**Engineering Non-pathogenic Bacteria for Auto-transporter-Driven Secretion of Functional Interferon**

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**Abstract**

In recent years, various strategies have been developed to enable the oral administration of protein-based drugs (biologics) with the aim of overcoming the degradation and inactivation of these drugs that can occur as they traverse the gastrointestinal tract (GIT). In this study, we investigated bacteria as a delivery vehicle for biologics, harnessing their ability to withstand the harsh gastric environment and deliver therapeutic drugs directly to the intestine. Specifically, we explored the use of the type 5 secretion system (T5SS) to secrete therapeutic cargoes under simulated gut conditions.Our research focused on EspC, a T5SS protein from enteropathogenic *Escherichia coli*, and its potential to secrete interferon-α (IFNα), a cytokine with immunomodulatory and antiviral properties widely used in the clinic. We demonstrated that EspC can facilitate the secretion of IFN when expressed in non-pathogenic bacteria. Moreover, this EspC-secreted IFN was able to activate the JAK-STAT pathway, upregulate IFN-stimulated genes, and induce a robust antiviral response in cells. Collectively, these findings provide proof of concept supporting the utilization of the EspC protein as a novel delivery platform for protein-based therapeutics.

**Keywords:** Antiviral; EspC; Interferon; Oral drug delivery; Protein secretion; Type V secretion system.

**Introduction**

Protein and peptide-based drugs (biologics) have emerged as a promising class of therapeutic agents due to their high selectivity, efficacy, and reduced side effects compared to small molecule drugs. The oral delivery of biologics, as well as other drugs, is usually favored over parenteral administration due to ease of administration, improved patient compliance, and reduced production costs. However, the oral administration of biologics is challenging due to the enzymatic and chemical/physical degradation of these drugs in the gastrointestinal tract (GIT) 1,2.

Several strategies have been developed to address the challenges associated with the oral administration of biologics, including chemical modifications such as PEGylation and pro-drug designs, nanoparticle-based delivery systems, and the co-administration of protease inhibitors and absorption-enhancing compounds alongside the therapeutic protein 1. However, these approaches often reduce the efficiency of these biologics and are associated with high production costs 1,3. Engineered bacteria have emerged as promising platforms for the oral delivery of therapeutic proteins and peptides. Recent studies have demonstrated the feasibility of utilizing these bacteria to express and secrete therapeutic molecules directly in the gastrointestinal tract 4,5. These bacteria are naturally designed to withstand the harsh conditions found in the stomach, allowing them to reach the small intestine and colon, where they can release the therapeutic proteins without the need for complex purification processes 6.

Bacterial secretion systems present a promising option for engineering bacteria to secrete therapeutic proteins 7. These secretion systems are normally used by bacteria to transport proteins across their cell membranes, and they play crucial roles in bacterial physiology, pathogenesis, and interactions with the environment 6,8. A prominent example of a bacterial secretion system that was previously utilized for biologic delivery is the type 3 secretion system (T3SS) 9-11. This secretion system, which is found in many Gram-negative pathogens, is a syringe-like apparatus comprising more than 20 different proteins that can inject effector proteins across the bacterial outer membrane and into host cells 12-15. The T3SS has been shown to mediate the secretion of recombinant proteins such as vaccine antigens, enzymes, transcription factors, and anticancer drugs 6,16. Our group previously demonstrated that we can utilize the T3SS of enteropathogenic *Escherichia coli* (EPEC) to secrete functional interferon-α2 (IFNα2) across the bacterial outer membrane and into the extracellular environment 17. While the T3SS is an exciting secretion system that has been shown to promote the secretion of various proteins 9-11, the complex is expressed mainly in pathogenic bacteria. While several groups have successfully transferred the T3SS into non-pathogenic strains 18, safety concerns persist.

In light of these issues, we attempted to use the type V secretion system (T5SS) as an alternative secretion system. The T5SS, also known as the autotransporter system, is a common secretion system in Gram-negative bacteria and is responsible for the secretion of various proteins, including enzymes and toxins 19-23. Autotransporters encode all the necessary components for their own secretion within a single protein. They include an N-terminal signal peptide (SP), which is recognized by the Sec-mechanism and facilitates the translocation of the protein across the inner membrane. In addition, they have a passenger domain (PD), which is the secreted portion of the protein, and a C-terminal translocator domain that forms a β-barrel pore across the outer membrane 21 (Figure 1). Another key autotransporter component is a short linker domain positioned between the PD and the β-barrel domains, which includes an α-helix and a disordered region that facilitates the passage of the PD through the β-barrel domain 24. It has also been demonstrated that T5SS-mediated PD secretion requires the interaction between the β-barrel domain and the BamA protein, which creates the lumen through which the PD passes 25,26. Since PD secretion is accomplished by a single protein together with the general bacterial secretory machinery such as the Sec and Bam complexes, it offers a straightforward method for introducing the T5SS into non-pathogenic bacteria. However, the T5SS may face limitations in its ability to secrete proteins that contain disulfide bonds or require certain post-translational modifications. These features could interfere with the translocation process or necessitate additional engineering to maintain the desired protein conformation 27,28.

Here, we sought to study whether EspC, a T5SS protein expressed in EPEC, can be used as a delivery system. EspC is a 110 kDa serine protease autotransporter that belongs to the serine protease autotransporters of Enterobacteriaceae (SPATE) subfamily 28. EspC, like many other autotransporters, is initially translocated into the periplasm via a Sec-dependent mechanism. During this process, the SP is cleaved, and the C-terminal β-barrel forms a pore in the outer membrane. This pore interacts with BamA to facilitate the transport of the PD across the membrane 25, where it is ultimately released into the external environment through cleavage next to the linker domain (Figure 1A). The PDs of SPATE proteins exhibit serine protease activity, leading to the initial hypothesis that they autonomously catalyze the C-terminal cleavage. However, experimental evidence demonstrates that disrupting or deleting the serine endopeptidase within the PD results in its detachment from the β-barrel component 28, thus suggesting that an alternative endopeptidase might also be involved in this C-terminal cleavage process.

In this study, we examined whether EspC can be employed to secrete a type I IFN. IFNs are a family of cytokines that play crucial roles in innate antiviral immunity, the modulation of adaptive immunity, and the induction of antiproliferative effects 29. All type I IFNs bind to a common cell surface receptor composed of two subunits, IFNAR1 and IFNAR2, which activates the JAK-STAT signaling pathway, leading to the upregulation of numerous interferon-stimulated genes (ISGs) that modulate the immune response 29-31. Among these type I IFNs, IFNα2 and IFNβ have been widely used in the clinic for the treatment of chronic viral infections such as hepatitis B, as well as various cancers including hairy cell leukemia, malignant melanoma, and AIDS-related Kaposi's sarcoma 32,33. Type I IFNs are an attractive therapeutic agent given their numerous roles in regulating the immune response. Their administration typically entails a regimen that involves frequent intramuscular or subcutaneous injections, reducing patient compliance. Here, we examined the ability of a highly potent version of IFNα2 containing three mutations along the protein sequence (H57Y, E58N, Q61S – referred to as YNS) to be secreted using the EspC protein. This YNS version elicited an enhanced signal transduction response, as demonstrated by its 60-fold and 3-fold greater binding affinity for IFNAR1 compared to wildtype IFNα2 and IFNβ, respectively, rendering it more therapeutically potent 33. In this study, we found that IFNα2 can be secreted via the EspC autotransporter when expressed in non-pathogenic bacteria. Overall, our results provide a proof of concept that the EspC protein can be employed as a novel delivery system for protein-based drugs.

**Materials and Methods**

**Bacterial strains** - Wildtype (WT) enteropathogenic *Escherichia coli* (EPEC) O127:H6 strain E2348/69, Δ*espC* mutant EPEC, which lacks the native EPEC T5SS EspC protein, and the Δ*lee* mutant EPEC, which includes the deletion of the entire locus of enterocyte effacement (LEE), were used to examine the expression and secretion of the various EspC proteins (Table 1). WT TOP10 *E. coli* andTOP10 *E. coli* Δ*dsbA* mutants in which the protein that catalyzes disulfide bond formation in *E. coli* was deleted were used to evaluate the T5SS activity of EspC in non-pathogenic strains (Table 1).

**Construction of EspC expression vectors** - To identify EspC domains that are non-essential for its autosecretory function, we generated two EspC versions: EspCN+C, which lacks the middle portion of the passenger domain (PD), and EspCC, which lacks both the N-terminus and the middle parts of the PD (Figure 2A). To do so, we first amplified the full-length *espC* gene from EPEC genomic DNA using the primer pair EspC\_F/EspC\_R (Table 2). In parallel, the pSA10 vector was amplified using the primer pair vector\_F/vector\_R (Table 2). The open plasmid and the fused PCR product were treated with DpnI, purified, and assembled using the Gibson assembly method 34,35 to create the pEspC vector. To create the EspCN+C construct, we amplified the pEspC vector using the primer pair N+C\_vector\_F/N+C\_vector\_R (Table 2). Those primers amplified the plasmid, including the EspC signal sequence, the C-part of the passenger domain, and the β-barrel domain. In parallel, we amplified the N-terminal part of the EspC passenger domain using the primer pair N+C\_insert\_F/N+C\_insert\_R (Table 2), which fused a Flag tag at the N-terminus of the PCR fragment. The open plasmid and the PCR fragment were treated with DpnI, purified, and assembled using the Gibson assembly method to create the EspCN+C vector. To construct the EspCC plasmid, we amplified the EspCN+C using the primer pair C\_vector\_F/C\_vector\_R (Table 2). The vector was then treated with DpnI, purified, and self-assembled in a Gibson assembly reaction to create the pEspCC vector. To construct the EspC+IFN plasmid, we amplified the pEspC vector using the primer pair IFN\_vector\_F/IFN\_vector\_R (Table 2), and the IFNYNS encoding sequence from the pT7T318U plasmid (Table 1) using the primer pair IFN\_insert\_F/IFN\_insert\_R (Table 2). The open plasmid and the PCR fragment were treated with DpnI, purified, and assembled using the Gibson assembly method to create the EspC+IFN vector. To create the EspCC+IFN vector, we amplified the EspCC vector using the primer pair C\_vector\_F/IFN\_C\_vector\_R (Table 2), and the IFNYNS coding sequence from the pT7T318U plasmid (Table 1), using the primer pair IFN\_insert\_F1/IFN\_insert\_R1 (Table 2). The PCR fragment was then re-amplified using primer pair IFN\_insert\_F1/IFN\_insert\_R2 (Table 2). The plasmid and the final PCR fragment were treated with DpnI, purified, and assembled using the Gibson assembly method to create the pEspCC+IFN vector. All constructs were verified by DNA sequencing and are listed in Table 1.

**Construction of the null Δ*espC* EPEC mutant strain** – The non-polar deletion of *espC* in the

WT EPEC O127:H6 strain E2348/69 was generated by using the *sacB*-based allelic exchange method 36. Briefly, PCR fragments of the flanking upstream and downstream regions of *espC* (0.9 and 1.32 kb, from the 5’ and 3’ ends of *espC*, respectively) were amplified from the EPEC genome with the corresponding primer pairs EspC\_up\_F/EspC\_up\_R and EspC\_down\_F/EspC\_down\_R (Table 2). The fragments were then annealed to each other using the primer pair EspC\_up\_R/EspC\_down\_F (Table 2) in an overlap extension PCR reaction. In parallel, the pRE112 suicide vector was linearized by PCR using the pRE11\_F/pRE112\_R primer pair. The linearized pRE112 plasmid and the annealed PCR product were treated with DpnI, purified, and assembled using the Gibson assembly method 34,35. The resulting pEspC\_del plasmid (Table 1) contained the flanking regions of *espC*, with 91% of *espC* having been deleted. The plasmid was then transformed into the *E. coli* SM10λpir conjugative strain to be introduced into WT EPEC. After a sucrose selection process, EPEC colonies that were susceptible to chloramphenicol were screened for the deletion of *espC* by PCR. The deletion of the *espC* gene was confirmed by sequencing.

**Type 5 secretion assay** – Bacteria (EPEC and TOP10 *E. coli*) were grown overnight in LB medium supplemented with the appropriate antibiotics in a shaker at 37°C. The overnight cultures were then diluted into pre-heated DMEM and grown statically for 6 h in a tissue culture incubator under 5% CO2 to an optical density at 600 nm (OD600) of 0.7. To examine secretion under gut-simulating conditions, overnight cultures were grown in synthetic fecal stool media (ChemBiozone) for 6 h in an anaerobic chamber (Whitley A35 anaerobic workstation with 10% CO2, 5% H2, and 85% N2). Due to the high density of the synthetic fecal stool media, we mixed it with DMEM at three dilution levels (1:1, 1:5, and 1:10). To induce protein expression in strains carrying the pSA10 vectors, IPTG (0.1 mM) was added to the bacterial cultures after 2 h incubation. The cultures were then centrifuged for 5 min at 1,500 × *g* to separate bacterial pellets from culture supernatants. The bacterial pellets were dissolved in SDS-PAGE sample buffer, and the supernatants containing the secreted proteins were collected and filtered through a Millipore 0.22-μm filter. The supernatants were normalized according to the bacterial OD600 values and treated with 10% (v/v) trichloroacetic acid (TCA) overnight at 4°C to precipitate the proteins. The samples were then centrifuged at 18,000 × *g* for 30 min at 4°C. The precipitates of the secreted proteins were dissolved in SDS-PAGE sample buffer, and any remaining TCA in the samples was neutralized using saturated Tris. Bacterial pellets and supernatants were analyzed for protein expression and secretion, respectively, via SDS-PAGE and western blotting.

**Western blotting** - Samples were subjected to SDS-PAGE and then transferred to nitrocellulose membrane (pore size: 0.45 μm; Cytiva Protran). The blots were blocked for 1 h with 5% (w/v) skim milk-PBST (0.1% Tween in phosphate-buffered saline) and then incubated with the primary antibody (diluted in 5% skim milk-PBST) for 1 h, at room temperature or overnight at 4°C. The membranes were then washed with PBST and incubated with the secondary antibody (diluted in 5% skim milk-PBST) for 1 h at room temperature. Chemiluminescence was detected with ECL reagents (Cyanagen). The following primary antibodies were used: mouse anti-Flag (Sigma) diluted 1:1,000; rabbit anti-phosphorylated STAT2 (Abcam Inc.), diluted 1:600; rabbit anti-IFNα2 (Abcam inc.), diluted 1:1000; mouse anti-DnaK (Abcam, Inc.), diluted 1:10,000, and mouse anti-Actin (MP biomedicals), diluted 1:10,000. Horseradish peroxidase-conjugated (HRP)-goat anti-mouse and HRP-goat anti-rabbit (Abcam Inc.), diluted 1:10,000, were used as the secondary antibodies. Western blots representative of at least three independent experiments are presented in the Results section.

**Quantification of secreted IFN levels** - Filtered supernatants from TOP10 *E. coli* ∆*dsbA* bacteria expressing either EspCC or EspCC+IFN were analyzed in triplicate using a commercial Human Interferon alpha2 ELISA kit (Abcam Inc.) according to the manufacturer's protocol to determine the IFN concentrations in these samples. Recombinant human IFNα2 was used as a protein standard.

**STAT2 phosphorylation assay** - HT-29 and Caco-2 cells at 70% confluence were incubated with filtered supernatant samples from TOP10 *E. coli* ∆*dsbA* expressing EspCC, EspCC+IFN, or EspC+IFN for 1 h in a tissue culture incubator (37°C, 5% CO2). The cells were then washed and lysed, and their protein extracts were subjected to SDS-PAGE and western blotting analyses using antibodies against phosphorylated STAT2 and actin (loading control). Recombinant IFNβ (2 nM) was used as a positive control, while untreated cells were used as a negative control. For the neutralization assay, supernatant samples from TOP10 *E. coli* ∆*dsbA* expressing the EspCC+IFN protein (0.5 nM) and samples of recombinant IFNα2 (0.5 nM, Abcam Inc.) were left untreated or mixed with the neutralizing anti-human IFNα2 antibody (R&D Systems) at a tenfold molar excess (5 nM) for 1 h at room temperature. The samples were then added to 70% confluent Caco-2 cells for 1 h at 37°C. The cells were then washed and lysed, and their protein extracts were subjected to SDS-PAGE and western blot analyses using antibodies against phosphorylated STAT2 and actin (loading control).

**Antiviral assay** – The antiviral activity induced by bacterial supernatants was evaluated as previously described 17. Briefly, 15,000 HeLa cells were grown overnight and then incubated for 4 h with serial dilutions of supernatant extracts collected from TOP10 *E. coli* Δ*dsbA* expressing either EspCC or EspCC+IFN. Thereafter, the cells were washed and transduced with a GFP-expressing lentivirus (VSV-G pseudotyped lentivirus with a pHR-CMV-GFP vector; Table 1) at a multiplicity of infection (MOI)of 1 37. Cells were harvested 48 h post-transduction and subjected to FACS analysis to identify GFP-positive cells. The percentage of GFP-expressing cells in the treated samples was calculated relative to the number of GFP-expressing cells in untreated HeLa cells. Commercial IFNβ was used as a positive control.

**RNA extraction and cDNA preparation** – Confluent HeLa cells were incubated for 7 h with filtered supernatants from TOP10 *E. coli* *∆dsbA* bacteria or TOP10 *E. coli* *∆dsbA* expressing EspCC or EspCC+IFN. Untreated HeLa cells and HeLa cells incubated with commercial IFNβ (2 nM; Pepro-Tech) were used as negative and positive controls, respectively. Following incubation, 1 × 106 cells were collected and subjected to RNA extraction using the TRIzol reagent (Invitrogen) and Direct-zol RNA Miniprep kit (Zymo Research) according to the manufacturer's protocol. Total RNA was resuspended in RNase-free water, and its quality was assessed by agarose gel electrophoresis. Up to 1 µg of RNA was taken from each sample for cDNA synthesis using the Protoscript II First Strand cDNA Synthesis Kit (NEB) with the oligo (dT)23 primer according to the manufacturer’s protocol.

**Quantitative PCR (qPCR)** –PCR primer pairs for specific antiviral gene transcripts were designed using the primer BLAST application (NCBI). To reduce the noise caused by DNA contamination, forward and reverse primers were selected from different exons. The primers used for this study are presented in Table 2. RT-qPCR reactions containing the cDNA of cells exposed to the various treatments, gene-specific primers, and SYBR Green I mix (Roche) were analyzed in triplicate using a QuantStudio cycler (Applied Biotechnologies, Thermo). To evaluate reaction efficiency, a standard curve was constructed for each primer pair by preparing 10-fold serial dilutions of the purified template. The conditions for amplification were: initial denaturation at 95°C for 6 min followed by 35 cycles of 95°C for 15 s, cooling to 58°C for 15 s, and 72°C for 15 s while monitoring fluorescence. Post-amplification melting-curve analyses were performed to confirm reaction specificity. Relative expression levels for each target gene were normalized to the actin housekeeping gene and compared using a double delta Ct analysis. Real-time data were reported as the fold change in relative expression levels.

**Statistical analysis** –Statistical analysis was performed using IBM SPSS Statistics 29.0. Statistical significance for the antiviral and RT-PCR assays was calculated using an independent 2-tailed t-test with assumed equal variances. Error bars represent the standard deviation.

**Results**

**Identification of the *espC* domains essential for autotransporter function.** To employ EspC as a secretion platform, we initially sought to identify the minimal *espC* sequence necessary for its autotransporter function. The SP and β-barrel domain (Figure 1B) have been established as crucial components for secreting the PD 21. However, whether other EspC domains are essential to this process is unclear. To investigate this unknown, we removed various segments along the PD and examined the secretion of the remaining portions. To detect protein secretion, it was necessary to retain a portion of the PD as a reporter protein, as removing the entire PD would have resulted in the secretion of only a very short peptide due to the cleavage downstream of the SP and upstream of the β-barrel domain, which would have been hard to detect. We, therefore, focused on three similarly-sized segments of the PD: the N-terminal (NH2: residues 55-381), middle (residues 382-708), and C-terminal portions (COOH; residues 709-1004) 38 (Figure 2). It has been suggested that the EspC serine endopeptidase motif, located in the middle part of the PD, can induce cellular damage 39 but is not essential for the release of the PD from the β-barrel domain 28. Thus, we omitted the middle part from the EspC designs and generated the EspCN+C and EspCC vectors (Figure 2). The EspCN+C constructs included the SP and β-barrel domains, along with the N- and C-terminal portions of the PD. To facilitate the detection of the secreted portion, following the cleavage of the SP and the β-barrel domains, we added a Flag tag downstream from the SP cleavage site. The additional construct, EspCC, included the SP and β-barrel domains with only the C-terminal portion of the PD (Figure 2). As with the EspCN+C construct, a Flag tag was added downstream of the SP sequence to facilitate the detection of C-part secretion. To examine the ability of the EspCN+C and EspCC constructs to support T5S, the plasmids were transformed into three EPEC strains - wildtype (WT) EPEC, the Δ*espC*a mutant in which the chromosomal *espC* gene had been deleted, and an EPEC mutant in which the entire locus of enterocyte effacement (LEE), which is crucial for EPEC pathogenicity, was deleted. This latter EPEC Δ*lee* mutant is therefore considered a non-pathogenic strain, making it better suited for protein drug delivery. It has previously been shown that EspC is naturally secreted at high concentrations when the bacteria are grown under T3SS-inducing conditions 40-42. Therefore, we grew these strains under similar conditions through static culture in tissue culture medium in a CO2 incubator. To examine the ability of the EspC constructs to express and secrete the PD portions, the bacterial pellets were separated from the culture supernatants by centrifugation, collected, and lysed. The supernatants were normalized according to the optical density (OD600nm) of the bacteria, filtered, and treated with trichloroacetic acid (TCA) to precipitate the proteins in these samples. We examined the bacterial pellets for protein expression and the supernatants for secretion of the PD portions through SDS-PAGE and western blotting with an anti-Flag antibody. We observed that both EspCN+C and EspCC were properly expressed in bacterial pellets (Figure 2), and their processed portions, following the cleavage of the SP and β-barrel domains, were secreted into the extracellular medium (Figure 2). These results indicated that the secretion of EspC is not dependent on the N-terminal or middle parts of the EspC PD.

**IFN disulfide bonds interfere with EspC-mediated secretion.** To examine whether the minimized EspCC design can support the secretion of a recombinant protein, we inserted the coding sequence for human IFNα2 into the EspCC construct.We used the IFNα2 YNS version, which reportedly exhibits enhanced activity 33. We found that the EspCC+IFN fusion protein was expressed in all EPEC strains (WT, Δ*espC*, and Δ*lee*). However, while the EspCC construct facilitated the secretion of the C-terminal portion into the culture medium, the fused construct consisting of the C-terminus and IFN was not secreted (Figure 3). A likely explanation for this lack of IFN secretion could be the presence of disulfide bonds in the IFN protein. Studies have shown that T5SS proteins typically contain a limited number of cysteine residues within their PDs, and when present, these residues are often located close to one another 21,27,43. It has previously been suggested that recombinant proteins with disulfide bonds, such as IFNα2, might pose a barrier to the autotransporters' secretion mechanism. Disulfide bond formation induces the partial folding of the precursor protein, which hinders its translocation across the outer membrane 44. We therefore hypothesized that the two pairs of disulfide bonds within the IFNα2, which are separated by more than 90 residues 45 might interfere with the ability of this construct to be secreted via the EspC autotransporter.

**IFN can be secreted via EspC when expressed in a Δ*dsbA* mutant strain**. To test our hypothesis that disulfide bond formation interferes with IFN secretion via the EspC autotransporter, we utilized a bacterial strain (TOP10 *E. coli*) that is deficient in its ability to create disulfide bonds due to the deletion of the *dsbA* gene. This gene encodes the periplasmatic protein that mediates disulfide bond formation in *E. coli* 46. In addition, we constructed a new vector, EspC-IFN, in which the IFN coding sequence was directly inserted between the SP and β-barrel domains, without the C-terminal portion of the PD (Figure 4A). This construct allowed us to investigate whether the C-terminal portion of the PD is crucial for EspC-mediated secretion. The EspC+IFN and EspCC+IFN plasmids were transformed into TOP10 *E. coli* WT and Δ*dsbA* mutant strains to determine their ability to support IFN secretion. We found that expression of the EspCC+IFN construct in the TOP10 *E. coli* Δ*dsbA* mutant facilitated significant secretion of IFN to the culture medium, while only minimal secretion was observed when the EspCC+IFN construct was expressed in WT TOP10 *E. coli* Top10. The high DnaK signal observed in the supernatant sample of the WT strain suggested that the minimal IFN signal resulted from limited bacterial lysis and not from the active secretion of IFN. Furthermore, we found that the expression of the EspC+IFN construct in the Δ*dsbA* mutant strain did not facilitate IFN secretion (Figure 4C). This suggests that the EspC+IFN construct, which lacks the C-terminal portion of the PD, is insufficient for secretion via the EspC autotransporter. It is therefore likely that the C-terminal portion of the PD contains crucial components that are required for EspC-mediated secretion. Additionally, we can conclude that TOP10 *E. coli* Δ*dsbA* allows effective protein secretion via the EspC protein when the transported cargo contains disulfide bonds.

**Secreted EspCC+IFN induces JAK-STAT signal transduction *in vitro***. After detecting IFN fused to the C-terminal portion of PD in the supernatant fraction of TOP10 *E. coli* Δ*dsbA*, we examined whether this secreted IFN was functional. The binding of IFN to its receptor, IFNAR, activates the JAK-STAT signaling pathway, resulting in phosphorylation of the cellular STAT2 protein 47. To examine the functionality of EspC-secreted IFN, we incubated Caco-2 and HT-29 cells with filtered supernatants fromTOP10 *E. coli* ∆*dsba* strains expressing EspCC, EspCC+IFN, or EspC+IFN. The cells were then collected and subjected to SDS-PAGE and western blotting analyses to detect phosphorylated-STAT2 (p-STAT2) as a means of detecting the ability of IFN to initiate the JAK-STAT response. We found that cells incubated with supernatants from bacteria that secrete the EspCC+IFN protein triggered STAT2 phosphorylation, much like cells incubated with commercial IFNβ (Figure 5). In contrast, no phosphorylation (in HT-29) or very low levels of phosphorylation (in Caco-2) were observed with supernatants of bacteriathat expressed either EspCC or EspC+IFN (Figure 5A, B). To validate that STAT2 phosphorylation was specifically induced by bacterially secreted IFN, we examined the ability of an anti-IFNα2 antibody to neutralize the effect of the IFN found in the supernatants of TOP10 *E. coli* ∆*dsba* expressing EspCC+IFN. Using the human Interferon alpha 2 ELISA kit, we first measured the concentration of IFN in the bacterial supernatant. Caco-2 cells were treated with bacterial supernatants containing IFN or commercial IFNα2 (0.5 nM). To assess antibody neutralization, these samples were pre-incubated with a 10-fold excess (5 nM) of anti-IFNa2 before application to the cells. As expected, we observed that the addition of recombinant IFNα2 (0.5 nM) induced robust STAT2 phosphorylation, while very low levels of p-STAT2 were observed in untreated cells or cells incubated with the supernatants of bacteria expressing EspCC (Figure 5C). Furthermore, we found that pre-incubation of commercial IFNα2 or EspCC+IFN-containing supernatants with the neutralizing antibody almost completely eliminated STAT2 phosphorylation (Figure 5C). These findings suggest that IFN can be secreted via the EspC platform, and although it is fused to the C-terminal portion of the PD, it is biologically active.

**EspC-secreted IFN induces ISG transcription**.To evaluate the effect of EspCC+IFN on gene regulation, we examined changes in the levels of transcriptional activity for three antiviral ISGs – *OAS2*, *MX2*, and *PKR*. Since gut epithelial cells, such as Caco-2 and HT-29, may be less sensitive to IFN stimulation 48, we incubated bacterial supernatants with HeLa cells. Samples of untreated HeLa cells or HeLa cells treated with commercial IFNβ (2 nM) served as negative and positive controls, respectively. Our results showed that the exposure of HeLa cells to a supernatant containing the EspCC+IFN protein induced the significant upregulation of antiviral ISGs at the mRNA level. However, the upregulation of *OAS2* and *MX2* was not as significant as that induced by commercial IFNβ (Figure 6). This difference can be attributed to the EspCC+IFN design, which resulted in secreted IFN that was fused to the C-terminal portion of the PD. This fusion may interfere with the efficient binding of IFN to IFNAR, thus leading to a reduced response. Incubation of HeLa cells with TOP10 *E. coli* Δ*dsbA* and TOP10 *E. coli* Δ*dsbA* expressing the EspCC protein also induced enhanced ISG transcription, albeit at levels significantly lower than those induced by commercial IFNβ or the supernatants containing EspCC+IFN (Figure 6). This more limited upregulation likely represents the cellular response to general bacterial components, such as LPS, which can trigger the autocrine and paracrine secretion of type I IFNs by cells 49. There was no substantial difference in gene regulation between samples of cells incubated with supernatants of TOP10 *E. coli* Δ*dsbA* versus supernatants of TOP10 *E. coli* Δ*dsbA* expressing EspCC (Figure 6). These results suggest that the C-terminal portion of PD does not trigger an antiviral response in cells. They also confirm that the observed upregulation of ISGs in HeLa cells incubated with EspCC+IFN supernatants was specifically induced by EspC-secreted IFN, rather than a response to general bacterial components present in the prepared supernatants.

**EspCC+IFN induces an antiviral effect in HeLa cells**. The activation of the IFN signaling pathway is essential to mount a robust defense response against viral infections. Upon activation, it upregulates the expression of antiviral ISGs (as observed above), which enhance cellular resistance to viral invasions. To assess the antiviral response *in vitro*, we used a GFP-expressing pseudovirus model to infect HeLa cells. This modified VSV-G pseudotyped virus was designed to efficiently transduce target cells but lacks the ability to complete the viral replication cycle or generate new progeny virions. Using this model, we tested whether bacterially secreted IFN could enhance the antiviral response. To do this, cells were pre-incubated for 4 h with concentrated or diluted supernatants fromTOP10 *E. coli* ∆*dsbA* expressing either EspCC+IFN or EspCC. The cells were then washed and infected with the GFP-expressing pseudovirus (MOI = 1) for 48 h. Thereafter, the cells were harvested and subjected to FACS analysis to determine the percentage of GFP-positive cells, which serves as an indication of viral infection. The percentage of GFP-positive cells treated with bacterial supernatants was normalized relative to the level of GFP-positive untreated HeLa cells. We found that cells treated with supernatants of bacteria expressing EspCC+IFN showed a reduction in viral entry (60%) compared to untreated cells, while the cells incubated with the supernatants of bacteria expressing EspCC exhibited a similar number of GFP-positive cells as the untreated cells upon viral infection (Figure 7A). These results indicate that EspCC+IFN can stimulate an antiviral response in HeLa cells and subsequently reduce viral entry into cells in a dose-dependent manner. To compare these results to commercial IFNβ, we plotted our results as a function of the IFN concentration found in the supernatants along with the percentage of GFP-positive cells after treatment with recombinant IFNβ (Figure 7B). The results suggested that the bacterially secreted EspCC+IFN triggers an antiviral response similar to that elicited by IFNβ. Representative immunofluorescence images showed that cells infected with viral particles without pre-treatment display a higher percentage of GFP-positive cells. In contrast, cells pre-incubated with the bacterial extract from *E. coli* Top10 ∆*dsbA* expressing EspCC+IFN exhibit a reduced percentage of GFP-positive cells comparable to that for cells pretreated with IFNβ (Figure S1). Cells pre-incubated with the bacterial extract fromTOP10 *E. coli* ∆*dsbA* expressing EspCC showed a minimal reduction in GFP-positive cells compared to untreated cells (Figure S1). These results indicate that EspCC+IFN can be secreted by non-pathogenic bacteria, bind to cellular IFNAR, and activate signal transduction, resulting in STAT2 phosphorylation and ISG upregulation, which initiates an antiviral response.

**EspCC+IFN can be secreted under simulated gut conditions.** To assess whether EspC-mediated secretion can occur in the GIT, we tested the secretion of EspCC+IFN under gut-simulating conditions *in vitro*. To that end, TOP10 *E. coli* Δ*dsbA* carrying either the EspCC or the EspCC+IFN plasmids were grown anaerobically in DMEM supplemented with various dilutions of synthetic fecal stool media (1:1, 1:5, and 1:10). In addition, the strains were grown anaerobically in full DMEM as a control, as this medium has been previously shown to induce EspC-medicated secretion under aerobic conditions. We observed that EspCC+IFN was secreted to varying degrees in all tested conditions (Figure 8). The strongest secretion occurred in bacteria grown in full DMEM, with a gradual reduction as the proportion of synthetic fecal material in the medium increased. Surprisingly, while IFN was consistently detected in the supernatants of bacteria carrying the EspCC+IFN plasmid under all growth conditions, EspCC+IFN expression was only clearly observed in the bacterial pellet when grown in full DMEM. Bacteria grown in the 1:10 dilution of synthetic fecal medium exhibited partial expression, whereas no expression was detected in the 1:1 and 1:5 dilutions (Figure 8). To investigate this discrepancy between protein secretion and expression, given that protein expression is a prerequisite for secretion, we analyzed the bacterial pellets for DnaK, a housekeeping protein used as a marker for bacterial growth. Western blotting revealed a pattern similar to that of IFN expression in the bacterial pellets, showing that samples grown in media with higher levels of synthetic fecal content had reduced levels of DnaK (Figure 8). Given that the plating of these cultures on LB agar showed a similar number of colony-forming units across all conditions (data not shown), we concluded that the reduced EspCC+IFN signal in the pellets was due to less effective detection and technical limitations rather than decreased protein expression. These limitations may stem from the presence of residual salts or metabolites, originating from the synthetic fecal medium, that interfere with antibody binding, as previously reported 50. Interestingly, this issue was not observed in the supernatant samples, likely because these samples were pretreated with TCA, which helps precipitate proteins and dissociate them from salts and metabolites 51. This suggests that while synthetic feces can complicate protein detection in bacterial lysates, it does not interfere with the ability to detect secreted proteins in the culture supernatants. In summary, our results indicate that EspCC+IFN can be secreted under gut-simulating conditions *in vitro*. This supports the feasibility of using an EspC-medicated system for the delivery of biologics *in vivo.*

**Discussion**

In recent years, several strategies have been developed for the oral administration of biologics with the aim of overcoming challenges related to the breakdown and inactivation of these drugs as they pass through the GIT 1. Several techniques have been developed for the oral delivery of biologics, however, those methods are costly and may reduce drug efficacy 1,3. To address these challenges, researchers, including our team, have explored using bacteria as a delivery platform for these drugs. This approach leverages the ability of some bacteria to survive the harsh conditions of the stomach and produce the drug directly in the GIT, using genetically engineered bacteria that express and secrete therapeutic proteins or peptides 4,5. Bacterial secretion systems, such as the T3SS, are a promising option for the engineering of bacteria to secrete biologics 9-11. However, some of these secretion systems are used as virulence mechanisms, expressed by pathogenic bacteria, or contain very complex machinery 9-11.

In this study, we present the use of the relatively simple secretion system, T5SS, as a novel method for secreting functional IFN using the EspC autotransporter. The EspC is a unique secretion apparatus because it relies on the expression of a single protein. We discovered that the full EspC sequence is not essential for recombinant protein secretion; notably, both the N-terminal and middle regions are dispensable for this process (Figure 2). These findings indicate that large segments of EspC’s passenger domain can be removed while preserving its capacity to secrete the cargo protein, unlike another *E. coli* autotransporter, Hemoglobin protease 52. However, fusing IFN to the C-terminal part of the EspC PD disrupted the ability of the PD to be secreted. To determine whether this inability resulted from disulfide bonds formed within the IFN protein 21,27, we examined the ability of EspCC+IFN to be secreted when expressed in the TOP10 *E. coli* Δ*dsbA* mutant, which is deficient in disulfide bond formation. The Δ*dsbA* mutant facilitated efficient IFN secretion via EspC (Figure 4), thus confirming that this strain can be used as a delivery platform for disulfide-bond-containing proteins using the EspC platform.

To establish that the EspCC-secreted IFN was functional, we examined its ability to activate the JAK-STAT signaling pathway and upregulate ISGs. We found that the supernatants of TOP10 *E. coli* Δ*dsbA* expressing EspCC+IFN induced the phosphorylation of STAT2 and the upregulation of antiviral genes (Figures 5 and 6). Interestingly, HeLa cells treated with commercial IFNβ exhibited significantly higher levels of *OAS2* and *MX2* transcription as compared to those treated with EspCC+IFN supernatants, although the IFN YNS version used in this study was previously reported to induce a stronger antiviral response in WISH cells than that elicited by IFNβ 33. We speculate that this reduced ISG upregulation induced by EspCC+IFN compared to the IFNβ is due to the presence of the ~30 kDa PD C-terminal fragment fused to the C-terminal region of IFN. This fusion may alter the affinity with which IFN binds to IFNAR, as previous studies have demonstrated that weaker binding of IFNα subtypes to the receptor results in the less pronounced upregulation of antiviral genes 53,54. Furthermore, the binding of IFNα2 to IFNAR has been shown to involve residues at the C-terminus of IFNα2 55. Therefore, it is probable that the fusion of the C-terminal portion of the PD at this critical region of IFN reduces its affinity for its cognate receptor. To address this issue, we plan to add a second serine protease cleavage site between IFN and the C-terminal portion of PD, which will result in the release of the mature form of IFN from the interfering C-terminal fragment 56. We speculate that this addition will enhance the biological activity of the secreted IFN. Despite the relatively mild upregulation of antiviral genes by the EspC-secreted IFN, it simulated a sufficient antiviral response that protected against the viral infection of cells (Figure 7). These results suggest that although EspCC+IFN may have a lower affinity for IFNAR, it can still induce a transcriptional response that is strong enough to activate a full antiviral cellular response 54. This aligns with a previous study showing that partial STAT activation is sufficient to confer complete antiviral protection 54, implying that antiviral gene transcription levels are not linearly linked to cellular resistance to viral infections.

Overall, this study introduces an innovative delivery method for IFN that holds the potential for further development for oral administration of the drug, offering a promising alternative to the current IFN parental treatment strategies and improving patient compliance 32,33. We have demonstrated that IFN secretion can be facilitated by non-pathogenic bacteria using the EspC protein and that secreted IFN can activate IFN signal transduction *in vitro*. To examine the ability of bacterially secreted IFN to cross the mucus layer and reach the epithelium and possibly the bloodstream, we plan to move to an animal model. For these analyses, we will construct a plasmid that encodes murine IFN, which shares ~50% similarly with human IFN and its corresponding receptors 57. This secreting bacteria will be used to evaluate IFN levels *in vivo* when given orally to mice. This will ideally validate the potential of using the EspC-based method as a promising platform for the delivery of various biologics, potentially encouraging its broader application in biotechnology and medicine.

**Figure legends**

Figure 1: **Schematic representation of EspC secretion pathway and domain organization**. (A) The EspC precursor protein is first recognized by the Sec machinery (embedded within the inner membrane and indicated in blue) through its N-terminal signal peptide (SP; light blue) and then transported into the periplasmic space, where the SP is cleaved. The β-barrel domain (gray) folds across the outer membrane and interacts with the Bam complex (light purple) to allow the passenger domain (pink) to pass through the pore and reach the bacterial surface. The passenger domain is then cleaved off (at the linker sequence - green) and released into the extracellular medium. (B) Schematic diagram of the EspC precursor. The protein contains an N-terminal signal peptide (SP; light blue), a passenger domain (pink), which is the secreted portion, a linker region (green) that contains an α-helix and a cleavage site, and the β-barrel domain (gray). The three sections of the passenger domain utilized in this study are shown.

Figure 2: The **EspCC construct supports type 5 secretion**. Domain organization of the truncated EspC protein versions (EspCN+C and EspCC) and their expected sizes (expressed precursors and the secreted versions) are presented. The constructs were transformed in EPEC wildtype (WT), Δ*espC* (deletion of the *epsC* gene), and Δ*lee* (deletion of the entire LEE pathogenicity island) strains and grown under gut-simulating conditions for 6 h. The cultures were then centrifuged to separate the supernatants (secreted fraction) from the bacterial pellets. The supernatants were normalized according to OD600 values, concentrated, and analyzed along the bacterial pellets via SDS-PAGE and western blotting with an anti-Flag antibody. The expression (upper panel, bacterial pellets) and EspC-mediated secretion (lower panel, supernatants) demonstrate that the truncated EspC versions are expressed and secreted.

Figure 3: **The EspCC-IFN construct is not secreted into EPEC culture supernatants**. Domain organization of the EspCC-IFN protein and its expected sizes, as a precursor and a secreted protein, are presented along the EspCC protein. The EspCC-IFN construct was transformed in EPEC wildtype (WT), Δ*espC*, and Δ*lee* strains, which were grown for 6 h. The cultures were centrifuged, and the supernatants and pellets were separated. The secreted fractions were normalized according to OD600 values, concentrated, and analyzed along with the bacterial pellets by SDS-PAGE and western blotting with an anti-Flag antibody. The expression (left panel, bacterial pellets) and EspC-mediated secretion (right panel, supernatants) demonstrated secretion of the cargo-free EspCC construct but not of the one fused to IFN.

Figure 4: **The EspCC+IFN construct supports** **IFN secretion when expressed in Δ*dsbA* mutant *E. coli***. (A) Domain organization of the EspCC+IFN and EspC+IFN proteins and their expected sizes, as precursors and secreted proteins, are presented along the EspCC protein. (B-C) The constructs were transformed into TOP10 *E. coli* wildtype (WT) and Δ*dsbA* mutant (deficient in disulfide bond formation) strains, which were grown for 6 h. The cultures were centrifuged to separate the supernatants and the pellets. Bacterial pellets (B) and normalized supernatants (C) were analyzed by SDS-PAGE and western blotting with anti-Flag, anti-IFNα2, and anti-DnaK antibodies. The results demonstrated that IFN can be secreted into the culture supernatant when expressed using the EspCC-IFN construct in the TOP10 *E. coli* Δ*dsbA* mutant.

Figure 5: **EspC-secreted IFN induces IFNAR signaling.** HT-29 (A) and Caco-2 (B) cells were incubated with the supernatants of bacterial cultures from TOP10 *E. coli* *∆dsbA* transformed with EspCC, EspCC+IFN, or EspC+IFN (protein schemes are presented in panel A). The cells were washed and lysed, and their protein extracts were subjected to SDS-PAGE and western blotting using antibodies against phosphorylated STAT2 (p-STAT) and actin (loading control). Cells incubated with commercial IFNβ (2 nM) were used as a positive control, while a sample of untreated cells was used as a negative control. A strong p-STAT signal was observed in cells that were incubated with the supernatants of TOP10 *E. coli* *∆dsbA* expressing the EspCC+IFN protein. (C) Caco-2 cells were incubated with supernatants from a culture of TOP10 *E. coli* *∆dsbA* expressing the EspCC+IFN protein (0.5 nM) or with commercial IFNα2 (0.5 nM) alone or following pre-incubation with an anti-IFNα2 antibody (5 nM) for 1 h. IFNα2 (0.5 nM) was used as a positive control, and untreated cells and cells incubated with supernatants from a culture of TOP10 *E. coli* *∆dsbA* expressing EspCC were used as negative controls.

Figure 6: **EspC-secreted IFN induces the transcription of interferon-stimulated antiviral genes**. HeLa cells were incubated with bacterial supernatants of *E. coli* Top10 Δ*dsbA* or Δ*dsbA* expressing either EspCC (Δ*dsbA* + EspCC) or EspCC+IFN (Δ*dsbA* + EspCC+IFN). Cells incubated with commercial IFNβ (2 nM) served as a comparative reference. Transcription levels of three interferon-stimulated genes (*MX2*, *OAS2*, and *PKR*) were determined by RT-PCR and are presented as fold induction relative to untreated cells. Results represent at least 4 independent biological replicates. Bars represent the standard error, \*P < 0.05, \*\*P < 0.01.

Figure 7: **Bacterially secreted EspCC+IFN exhibits antiviral activity.** HeLa cells were incubated with extracts of bacterial supernatants from TOP10 *E. coli* ∆*dsbA* expressing either EspCC or EspCC+IFN for 4 h before being washed and transduced with a GFP-expressing pseudovirus at an MOI of 1. Cells were harvested 48 h post-transduction and subjected to FACS analysis to assess GFP expression. The results are presented as the percentage of GFP-positive cells relative to GFP-positive cells in the untreated control sample, which was not pre-incubated with bacterial supernatants. The antiviral activity is presented as a function of the volume of the bacterial extracts(A)or IFN concentration (B). The antiviral activity of recombinant IFNβ is also depicted in (B), serving as a comparative reference for the activity of bacteria-secreted IFN and showing that bacterially secreted EspCC+IFN triggers an antiviral response similar to that elicited by IFNβ. Bars represent the standard deviation, \*\*P<0.01, \*\*\*P<0.001.

Figure 8: **EspCC+IFN is secreted under gut-simulating conditions.** TOP10 *E. coli* Δ*dsbA* expressing either EspCC or EspCC+IFN proteins were grown anaerobically in full DMEM and in various dilutions of synthetic fecal stool media (1:1, 1:5, and 1:10) for 6 h. The cultures were centrifuged to separate the supernatants and the pellets. Bacterial pellets (upper panel) and normalized supernatants (lower panel) were analyzed by SDS-PAGE and western blotting with anti-Flag and anti-DnaK antibodies, demonstrating that IFN can be secreted into the culture supernatant under gut-simulating conditions.

















**Table 1: Strains and plasmids used in this study**

|  |  |  |
| --- | --- | --- |
| **Strain/Plasmid** | **Description** | **Reference** |
| **Strains** | WT EPEC | EPEC strain E2348/69, streptomycin-resistant | 58 |
| EPEC Δ*espC* | Genomic deletion of *espC*, streptomycin-resistant | This study |
| EPEC Δ*lee* | Genomic deletion of the locus of enterocyte effacement (LEE), streptomycin-resistant | 59 |
| *E. coli* Top10  | *E. coli* K12 F–*mcr*A Δ(*mrr*-*hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K λ–*rps*L(StrR) *end*A1 *nup*G. Streptomycin-resistant | Thermo Fisher |
| *E. coli* Top10 Δ*dsbA* | *E. coli* K12 F–*mcr*A Δ(*mrr*-*hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K λ–*rps*L(StrR) *end*A1 *nup*G Δ*dsbA*. Streptomycin-resistant | **60** |
| **Plasmids** | pT7T318U | Cloned human IFNα2 gene with YNS mutations. | 33 |
| pRE112 | Suicide vector for allelic exchange, chloramphenicol resistance. | [Edwards, 1998 #252] |
| pEspC (pSA10) | Full-length EspC encoding sequence within the pSA10 vector. | This study |
| pEspCN+C | EspC SP, N- and C-terminal portions of the PD, and the β-barrel domains encoded on the pSA10 plasmid, carbenicillin resistant.  | This study |
| pEspCC | EspC SP, C-terminal portion of the PD, and the β-barrel domains encoded on the pSA10 plasmid, carbenicillin resistant.  | This study |
| pEspC+IFN | Human IFNα2 gene encoded between the EspC SP and the β-barrel domains. pSA10 plasmid, carbenicillin resistant.  | This study |
| pEspCC+IFN | Human IFNα2 gene encoded between the EspC SP and the C-terminal portion of the PD and the β-barrel domains. pSA10 plasmid, carbenicillin resistant. | This study |
| pEspC\_del | pRE112 containing the flanking regions of espC, chloramphenicol resistance.  | This study |
| pHR-CMV-GFP | GFP-expressing lentivirus | 37 |

**Table 2: Sequences of primers used in this study**

|  |  |  |
| --- | --- | --- |
| Construct/gene | Primer name | Sequence |
| pEspC (pSA10) | EspC\_F | CACACAGGAAACAGATGAATAAAATATACGCATTAAAATATTGTCAC |
| EspC\_R | GATCCCCGGGAATTTCAGAAAGAATAACGGAAGTTAGC |
| Vector\_F | AATTCCCGGGGATCCGTCG |
| Vector\_R | CTGTTTCCTGTGTGAAATTGTTATCCG |
| pEspCN+C (pSA10) | N+C\_vector\_F | AATCACTCATTACTGGATATTGG |
| N+C\_vector\_R | GTCGTCATCGTCTTTGTAGTCAGCAGCCTGAGATGC |
| N+C\_insert\_F | ACAAAGACGATGACGACAAGCTAAATATTGATAATGTATGGGCTAG |
| N+C\_insert\_R | CCAATATCCAGTAATGAGTGATTCTTGAATGTTTTATTATTACCAGTG |
| pEspCC (pSA10) | C\_vector\_F | AATCACTCATTACTGGATATTGGTAATAAATTTACC |
| C\_vector\_R | AGTAATGAGTGATTCTTGTCGTCATCGTCTTTGTAGT |
| pEspC+IFN(pSA10) | IFN\_vector\_F | CTGATGCCGGTGCCTCG |
| IFN\_vector\_R | TTGAGCAGCCTGAGATGCG |
| IFN\_insert\_F | CATCTCAGGCTGCTCAAATGTGTGATCTGCCG |
| IFN\_insert\_R | GGCACCGGCATCAGATTCCTTACTTCTTAAACTTTCTTGC |
| pEspCC+IFN(pSA10) | C\_vector\_F | AATCACTCATTACTGGATATTGGTAATAAATTTACC |
| IFN\_C\_vector\_R | CTTGTCGTCATCGTCTTTGTAGTC |
| IFN\_insert\_F1 | CTACAAAGACGATGACGACAAGATGTGTGATCTGCCGCAG |
| IFN\_C\_insert\_R1 | GTAATGAGTGATTTTCCTTACTTCTTAAACTTTCTTGC |
| IFN\_C\_insert\_R2 | TTACCAATATCCAGTAATGAGTGATTTTCCTTACTTC |
| pEspC\_del(pRE112) | EspC\_up\_F | CGCGGCAGCCCCGCGCGAGCCGGGC |
| EspC\_up\_R | CAAGCTTCTTCTAGAGGTACCGGCGTTTTCGCAACCAGTGAACGGTCAC |
| EspC\_down\_F | GAGCTCGATATCGCATGCCCCCTTTCTGGCGTAAGCCCCCTG |
| EspC\_down\_R | GCTCGCGCGGGGCTGCCGCGAGCGGC |
| pRE112\_F | GCATGCGATATCGAGCTC |
| pRE112\_R | GGTACCTCTAGAAGAAGCTTG |
| *ACTIN* | Actin\_F | TCCATCATGAAGTGTGACGT |
| Actin\_R | CTCAGGAGGAGGAATGATCT |
| *OAS2* | OAS\_F | AAGTCAGCTTTGAGCCTCCC |
| OAS\_R | CCAGAACTCAGCTGACCCAG |
| *MX2* | MX\_F | TTTTAACCCTCTGGGGACGC |
| MX\_R | TAGCGGTCTCACTCTGCTCT |
| *PKR* | PKR\_F | TCTTCGCTGGTATCACTCGTC |
| PKR\_R | TTCTTCCCGTATCCTGGTTGG |

**References:**

1. Mehrotra, S., Kalyan Bg, P., Nayak, P.G., Joseph, A., and Manikkath, J. (2024). Recent Progress in the Oral Delivery of Therapeutic Peptides and Proteins: Overview of Pharmaceutical Strategies to Overcome Absorption Hurdles. Adv Pharm Bull *14*, 11-33. 10.34172/apb.2024.009.

2. Wright, L., Barnes, T.J., and Prestidge, C.A. (2020). Oral delivery of protein-based therapeutics: Gastroprotective strategies, physiological barriers and in vitro permeability prediction. Int J Pharm *585*, 119488. 10.1016/j.ijpharm.2020.119488.

3. Cao, S.J., Xu, S., Wang, H.M., Ling, Y., Dong, J., Xia, R.D., and Sun, X.H. (2019). Nanoparticles: Oral Delivery for Protein and Peptide Drugs. AAPS PharmSciTech *20*, 190. 10.1208/s12249-019-1325-z.

4. Hosseinidoust, Z., Mostaghaci, B., Yasa, O., Park, B.W., Singh, A.V., and Sitti, M. (2016). Bioengineered and biohybrid bacteria-based systems for drug delivery. Adv Drug Deliv Rev *106*, 27-44. 10.1016/j.addr.2016.09.007.

5. Riglar, D.T., and Silver, P.A. (2018). Engineering bacteria for diagnostic and therapeutic applications. Nat Rev Microbiol *16*, 214-225. 10.1038/nrmicro.2017.172.

6. Claesen, J., and Fischbach, M.A. (2015). Synthetic microbes as drug delivery systems. ACS Synth Biol *4*, 358-364. 10.1021/sb500258b.

7. Burdette, L.A., Leach, S.A., Wong, H.T., and Tullman-Ercek, D. (2018). Developing Gram-negative bacteria for the secretion of heterologous proteins. Microb Cell Fact *17*, 196. 10.1186/s12934-018-1041-5.

8. Green, E.R., and Mecsas, J. (2016). Bacterial Secretion Systems: An Overview. Microbiol Spectr *4*. 10.1128/microbiolspec.VMBF-0012-2015.

9. Chamekh, M., Phalipon, A., Quertainmont, R., Salmon, I., Sansonetti, P., and Allaoui, A. (2008). Delivery of biologically active anti-inflammatory cytokines IL-10 and IL-1ra in vivo by the Shigella type III secretion apparatus. J Immunol *180*, 4292-4298. 10.4049/jimmunol.180.6.4292.

10. Shi, L., Yu, B., Cai, C.H., and Huang, J.D. (2016). Angiogenic inhibitors delivered by the type III secretion system of tumor-targeting Salmonella typhimurium safely shrink tumors in mice. AMB Express *6*, 56. 10.1186/s13568-016-0226-8.

11. Walker, B.J., Stan, G.V., and Polizzi, K.M. (2017). Intracellular delivery of biologic therapeutics by bacterial secretion systems. Expert Rev Mol Med *19*, e6. 10.1017/erm.2017.7.

12. Deng, W., Marshall, N.C., Rowland, J.L., McCoy, J.M., Worrall, L.J., Santos, A.S., Strynadka, N.C.J., and Finlay, B.B. (2017). Assembly, structure, function and regulation of type III secretion systems. Nat Rev Microbiol *15*, 323-337. 10.1038/nrmicro.2017.20.

13. Ham, H., and Orth, K. (2012). The role of type III secretion system 2 in Vibrio parahaemolyticus pathogenicity. J Microbiol *50*, 719-725. 10.1007/s12275-012-2550-2.

14. Marteyn, B., Gazi, A., and Sansonetti, P. (2012). Shigella: a model of virulence regulation in vivo. Gut Microbes *3*, 104-120. 10.4161/gmic.19325.

15. Sal-Man, N., Setiaputra, D., Scholz, R., Deng, W., Yu, A.C., Strynadka, N.C., and Finlay, B.B. (2013). EscE and EscG are cochaperones for the type III needle protein EscF of enteropathogenic Escherichia coli. J Bacteriol *195*, 2481-2489. 10.1128/JB.00118-13.

16. Bai, F., Li, Z., Umezawa, A., Terada, N., and Jin, S. (2018). Bacterial type III secretion system as a protein delivery tool for a broad range of biomedical applications. Biotechnol Adv *36*, 482-493. 10.1016/j.biotechadv.2018.01.016.

17. Rostovsky, I., Wieler, U., Kuzmina, A., Taube, R., and Sal-Man, N. (2024). Secretion of functional interferon by the type 3 secretion system of enteropathogenic Escherichia coli. Microb Cell Fact *23*, 163. 10.1186/s12934-024-02397-y.

18. Gonzalez-Prieto, C., and Lesser, C.F. (2018). Rationale redesign of type III secretion systems: toward the development of non-pathogenic E. coli for in vivo delivery of therapeutic payloads. Curr Opin Microbiol *41*, 1-7. 10.1016/j.mib.2017.10.011.

19. Beriotto, I., Icke, C., Sevastsyanovich, Y.R., Rossiter, A.E., Romagnoli, G., Savino, S., Hodges, F.J., Cole, J.A., Saul, A., MacLennan, C.A., et al. (2023). Efficient Autotransporter-Mediated Extracellular Secretion of a Heterologous Recombinant Protein by Escherichia coli. Microbiol Spectr *11*, e0359422. 10.1128/spectrum.03594-22.

20. Fan, E., Chauhan, N., Udatha, D., Leo, J.C., and Linke, D. (2016). Type V Secretion Systems in Bacteria. Microbiol Spectr *4*. 10.1128/microbiolspec.VMBF-0009-2015.

21. Henderson, I.R., Navarro-Garcia, F., and Nataro, J.P. (1998). The great escape: structure and function of the autotransporter proteins. Trends Microbiol *6*, 370-378. 10.1016/s0966-842x(98)01318-3.

22. Meuskens, I., Saragliadis, A., Leo, J.C., and Linke, D. (2019). Type V Secretion Systems: An Overview of Passenger Domain Functions. Front Microbiol *10*, 1163. 10.3389/fmicb.2019.01163.

23. Sevastsyanovich, Y.R., Leyton, D.L., Wells, T.J., Wardius, C.A., Tveen-Jensen, K., Morris, F.C., Knowles, T.J., Cunningham, A.F., Cole, J.A., and Henderson, I.R. (2012). A generalised module for the selective extracellular accumulation of recombinant proteins. Microb Cell Fact *11*, 69. 10.1186/1475-2859-11-69.

24. Drobnak, I., Braselmann, E., Chaney, J.L., Leyton, D.L., Bernstein, H.D., Lithgow, T., Luirink, J., Nataro, J.P., and Clark, P.L. (2015). Of linkers and autochaperones: an unambiguous nomenclature to identify common and uncommon themes for autotransporter secretion. Mol Microbiol *95*, 1-16. 10.1111/mmi.12838.

25. Doyle, M.T., and Bernstein, H.D. (2021). BamA forms a translocation channel for polypeptide export across the bacterial outer membrane. Mol Cell *81*, 2000-2012 e2003. 10.1016/j.molcel.2021.02.023.

26. Ieva, R., and Bernstein, H.D. (2009). Interaction of an autotransporter passenger domain with BamA during its translocation across the bacterial outer membrane. Proc Natl Acad Sci U S A *106*, 19120-19125. 10.1073/pnas.0907912106.

27. Dautin, N. (2021). Folding Control in the Path of Type 5 Secretion. Toxins (Basel) *13*. 10.3390/toxins13050341.

28. Stein, M., Kenny, B., Stein, M.A., and Finlay, B.B. (1996). Characterization of EspC, a 110-kilodalton protein secreted by enteropathogenic Escherichia coli which is homologous to members of the immunoglobulin A protease-like family of secreted proteins. J Bacteriol *178*, 6546-6554. 10.1128/jb.178.22.6546-6554.1996.

29. Snell, L.M., McGaha, T.L., and Brooks, D.G. (2017). Type I Interferon in Chronic Virus Infection and Cancer. Trends Immunol *38*, 542-557. 10.1016/j.it.2017.05.005.

30. Lazear, H.M., Schoggins, J.W., and Diamond, M.S. (2019). Shared and Distinct Functions of Type I and Type III Interferons. Immunity *50*, 907-923. 10.1016/j.immuni.2019.03.025.

31. McNab, F., Mayer-Barber, K., Sher, A., Wack, A., and O'Garra, A. (2015). Type I interferons in infectious disease. Nat Rev Immunol *15*, 87-103. 10.1038/nri3787.

32. Gibbert, K., Schlaak, J.F., Yang, D., and Dittmer, U. (2013). IFN-alpha subtypes: distinct biological activities in anti-viral therapy. Br J Pharmacol *168*, 1048-1058. 10.1111/bph.12010.

33. Kalie, E., Jaitin, D.A., Abramovich, R., and Schreiber, G. (2007). An interferon alpha2 mutant optimized by phage display for IFNAR1 binding confers specifically enhanced antitumor activities. J. Biol. Chem. *282*, 11602-11611. 10.1074/jbc.M610115200.

34. Gibson, D.G., Benders, G.A., Andrews-Pfannkoch, C., Denisova, E.A., Baden-Tillson, H., Zaveri, J., Stockwell, T.B., Brownley, A., Thomas, D.W., Algire, M.A., et al. (2008). Complete chemical synthesis, assembly, and cloning of a Mycoplasma genitalium genome. Science *319*, 1215-1220. 10.1126/science.1151721.

35. Gibson, D.G., Young, L., Chuang, R.Y., Venter, J.C., Hutchison, C.A., 3rd, and Smith, H.O. (2009). Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat Methods *6*, 343-345. 10.1038/nmeth.1318.

36. Edwards, R.A., Keller, L.H., and Schifferli, D.M. (1998). Improved allelic exchange vectors and their use to analyze 987P fimbria gene expression. Gene *207*, 149-157. 10.1016/s0378-1119(97)00619-7.

37. Krasnopolsky, S., Kuzmina, A., and Taube, R. (2020). Genome-wide CRISPR knockout screen identifies ZNF304 as a silencer of HIV transcription that promotes viral latency. PLoS Pathog. *16*, e1008834. 10.1371/journal.ppat.1008834.

38. Tejeda-Dominguez, F., Huerta-Cantillo, J., Chavez-Duenas, L., and Navarro-Garcia, F. (2017). A Novel Mechanism for Protein Delivery by the Type 3 Secretion System for Extracellularly Secreted Proteins. mBio *8*. 10.1128/mBio.00184-17.

39. Navarro-Garcia, F., Serapio-Palacios, A., Vidal, J.E., Salazar, M.I., and Tapia-Pastrana, G. (2014). EspC promotes epithelial cell detachment by enteropathogenic Escherichia coli via sequential cleavages of a cytoskeletal protein and then focal adhesion proteins. Infect Immun *82*, 2255-2265. 10.1128/IAI.01386-13.

40. Deng, W., Li, Y., Hardwidge, P.R., Frey, E.A., Pfuetzner, R.A., Lee, S., Gruenheid, S., Strynakda, N.C., Puente, J.L., and Finlay, B.B. (2005). Regulation of type III secretion hierarchy of translocators and effectors in attaching and effacing bacterial pathogens. Infect. Immun. *73*, 2135-2146. 10.1128/IAI.73.4.2135-2146.2005.

41. Elliott, S.J., Sperandio, V., Giron, J.A., Shin, S., Mellies, J.L., Wainwright, L., Hutcheson, S.W., McDaniel, T.K., and Kaper, J.B. (2000). The locus of enterocyte effacement (LEE)-encoded regulator controls expression of both LEE- and non-LEE-encoded virulence factors in enteropathogenic and enterohemorrhagic Escherichia coli. Infect Immun *68*, 6115-6126. 10.1128/IAI.68.11.6115-6126.2000.

42. Kenny, B., Abe, A., Stein, M., and Finlay, B.B. (1997). Enteropathogenic Escherichia coli protein secretion is induced in response to conditions similar to those in the gastrointestinal tract. Infect Immun *65*, 2606-2612. 10.1128/iai.65.7.2606-2612.1997.

43. Abadie, V., Kim, S.M., Lejeune, T., Palanski, B.A., Ernest, J.D., Tastet, O., Voisine, J., Discepolo, V., Marietta, E.V., Hawash, M.B.F., et al. (2020). IL-15, gluten and HLA-DQ8 drive tissue destruction in coeliac disease. Nature *578*, 600-604. 10.1038/s41586-020-2003-8.

44. Jong, W.S., ten Hagen-Jongman, C.M., den Blaauwen, T., Slotboom, D.J., Tame, J.R., Wickstrom, D., de Gier, J.W., Otto, B.R., and Luirink, J. (2007). Limited tolerance towards folded elements during secretion of the autotransporter Hbp. Mol Microbiol *63*, 1524-1536. 10.1111/j.1365-2958.2007.05605.x.

45. Klaus, W., Gsell, B., Labhardt, A.M., Wipf, B., and Senn, H. (1997). The three-dimensional high resolution structure of human interferon alpha-2a determined by heteronuclear NMR spectroscopy in solution. J Mol Biol *274*, 661-675. 10.1006/jmbi.1997.1396.

46. Bardwell, J.C., McGovern, K., and Beckwith, J. (1991). Identification of a protein required for disulfide bond formation in vivo. Cell *67*, 581-589. 10.1016/0092-8674(91)90532-4.

47. Li, Y., Batra, S., Sassano, A., Majchrzak, B., Levy, D.E., Gaestel, M., Fish, E.N., Davis, R.J., and Platanias, L.C. (2005). Activation of mitogen-activated protein kinase kinase (MKK) 3 and MKK6 by type I interferons. J Biol Chem *280*, 10001-10010. 10.1074/jbc.M410972200.

48. Van Winkle, J.A., Constant, D.A., Li, L., and Nice, T.J. (2020). Selective Interferon Responses of Intestinal Epithelial Cells Minimize Tumor Necrosis Factor Alpha Cytotoxicity. J Virol *94*. 10.1128/JVI.00603-20.

49. Ciesielska, A., Matyjek, M., and Kwiatkowska, K. (2021). TLR4 and CD14 trafficking and its influence on LPS-induced pro-inflammatory signaling. Cell Mol Life Sci *78*, 1233-1261. 10.1007/s00018-020-03656-y.

50. Torkashvand, F., and Vaziri, B. (2017). Main Quality Attributes of Monoclonal Antibodies and Effect of Cell Culture Components. Iran Biomed J *21*, 131-141. 10.18869/acadpub.ibj.21.3.131.

51. Goldring, J.P. (2015). Methods to Concentrate Proteins for Protein Isolation, Proteomic, and Peptidomic Evaluation. Methods Mol Biol *1314*, 5-18. 10.1007/978-1-4939-2718-0\_2.

52. Jong, W.S., Soprova, Z., de Punder, K., ten Hagen-Jongman, C.M., Wagner, S., Wickstrom, D., de Gier, J.W., Andersen, P., van der Wel, N.N., and Luirink, J. (2012). A structurally informed autotransporter platform for efficient heterologous protein secretion and display. Microb Cell Fact *11*, 85. 10.1186/1475-2859-11-85.

53. Jaitin, D.A., Roisman, L.C., Jaks, E., Gavutis, M., Piehler, J., Van der Heyden, J., Uze, G., and Schreiber, G. (2006). Inquiring into the differential action of interferons (IFNs): an IFN-alpha2 mutant with enhanced affinity to IFNAR1 is functionally similar to IFN-beta. Mol Cell Biol *26*, 1888-1897. 10.1128/MCB.26.5.1888-1897.2006.

54. Kalie, E., Jaitin, D.A., Podoplelova, Y., Piehler, J., and Schreiber, G. (2008). The stability of the ternary interferon-receptor complex rather than the affinity to the individual subunits dictates differential biological activities. J Biol Chem *283*, 32925-32936. 10.1074/jbc.M806019200.

55. Quadt-Akabayov, S.R., Chill, J.H., Levy, R., Kessler, N., and Anglister, J. (2006). Determination of the human type I interferon receptor binding site on human interferon-alpha2 by cross saturation and an NMR-based model of the complex. Protein Sci *15*, 2656-2668. 10.1110/ps.062283006.

56. Dautin, N., Barnard, T.J., Anderson, D.E., and Bernstein, H.D. (2007). Cleavage of a bacterial autotransporter by an evolutionarily convergent autocatalytic mechanism. EMBO J *26*, 1942-1952. 10.1038/sj.emboj.7601638.

57. Harari, D., Abramovich, R., Zozulya, A., Smith, P., Pouly, S., Koster, M., Hauser, H., and Schreiber, G. (2014). Bridging the species divide: transgenic mice humanized for type-I interferon response. PLoS One *9*, e84259. 10.1371/journal.pone.0084259.

58. Iguchi, A., Thomson, N.R., Ogura, Y., Saunders, D., Ooka, T., Henderson, I.R., Harris, D., Asadulghani, M., Kurokawa, K., Dean, P., et al. (2009). Complete genome sequence and comparative genome analysis of enteropathogenic *Escherichia coli* O127:H6 strain E2348/69. J. Bacteriol. *191*, 347-354. 10.1128/JB.01238-08.

59. Shaulov, L., Gershberg, J., Deng, W., Finlay, B.B., and Sal-Man, N. (2017). The Ruler Protein EscP of the Enteropathogenic Escherichia coli Type III Secretion System Is Involved in Calcium Sensing and Secretion Hierarchy Regulation by Interacting with the Gatekeeper Protein SepL. mBio *8*. 10.1128/mBio.01733-16.

60. Wilmaerts, D., Dewachter, L., De Loose, P.J., Bollen, C., Verstraeten, N., and Michiels, J. (2019). HokB Monomerization and Membrane Repolarization Control Persister Awakening. Mol Cell *75*, 1031-1042 e1034. 10.1016/j.molcel.2019.06.015.