cytosol. Once cytosolic, effectors target and usurp a variety of host cell processes to promote the replication and survival of the pathogens. Here, we briefly summarize the history of the rationale redesign of these machines that is taking place to transform these essential virulence determinants into novel therapeutics focusing on the recent development of the first non-pathogenic *Escherichia coli* strains that have been engineered to encode functional and programmable T3SSs.

Repurposing T3SS as antigen delivery devices

Efforts to repurpose bacterial T3SSs into therapeutics began at the end of the twentieth century in 1998 when Rüssmann and colleagues converted the SPI-1 T3SS of an attenuated strain of *Salmonella enterica* serovar Typhimurium into an antigen delivery vector [3]. Prior to this work, *Salmonella* and other attenuated pathogens were being pursued for vaccine development because in addition to being relatively inexpensive to propagate and administer, when administered orally they stimulate the development of strong mucosal, humoral (antibody) and cellular (e.g. cytotoxic T cell) responses. However, a major challenge, at least when using attenuated *Salmonella*, was how to ensure the loading of heterologous antigens into the major class I (MHC-I) antigen presentation pathway, which requires the presence of the antigens in the cell cytosol. An issue because, upon contact to the host cell, *Salmonella* rapidly invade and establish residence within modified phagosomes, preventing access to cytosolic MHC-I. To target the delivery of heterologous antigens to the cytosol, Rüssmann and colleagues fused viral epitopes to SptP, one of multiple *Salmonella* SPI-1 type III secreted effectors injected into the host cell cytosol, even when the bacteria are confined to phagosomes. Remarkably they observed that mice vaccinated with the antigen secreting attenuated strain developed class I-restricted cytotoxic T cells against the heterologous viral antigens. Furthermore, the mice were protected against the development of lethal viral infections, thus demonstrating the potential of reengineered T3SSs as novel therapeutics.

Over the next twenty years, vaccination with similarly modified T3SSs of attenuated *S. Typhimurium*, *S. Typhi*, *Pseudomonas aeruginosa* and *Yersinia pseudotuberculosis* strains were demonstrated to provide efficacy as heterologous antigen delivery vectors in promoting immune protection against viral, bacterial and even parasitic infections [4–10]. Furthermore, as initially suggested by Rüssmann and colleagues, mice vaccinated with strains that inject tumor specific antigens developed tumor-specific cytotoxic T cells which, depending on the timing of vaccination, altered the development or promoted clearance of tumors and metastatic foci [11–18].

From virulence attenuation to inhibition of replication

In the case of *Salmonella* T3SS heterologous antigen delivery strains, attenuation has primarily been achieved through the use of strains either that are defective in the biosynthesis of essential nutrients [3,4,10,13,15,16,19] or that lack a major virulence regulatory factor [5,6]. While in the case of the similarly repurposed *Pseudomonas* strains, one that no longer secretes any effectors has been used [12]. However, although these strains do not cause infections when administered to mice, given concerns that they might cause disease in humans, particularly those that are immunocompromised, strategies that completely impair bacterial replication have more recently been pursued.

For example, adopting an adaptation initially described for *Listeria*-based vaccines [20], Le Gouëllec and colleagues developed a killed but metabolically active (KBMA) effector-less strain of *Pseudomonas* [14]. This strain was generated through the removal of the *uvrAB* operon, which is essential for nucleotide excision repair. Hence, the resultant strain is replication incompetent when exposed to DNA cross-linking agents. Notably, human antigen presenting cells exposed to this KBMA strain receive higher levels of secreted antigens but exhibit lower levels of cytotoxicity than those exposed to the isogenic live strain [14]. Although these bacteria are replication incompetent, when a KBMA strain that injects tumor antigens was administered to mice, the mice developed cytotoxic T cells that infiltrate tumors [11,14]. In addition, after receiving multiple rounds of this strain, the mice developed a long-acting immune response that protected them against late tumor challenge [11].

Carleton and colleagues also recently described another approach to develop a replication incompetent T3SS-based heterologous antigen delivery system [21]. In their case, they engineered *minD Salmonella*, a strain that undergoes high rates of aberrant cell division, to overexpress components of the *Salmonella* SPI-1 T3SS. In this way, it becomes possible to efficiently generate *Salmonella* minicells, variants of *Salmonella* that lack chromosomes that express the SPI-1 T3SS at levels similar to wild type (WT) *Salmonella*. They then demonstrated that mice that received dendritic cells, pre-loaded *ex vivo* with antigens via the minicells, developed antigen-specific CD8+ T cells and exhibited significant protection from an infectious challenge. In future work, it will be interesting to see how these replication incompetent heterologous antigen delivery systems work when used as human vaccines.

Transkingdom secretion systems can be engineered to deliver a variety of therapeutic proteins and DNA into mammalian cells

Following the initial establishment of T3SSs as antigen delivery systems, they were demonstrated to be capable of injecting a variety of proteins of therapeutic value, including anti-inflammatory cytokines [22], camelid-derived single domain antibodies [23,24] and transcription activator-like effector nucleases (TALENs) [25,26]. Furthermore, the Jin lab described protocols for how the repeated delivery of mammalian reprogramming factors via the T3SS of an attenuated effector-less *Pseudomonas* strain can be used to promote the transdifferentiation of mouse embryonic fibroblasts into myocytes [27] and murine embryonic stem cells into cardiomyocytes [28]; thus, expanding upon the potential clinical applications of repurposed T3SSs. In each of these cases, as with the heterologous antigens,

the protein of interest was modified with an N-terminal type III secretion signal sequence, a sequence which is characterized by its unstructured nature [29], likely accounting for why the modified therapeutic proteins retain function.

While repurposed T3SSs are likely limited to protein delivery, work is underway to develop type IV secretion systems (T4SSs) as therapeutic DNA delivery vectors [30]. The T4SSs of bacterial pathogens share a high degree of homology and likely evolved from DNA conjugative systems; however, while it is well established that these systems inject effectors into mammalian host cells, whether they also deliver DNA during the course of an infection is less clear. However, there is evidence that at least the T4SSs of *Bartonella henselae* [31,32], *Legionella pneumophila* and *Coxiella burnetii* [33] can deliver DNA plasmids into human cells, albeit with poor efficiency. T4SSs will need to be extensively modified to optimize DNA transfer before they are ready for therapeutic development; however, given that they transfer DNA via a conjugative-like process, which does not discriminate between DNA length or sequence, the potential clinical applications for these systems are quite broad.

Development of programmable non-pathogenic type III secretion competent strains of *E. coli*

While the T3SSs of both *Vibrio parahaemolyticus* and *Yersinia pestis* have been demonstrated to be functional when transferred into laboratory strains of *E. coli* [34,35], a major advance in the use of T3SSs as therapeutic vectors is the recent development of non-pathogenic strains of *E. coli* that express a functional, programmable T3SS [36,37]. Like KBMA *Pseudomonas* and *minD* *Salmonella*-derived minicells, these bacteria are completely avirulent. However, in contrast they are replication competent, thus offering new opportunities for the development of novel T3SS based applications for the *in vivo* delivery of therapeutic payloads.

The genes that encode the structural components needed to form the *Shigella* T3SS along with four effectors are encoded in adjacent operons within a 31 kb region of a large 220 kb virulence plasmid (VP) [38]. In order to selectively transfer this region of DNA into laboratory strains of *E. coli*, Reeves and colleagues developed a recombineering approach to capture large regions of the *Shigella* VP onto autonomously replicating plasmids (Figure 1a) [36]. By engineering the captured operons to be flanked by synthetic sequences, after introducing this plasmid into *E. coli* they were able to direct its integration into a site on the *E. coli* chromosome previously engineered to contain a corresponding synthetic landing pad sequence [39,40]. The introduction of these operons alone is not sufficient to develop a type III secretion competent strain. Rather it requires the expression of a master transcription regulator of the system, i.e., VirB. By placing the expression of VirB under the control of a regulatable (IPTG-inducible) promoter, Reeves and colleagues were able to develop mT3_ *E. coli*, a non-pathogenic strain that expresses a functional programmable T3SS. Furthermore, they established a platform to identify type III secretion signal sequences that promote the recognition of a variety of proteins including mammalian reprogramming factors and TALENs as substrates of mT3_ *E. coli* that are injected into human cells.

mT3_ *E. coli*, like WT *Shigella*, invade mammalian cells [36], not surprisingly, as three of the four effectors present in mT3_ *E. coli* play a role in host cell invasion [41]. However, unlike WT *Shigella*, once inside host cells mT3_ *E. coli* do not replicate or trigger host cell death. Nevertheless, to avoid potential issues associated with host cell invasion, in 2016, Du and colleagues developed mT3_eff_ *E. coli*, a variant of mT3_ *E. coli* devoid of all effectors [42]. mT3_eff_ *E. coli* express the T3SS at levels equivalent to WT *Shigella*, but does not invade mammalian cells nor adhere to HeLa cells. However, by further engineering mT3_eff_ *E. coli* to express AfaI, an adhesin from uropathogenic *E. coli*, Du and colleagues developed a variant of mT3_eff_ *E. coli* that attaches to cells, demonstrating that the binding specificity of mT3_eff_ *E. coli* can likely be engineered to enable the delivery of therapeutic payloads into a variety of cell types.

Soon after the publication of the work describing mT3_ *E. coli*, Ruano-Gallego and colleagues reported the successful introduction of a programmable version of the enteropathogenic *E. coli* (EPEC) T3SS into another non-pathogenic strain, *E. coli* K-12 [37]. The 27 proteins needed to form the EPEC T3SS are encoded on the chromosome within a pathogenicity island composed of five operons and a few smaller transcriptional units. As outlined in Figure 1b, given the relatively small size of these operons, Ruano-Gallego and colleagues were able to amplify each from EPEC using the polymerase chain reaction. During this step, the endogenous promoter and ribosome binding site (RBS) of each operon was exchanged for an IPTG-regulated promoter plus a strong RBS, thus generating programmable versions of each operon. Furthermore, the organization of the operons enabled the investigators to avoid including genes encoding effectors. Each amplified fragment was introduced into a suicide plasmid engineered such that when the operon was introduced into the *E. coli* chromosome, a gene encoding one of five major *E. coli* adhesins was removed. The five operons were introduced into a strain of *E. coli* K-12 previously engineered to no longer express any type I fimbriae. Notably, by rationally determining which operon to place in each locus, the investigators generated SIEC (synthetic injector *E. coli*) which express the EPEC T3SS at levels equivalent to that of the native pathogen. Using this strategy, the investigators predict that they have generated a strain that will not bind to mammalian cells, preventing non-specific protein injection.

While Ruano-Gallego and colleagues did not investigate whether SIEC injects heterologous proteins into mammalian cells, they did demonstrate that SIEC delivers Tir, a native EPEC effector, into cells. Tir injection was assayed via a functional assay. Specifically, SIEC was engineered to express Tir, which once injected localizes to the plasma membrane of host cells, as well as intimin, the EPEC adhesin to which Tir binds. The binding of Tir to intimin results in the formation of actin pedestals beneath sites of SIEC attachment. Thus, this intimin-Tir interaction likely serves to stabilize interactions between SIEC and target cells, raising the question of whether SIEC that lack intimin and Tir stably dock onto and deliver other proteins into mammalian cells. While this remains to be demonstrated, in another recent elegant study from the Fernández group, Piñero-Lambea and coworkers described the development of designer synthetic adhesins [43], which could be used to target SIEC to bind to specific cell types. These synthetic adhesins are derivatives of intimin, where the outer domain of the protein is replaced with a VHH, the variable domain of a camelid-derived heavy chain only antibody. As these VHH can presumably be raised to recognize and bind to

cell-specific outer membrane proteins with exquisite sensitivity, in the future they can be used to engineer strains of mT3_ *E. coli* or SIEC to target the *in vivo* sites of delivery of therapeutic payloads.

Conclusions and Future Directions

Over the past twenty years a variety of strategies have been undertaken to transform T3SSs from agents of virulence into therapeutic vectors for the delivery of heterologous antigens as well as proteins of direct therapeutic value. However, although they have been proven to be effective in mice models, safety concerns arise for their use in humans. To overcome that, non-pathogenic *E. coli* that encode a programmable T3SS have been recently developed, opening up new possibilities for their use *in vivo*. As summarized in Figure 2, mT3_ *E. coli*, mT3_ eff_ *E. coli* and SIEC share several traits that support their further development as vectors for the delivery of therapeutic payloads. First, the introduction of the operons encoding the T3SSs onto the *E. coli* chromosome, not only improves the stability, but alleviates the need for antibiotic selection when using the bacteria in animals or humans. Second, both systems were shown to be regulatable (Figure 1). Although there is evidence that IPTG can be used to control expression of genes within murine models [44–46], in the future the IPTG-inducible promoters can be exchanged for those identified to be induced specifically under diseased states. This, together with the use of synthetic adhesins to direct attachment of the engineered bacteria to specific cell types, would allow the control of the expression of these programmable T3SSs in space and time. However, the behavior of these strains in animal models has yet to be tested. Will they maintain and exhibit sufficient activity *in vivo*? Will therapeutic payloads retain activity when engineered to be recognized as secreted substrates? Although there are likely going to be new hurdles to address, they may be overcome by taking advantage of insights from synthetic biology approaches, such as the recent refactoring of the *Salmonella* SPI-1 T3SS [47]. Regardless, efforts toward optimizing these engineered systems not only have the potential of developing affordable new targeted therapies for a variety of human diseases, but also will likely lead to additional insights regarding the biology of these secretion systems and their roles in virulence.

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- Bacterial pathogens use secretion systems to inject proteins into host cells.
- These secretion systems can be repurposed to deliver therapeutic payloads.
- Multiple strategies have been pursued to avoid issues with pathogen virulence.
- Secretion systems work when transferred into non-pathogenic *E. coli*.
- These bacteria have potential for *in vivo* targeted delivery of therapeutics.

Figure 1. Schematic representations of the general strategies used to develop mT3_ *E. coli* and SIEC

(a) To develop mT3_ *E. coli*, the 31 kb region of *Shigella* VP that encodes the VirB-regulated *ipa* and *mxi/spa* operons was first captured via recombineering onto an autonomously replicating plasmid. The plasmid was engineered to introduce flanking synthetic landing pad sequences which could be used to introduce this region of DNA into the *E. coli* chromosome. The Expression of the T3SS is under the control of IPTG-regulated VirB (blue arrow). (b) To develop SIEC, multiple small operons that encode essential components of the EPEC T3SS (*LEE1*, *LEE2*, *LEE3*, *LEE4* and *escD*) were each amplified via PCR at

which point the fragments were engineered to be expressed via an IPTG-inducible promoter. Each operon was introduced into the chromosome resulting in a strain that expresses the T3SS in an IPTG-dependent manner. Blue, virB regulation; red, IPTG regulation; green, protein of therapeutic value; purple, Tir effector; orange, landing pad sequences.

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Figure 2. Variables to consider when repurposing T3SSs for *in vivo* delivery of therapeutic payloads

(1) Utilize non-pathogenic bacterial strains to alleviate concerns regarding the development of infections in patients, including immunocompromised individuals, (2) introduce operons encoding the T3SS into the bacterial chromosome to alleviate the need for antibiotic selection, (3) define conditions under which the T3SS is expressed through choice of promoters (what can require the presence of a master regulator) (4) outfit bacteria to deliver functional variants of proteins of therapeutic value engineered to be recognized as type III secreted substrates (5) introduce synthetic adhesins into the bacteria that target delivery of proteins into specific cell types. Blue, master regulator; green, protein of therapeutic value; purple, synthetic adhesin.