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Co-Expression of a Chimeric Protease Inhibitor Secreted by a Tumor-Targeted *Salmonella* Protects Therapeutic Proteins from Proteolytic Degradation

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Abstract

Sunflower trypsin inhibitor (SFTI) is a 14-amino-acid bicyclic peptide that contains a single internal disulfide bond. We initially constructed chimeras of SFTI with N-terminal secretion signals from the *Escherichia coli* OmpA and *Pseudomonas aeruginosa* ToxA, but only detected small amounts of protease inhibition resulting from these constructs. A substantially higher degree of protease inhibition was detected from a C-terminal SFTI fusion with *E. coli* YebF, which radiated more than a centimeter from an individual colony of *E. coli* using a culture-based inhibitor assay. Inhibitory activity was further improved in YebF-SFTI fusions by the addition of a trypsin cleavage signal immediately upstream of SFTI, and resulted in production of a 14-amino-acid, disulfide-bonded SFTI free in the culture supernatant. To assess the potential of the secreted SFTI to protect the ability of a cytotoxic protein to kill tumor cells, we utilized a tumor-selective form of the *Pseudomonas* ToxA (OTG-PE38K) alone and expressed as a polycistronic construct with YebF-SFTI in the tumor-targeted *Salmonella* VNP20009. When we assessed the ability of toxin-containing culture supernatants to kill MDA-MB-468 breast cancer cells, the untreated OTG-PE38K was able to eliminate all detectable tumor cells, while pretreatment with trypsin resulted in the complete loss of anti-cancer cytotoxicity. However, when OTG-PE38K was co-expressed with YebF-SFTI, cytotoxicity was completely retained in the presence of trypsin. These data demonstrate SFTI chimeras are secreted in a functional form and that co-expression of protease inhibitors with therapeutic proteins by tumor-targeted bacteria has the potential to enhance the activity of therapeutic proteins by suppressing their degradation within a proteolytic environment.

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Conflict of Interest

DB has financial interest in Aviex Technologies and Magna Therapeutics, and receives royalties from Yale University. DB and DQ have interest in a patent application that includes subject materials from this report.

Supplementary data for this paper are available on-line only at <http://jmb.or.kr>.

Keywords

Protease inhibitors; sunflower trypsin inhibitor (SFTI); YebF; VNP20009; tumor-targeted *Salmonella*; OTG-PE38K

Introduction

Production of heterologous proteins by prokaryotic and eukaryotic microbes is often limited by proteolysis. Two genetic approaches to limiting proteolytic degradation in heterologous protein expression systems that have been employed are the elimination of endogenous proteases through deletion mutations [1], or by generating protease resistant forms by altering protease sensitive regions of the heterologous protein [2]. Co-expression of secreted protease inhibitors has not been previously explored as a mechanism to protect heterologous proteins from proteolytic degradation.

Protease inhibitors have a wide range of therapeutic and biotechnological applications, from their uses in reducing blood pressure and treating HIV infections [3], to their application in protein purification [4]. Due to these therapeutic applications and their widespread use as a tool in biotechnology, large-scale production of protease inhibitors has been investigated using microbial systems. Examples include the surface display of aprotinin by *Escherichia coli*, which was engineered as a fusion to the diffuse adherence protein AIDA-I from pathogenic *E. coli* [5]. Under reducing conditions the fusion was transported to the cell surface in a functional form. Another example is α_1 -proteinase inhibitor, which is used in a replacement therapy for human α_1 -proteinase inhibitor deficiency associated with progressive emphysema. α_1 -Proteinase inhibitor has been cloned and expressed as a functional form in the fungus *Aspergillus niger* [6, 7]. The purified protein produced by the fungus is glycosylated similarly to the endogenous human protein and retains most of the inhibitory activity associated with its human counterpart isolated from plasma, and thus has potential as a production source for use in human therapy. Microbially produced protease inhibitors are also being explored as components of live therapeutic vectors. Elafin, a human inhibitor of neutrophil elastase, has been cloned and expressed in a secreted form by the gram-positive bacteria *Lactococcus lactis* and *Lactobacillus casei* as a potential treatment of Crohn's disease [8].

Several genera of live bacteria including *Salmonella* are being developed for their ability to target solid tumors by systemic administration [9, 10]. Attenuated strains of *Salmonella* preferentially replicate in murine tumors, accumulate to levels 1000 times greater than any other tissue and show strong antitumor effects [11–13]. The attenuated strain VNP20009 was extensively evaluated preclinically where it was shown to be both safe and effective against murine tumor models [14, 15] and has been evaluated in human clinical trials for safety and efficacy against melanoma and renal tumors [16]. Although a safe dose of the bacteria was established in humans and the bacteria were detected within some tumors, no antitumor activity was associated with colonized human tumors. This has indicated the need to develop mechanisms to increase the bacterial cytotoxicity towards cancer cells in order to enhance their therapeutic effect. Examples of such approaches have included bacterial

expression of prodrug converting enzymes [17–21], cytokines such as IL1 β [22] IL2 [23], LIGHT [24], CLL21 [25], IL18 [26], TNF α [27], TRAIL [28], antiangiogenic proteins such as endostatin [29, 30] and thrombospondin [31], delivery of the anti-immune suppressive siRNAs for IDO [32, 33], STAT [30, 35, 35] and bcl2 [36], expression of cytotoxic and apoptosis inducing proteins [37–42] and expression of immunotherapeutic proteins such as flagellin [43, 44]. Other approaches have also included the development of strains that are intrinsically more toxic to tumor cells, such as the strain A1-R [45–47], which does not express any foreign proteins.

Sunflower trypsin inhibitor (SFTI) is the smallest known member of the Bowman-Birk family of inhibitors [48], and is capable of inhibiting trypsin and chymotrypsin as well as tumor-associated matriptases [49]. Inhibition of matriptase has recently been shown to abrogate tumor progression [50]. SFTI is composed of a 14-amino-acid chain, GRCKTSIPPICFPD, which is cyclized head to tail and is disulfide bonded at cysteines 3 and 11. Interestingly, in one study, the open chain (non-head-to-tail cyclized), disulfide-bonded form was found to be a more potent inhibitor than the bicyclic form [51].

In the present study we explore construction of fusion proteins between the sunflower trypsin inhibitor (SFTI) with bacterial N-terminal hydrophobic secretion signal peptides from OmpA and ToxA and C-terminal fusions with the YebF carrier protein [52] in order to generate gram-negative bacteria with the ability to secrete biologically active protease inhibitors. The N-terminal fusions were also co-expressed with the colicin E3 lysis peptide, which releases proteins from the periplasm and has significantly improved secretion of a therapeutic protein [41]. The C-terminal fusions were further modified with the protein cleavage signal consisting of leucine and lysine (LK) amino acids to effect release of SFTI from the YebF carrier. We further tested the tumor-targeted *Salmonella* VNP20009's ability to secrete a chimeric protease inhibitor as a means of protecting the therapeutic potential of proteins secreted within a proteolytic environment such as that of a tumor [53]. We demonstrate in vitro that a polycistronic construct expressing the therapeutic chimeric EGFR-targeted *Pseudomonas* ToxA OTG-PE38K [41] and a secreted protease inhibitor is capable of protecting the tumor-selective toxin from a proteolytic challenge by trypsin.

Materials and Methods

Strains, Plasmids and Culture Media

The *Escherichia coli* strain EC100 (Epicentre, Madison, WI; *F*[−] *mcrA* Δ (*mrr-hsdRMS-mcrBC*) Φ 80*dlacZ* Δ *M15* Δ *lacX74* *recA1* *endA1* *araD139* Δ (*ara, leu*)7697 *galU* *galK* λ [−] *rpsL* (*Str*^R) *nupG*) was used for cloning and the *E. coli* strain JW2197–1 Δ (*araD-araB*)567 Δ *lacZ478* λ ::*rrnB-3*) λ [−] Δ *eco-762::kan* *rph-1* Δ (*rhaD-rhaB*)568 *hsdR51*), which is deleted in the periplasmic protease inhibitor ecotin [54] was used for expression in order to avoid any endogenous protease inhibitor activity. A plasmid encoding YebF, pAES40, was purchased from Athena Environmental Sciences, Inc. (Baltimore, MD). pTet99a is a tetracycline inducible plasmid derived from pTrc99a (GenBank [U13872.1](#) [55]) by substituting a chemically synthesized tet operon consisting of bases 225 to 928 of GenBank [AY528506.1](#) (modified with A618G and T627A and flanked by NdeI and NotI upstream and NcoI downstream restriction sites; Genscript, USA), and cloned into the NdeI and NcoI sites of

pTrc99a to replace the pTrc promoter (pDB221; Table 1). The plasmid pAra99a is a derivative of pTrc99a described by Quintero *et al.* [41] for arabinose inducible expression. *Salmonella* enterica serotype Typhimurium strains used were JR501 (*hsdSA29 hsdSB121 hsdL6 metA22 metE551 trpC2 ilv-452 H1-b H2-e,n,x* (cured of Fels 2) *fla-66 nml rpsL120 xyl-404 galE719* (*Salmonella* Genetic Stock Center, Calgary, Canada, strain 1593 [56], which is a restriction (–) methylation (+) strain, and attenuated *Salmonella* enterica serotype Typhimurium strain VNP20009 from the American Type Culture Collection (Manassas, VA; strain 202165, a.k.a. 41.2.9 or YS1646 [15]) a *purl*, *msbB*, *Suwwan* strain for which the complete sequence has been determined [57]. All strains were preserved frozen in 15% glycerol at –70°C. Bacteria were grown on LB and CTY with 100 or 50 µg/ml ampicillin respectively, as previously described [41], or on casein protease inhibitor detection medium described below. Induction was conducted by growing a fresh culture overnight, diluting the culture 1:5 in fresh media and allowing 2 h preinduction period, followed by addition of the inducing agent, of either 0.5 µg/ml of anhydrotetracycline hydrochloride (Acros Organics, Geel, Belgium) or 1.0 µg/ml of doxycycline HCl (Alfa Aesar, Ward Hill, MA) for pTet99a constructs (Note: anhydrotetracycline is an inducer but does not have antibiotic properties, while doxycycline at 0.5 µg/ml is below the minimum inhibitory concentration (MIC)), or 1 mg/ml arabinose for pAra99a-expressed constructs.

PCR and Cloning

PCRs were conducted as previously described (39) using a high-fidelity polymerase (Phusion Hot Start II, Fermentas/Thermo Fisher, USA). Briefly, thermocycler settings typically consisted of one cycle of 98°C for 3 min followed by 20–35 cycles of 98°C for 10 sec, variable annealing temperatures (see Table 1) for 30 sec and 72°C for 15–30 sec/Kb, with a final extension of 72°C for 5 min. All primers were purchased from IDT (USA). Products were separated from the polymerase and oligonucleotides using Zymoclean (Zymo Research, Irvine, CA), restriction digests were performed as indicated in Table 1, and separated by gel electrophoresis. The products were extracted from the gel by centrifugal filtration (Merck Millipore, Cork, Ire., Ultrafree MC 0.45 µm PVDF membrane cartridge) and ligated into the appropriate vector. Ligations were transformed into chemically competent EC100 grown on LB-Amp plates, and DNA sequences were determined by Sanger sequencing (Sequetech, USA) using the primers indicated in Table 1. Each DNA construct was determined to be complete and accurate before proceeding to subsequent steps. Electrocompetent JW2197–1 were transformed by electroporation as well as electrocompetent JR501 cells (restriction (–) and methylation (+)), which are used to generate appropriately methylated intermediate clones [56, 58]. The JR501 derived plasmids were then transformed into the attenuated *Salmonella* enterica serotype Typhimurium strain VNP20009 by electroporation.

Cloning of N-terminal Hydrophobic Secretion and YebF Fusion Constructs

The PCR and gene assembly primers used are shown in Table 1. The OmpA signal sequence fused in-frame to SFTI was generated by PCR with oligonucleotides designed online using DNA Works [59], with a first round using the four oligonucleotides 2a–2d, and a second round using primers 2F & 2R, and cloned into pTet99a to generate OmpA-SFTI (pDQ71). The *Pseudomonas* exotoxin A (ToxA) signal sequence (SS) fused in-frame with SFTI was

generated by PCR using the four oligonucleotides 3a-3d, followed by a second round using 3F & 3R, and cloned into pTet99a to generate ToxA-SFTI (pDQ70). The ColE3 lysis protein, including its ribosomal binding site (RBS), bases 2660–2815 of the complete colicin E3 plasmid (GenBank [KM287568](#) [60]), was subcloned from pAra99a-RBS-ColE3-lysis construct [41] using *Xba*I and *Pst*I into OmpA-SFTI and ToxA-SFTI resulting in OmpA-SFTI E3 lysis (pDQ75) and the ToxA-SFTI E3 lysis (pDQ76). A YebF PCR product was generated using the primers 6F and 6R with the pAES40 as the template and cloned into pTet using the *Nco*I and *Xba*I restriction sites to result in YebF (pDQ84). SFTI with a trypsin cleavage site consisting of the amino acids leucine and lysine (LK) immediately upstream of SFTI was cloned into the pAES40 vector using the self-annealing primers 7F and 7R digested with *Kpn*I and *Xba*I to result in YebF-^{LK}SFTI (pDQ38). YebF-^{LK}SFTI was regenerated by PCR using the primers 8F and 8R and cloned into pTet99a using *Nco*I and *Xba*I to result in pDQ44. The hexahistidine tag containing YebF-^{LK}SFTI-6H and YebF-SFTI-6H were generated by PCR using pAES40 YebF-^{LK}SFTI (pDQ38) as a template with the primers 9F and 9R, and 10F and 10R which were cloned into pTet using *Kpn*I and *Xba*I forming pDQ92 and DQ93. YebF-SFTI was generated by PCR using the primers 11F and 11R with YebF-SFTI-6H as a template and cloned into pTet99a (pDQ221).

A therapeutic chimeric EGFR-targeted ToxA expression construct [41] that consists of an arabinose inducible promoter expressing an OmpA signal sequence followed in-frame by the TGF α targeting ligand of epidermal growth factor receptor (EGFR), a long flexible linker consisting of the amino acids GGGGS repeated three times, and the truncated *Pseudomonas* ToxA, known as PE38, which has its C-terminal REDLK endoplasmic reticulum retention signal substituted with KDEL, OTG-PE38K (pDQ121) [41] was used for the protection assay. The wild-type toxin pAra-ToxA (pDQ14) described by Quintero *et al.* [41] was also utilized. An arabinose inducible OTG-PE38K polycistronic with YebF-^{LK}SFTI having an independent ribosomal binding site (RBS) was generated by PCR using the primers 13F and 13R with pAES40-SFTI as the template, with the product digested with *Bam*HI and *Xba*I and cloned into the *Bgl*II and *Xba*I sites of OTG-PE38K (pDQ121) [41] to generate OTG-PE38K RBS YebF-^{LK}SFTI (pDQ169). A YebF construct expressed by arabinose induction was generated by subcloning from pDQ84 into pAra99a using *Nco*I and *Xba*I, resulting in pDQ195. A YebF-^{LK}SFTI construct expressed by arabinose induction was generated by subcloning from pDQ44 into pAra99a using the enzymes *Nco*I and *Xba*I resulting in pDB573. The arabinose inducible constructs were transformed into and expressed in *Salmonella* VNP20009. A brief summary of the constructs and the functions of their components is presented in Table 2.

Plate-Based Assay for Secreted Protease Inhibitors

A culture-based protease inhibitor assay [61] with 0.5% casein was utilized in which the appropriate inducer was incorporated into the media prior to inoculation with bacteria and then grown overnight. Studies in *E. coli* to establish the protease inhibitory properties of the different constructs were done in the protease inhibitor (ecotin) minus strain JW2197–1 to eliminate any potential for protease inhibition due to possible release of periplasmic ecotin. Bacterial growth was washed off and the plates were treated with 1 ml of 0.625 mg/ml trypsin (MP Biomedical 1:250, Solon, OH) and antibiotics, then allowed to digest overnight.

The plates were then stained with the protein dye Ponceau S (0.1% Ponceau S (Sigma Aldrich; USA) in 5% acetic acid (ThermoFisher) in water) for 1 h, and finally destained in dH₂O to observe zones of protease inhibition.

N_α-Benzoyl-DL-Arginine p-Nitroanilide Hydrochloride (BAPNA) Cleavage Inhibition Assay

Protease activity was measured using trypsin cleavage of the substrate N_α-benzoyl-DL-arginine p-nitroanilide hydrochloride (BAPNA; Acros Organics; Geel, Belgium), which results in the liberation of p-nitroanilide that is measured photometrically at 405 nm based upon the protocol from Sigma Aldrich, except that a Tris-HCl (Fisher Scientific; USA) buffer was used as in Rascón *et al.* [62], and the assay was adjusted to a smaller scale and measured in a 96-well format. Trypsin units were defined as the amount of trypsin that cleaves the BAPNA substrate, yielding 1 μmol of p-nitroanilide per minute at room temperature. A synthetic, 14-amino-acid, disulfide-bonded, non-head-to-tail cyclized form, SFTI peptide was synthesized by Genscript (USA) and used as a standard. The peptide was trifluoroacetate-free with greater than 95% purity. Trypsin was then used to create a standard inhibition curve using dilutions of the synthetic SFTI standard, where one inhibitor unit was defined as the amount of inhibitor that inhibited one trypsin unit by 50%. Inhibitory activity of supernatants from *E. coli* JW2197–1 or VNP20009 harboring YebF, YebF-SFTI or YebF-LK^{SFTI} were assessed for protease inhibition utilizing the standard inhibition curve. A media-only background measurement was subtracted from the inhibition measurement for each supernatant. Each assay was done in duplicate and repeated three times.

Ammonium Sulfate Precipitation and Desalting

Ammonium sulfate precipitation was used to concentrate the secreted YebF protease inhibitor chimeric fusions from culture supernatants of *E. coli* for reverse zymography (described below). Induced cultures (100 ml, or if a smaller volume subsequent, then steps scaled appropriately) were grown overnight and cleared by centrifugation. Ammonium sulfate powder (Fisher Scientific) was slowly added to the supernatants at 4°C to 50% saturation with constant stirring, and allowed to stand 4 h to overnight. The 50% saturated supernatant was centrifuged at 8,000 × *g* for 30 min at 4°C to remove the precipitate, which was determined to have no inhibitory activity by the plate-based inhibitor assay. The 50% saturated supernatant was then brought to 80% saturation with ammonium sulfate at 4°C, and again allowed to stand overnight, followed by collection of the precipitate by centrifugation. The 50–80% fraction was screened by the plate-based inhibition assay and was shown to have potent inhibitory activity.

The pellets were dissolved in 700 μl of ultrapure PBS (HyClone Laboratories, Inc, USA), and were passed through a 50 mM ammonium bicarbonate-(Fisher Scientific) equilibrated G10 column (GE Healthcare, Ruckinghamshire, UK) by gravity according to the manufacturer's protocol, and collected as droplet fractions. Protease activity of the droplets was initially assayed using 2 μl of each fraction spotted onto a protease inhibition plate and developed as described above. The fractions representing the maximal activity were assayed by reverse zymography.

Reverse Zymography

Reverse zymography [63] was performed using SDS-PAGE with a Tris-tricine buffer system for low molecular weight peptides [64]. An 18% polyacrylamide gel (Fisher Scientific) was co-polymerized with 0.1% (w/v) gelatin, 0.75 M Tris-HCl (pH 8.4) (Fisher Scientific) and 0.1% (w/v) SDS (MP Biomedicals) and run at a constant current of 75 V. Due to the very low molecular weight of the SFTI peptide (approx. 1.5 kDa), the $10 \times 8 \times 0.075$ cm gel was only run $\sim\frac{1}{2}$ of the gel length and immediately washed with 2.5% Triton X-100 (Sigma Aldrich) to remove SDS, washed with water and then digested with trypsin-containing buffer ((4.5 mg/ml trypsin in 10 mM Tris-HCl pH 7.6, 200 mM NaCl (Fisher Scientific), 10 mM CaCl_2 , 0.02% (w/v) Brij-35 (Fisher Scientific)) for 3 h and then stained with Coomassie Brilliant Blue R250 (Protea Biosciences; Morgantown, WV).

Affinity Purification, Liquid Chromatography and Mass Spectrometry

Biologically active protease inhibitory peptides were affinity purified from induced culture supernatants using agarose resin immobilized trypsin (Pierce/ThermoFisher) having a specific activity of 200 TAME units per ml of gel supplied as a 50% slurry. A 250 μl aliquot of the slurry was added to 25 ml of induced culture supernatant and allowed to bind for 2 h with gentle rocking. The agarose resin was separated from the supernatant by centrifugation at $3,200 \times g$ for 30 min. All subsequent liquid reagents were filtered through a $0.2 \mu\text{m}$ high protein binding nitrocellulose filter to remove any contaminating proteins prior to use. The pellet was resuspended with 100 mM ammonium bicarbonate followed by re-centrifugation, repeated a total of 5 times. The washed resin was then resuspended in 20 mM Tris-HCl pH 8.5 and then washed with MilliQ water twice. The peptides were batch eluted with 1 volume of 0.01 N HCl with 0.3 M KCl, pH 1.8, and then centrifuged to allow removal of the supernatant, which was then neutralized with the ammonium bicarbonate 1:5 (v/v), followed by re-addition of the elution buffer to the pellet, repeated a total of 5 times. The BAPNA assay was used to detect protease inhibitory activity and the eluted proteins were analyzed by reverse phase liquid chromatography followed by electrospray ionization mass spectrometry (ESI-MS) using a Q-Exactive-Orbitrap spectrometer (ThermoFisher) in order to provide precise molecular weights.

Trypsin Protection and Cytotoxicity Assays

In an initial study, the wild-type ToxA expressed by VNP20009 was used to demonstrate sensitivity to trypsin and protection by a SFTI culture supernatant (data not shown). Subsequently, the tumor-selective toxin OTG-PE38K was used to assess the ability of the secreted form of SFTI to protect this agent from proteolytic degradation by trypsin. The OTG-PE38K is the same construct described by Quintero *et al.* [41], which is inducible by arabinose. The OTG-PE38K and OTG-PE38K RBS YebF- L^{K} SFTI constructs expressed in VNP20009 were used to generate the induced culture supernatants tested after overnight growth with induction ($\text{OD}_{600} = 2.4$), with arabinose induced YebF and YebF- L^{K} SFTI supernatants assessed as comparators. The trypsin challenges consisted of 2 h digests of the supernatants with 400 ng/ml trypsin at 37°C , followed by cytotoxicity assays using EGFR positive MDA-MB-468 breast cancer cells seeded as 2500 cells per well in 96-well plates. Cell survival was assessed using the MTT (methylthiazol diphenyltetrazolium; Calbiochem,

USA) assay [65] at 72 h as previously described [41]. Data were normalized to a no-treatment control (100%).

Statistical Analysis

The results were analyzed using a one-way ANOVA followed by post hoc Tukey HSD test and one-way t-tests using Prism software 6.0f (Graphpad, USA).

Results

Protease Inhibition by SFTI Expressed as Fusions to Hydrophobic Secretion Signals

Diagrams of the constructs generated using N-terminal hydrophobic signal sequences from OmpA and ToxA, with and without co-expression of the ColE3 lysis protein, are shown in Fig. 1, left panel. The arrangement of the *E. coli* strain JW2197–1 colonies on the protease detection plate prior to development are shown in Fig. 1, top right panel, and the post-development image showing the absence or presence of protease inhibition (red zones) is shown in Fig. 1, lower right panel. The protease inhibition assay shows that the induced pTet99a empty vector colony has no protease inhibitory activity (colony 1), while the OmpA-SFTI (colony 2) and ToxA-SFTI (colony 3) show faint red Ponceau S staining zones of inhibition. The OmpA-SFTI E3 lysis (colony 4) shows a greater zone of inhibition than the OmpA-SFTI alone, but the ToxA-SFTI E3 lysis (colony 5) does not as compared to ToxA-SFTI alone.

Protease Inhibition by SFTI Expressed as YebF Fusions With and Without a Trypsin Cleavage Signal

Diagrams of the constructs generated using C-terminal fusions of SFTI to YebF, with and without the trypsin cleavage signal LK, are shown in Fig. 2, panel A. The results in Fig. 2B show no staining for the pTet99a empty vector or for YebF, and strong staining for the YebF-SFTI, indicating the presence of protease inhibition when expressed in *E. coli*. The Ponceau S stained protease inhibition results from *Salmonella* VNP20009 strain are shown in Fig. 2C. Compared with the *E. coli* results in Fig. 2B, the *Salmonella* staining for the YebF-SFTI (Fig. 2C) is not as strong. However, when the YebF-SFTI is compared to YebF^{LK}SFTI (both in VNP20009) as shown in Fig. 2D, the addition of the trypsin cleavage signal (LK) resulted in an observably larger zone of inhibition. Colony growth before protease inhibition staining is shown in Fig. S1. The zone of inhibition was also larger in *E. coli* for the YebF^{LK}SFTI-6H;pDQ92) as compared with the hexahistidine fusion to SFTI without the LK trypsin signal (YebF-SFTI-6H; pDQ93; Fig. S3). When culture supernatants were assayed for protease inhibition by the BApNA assay, the total inhibitory activity was also greater for the YebF^{LK}SFTI construct in both the *E. coli* strain JW2197–1 and in VNP20009 (Fig. 3), with the standard curve having R² value of 0.9916 (Fig. 3A), and mean values with SEM shown in Fig. 3B ($p < 0.0001$; ****). These data also show that protease inhibition was greater in the *E. coli* strain, which is in agreement with the semi-quantitative result shown in Fig. 2.

Composition of the SFTI Peptides Produced as YebF Fusions

The larger size of the inhibition zone produced by the plate assay is consistent with either a greater amount of inhibitor being produced by the YebF-LK-SFTI construct, or a smaller product being produced, as it has also been shown that diffusion of protease inhibitors on casein plates correlates inversely with the size of the inhibitor [61]. As an initial assessment of the size of the SFTI peptides produced, reverse zymography of ammonium sulfate precipitated material was used. As shown in Fig. 4 top panel, the chemically synthesized SFTI peptide runs as a small diffuse band below 1.7 kDa, while the YebF-SFTI products ran at slightly higher positions with a major lower molecular weight band and a minor higher molecular weight band. However, the YebF-LK-SFTI-derived peptide ran at what appeared to be the same size as the chemically synthesized peptide, suggesting cleavage from the YebF carrier was occurring. In order to more accurately determine the composition of the peptides produced, mass spectrometry of affinity-purified peptides was used (Fig. 4; bottom panel). As shown in Fig. 4, bottom panel section A, no significant peptides bound to the trypsin resin were detected in the empty vector control. Fig. 4, bottom panel section B shows the mass spectrometry result from the synthetic, disulfide-bonded peptide, having a molecular weight of 1530.73 Daltons as determined by mass spectrometry, which closely matches the theoretical molecular weight of 1531.81 Daltons at pH 7.0. As shown in Fig. 4, bottom panel section C, the YebF-SFTI construct resulted in multiple products ranging in sizes from approximately 2519 to 2831 Daltons, which would correspond to peptides of 24 and 25 amino acids, with additional nonspecific products that also bound to the trypsin resin. However, as shown in Fig. 4; bottom panel section D, the YebF-LK-SFTI construct resulted in a primary product of 1530.74 Daltons, which most closely matches the purified disulfide-bonded SFTI from Fig. 5B and had a nearly identical empirical molecular weight of 1530.73 Daltons. In addition, there was a small amount of an approximately 1548 Dalton peak that may represent a secondary oxidation product.

Protection of a Cancer Cell-Selective Cytotoxin from Proteolytic Degradation

The culture supernatants of VNP20009 harboring four different constructs were tested for their anticancer cytotoxicity against MDA-MB-468 breast cancer cells. Protease inhibition of the constructs is shown in Fig. S4. The two toxin-containing constructs expressed in *Salmonella* VNP20009 are shown in Fig. 5A, which consist of the OTG-PE38K and the OTG-PE38K RBS YebF-LK-SFTI. The YebF and YebF-LK SFTI (Fig. 5B, 1 and 2) had little effect on the MDA-MB-468 breast cancer cells. The OTG-PE38K was able to eliminate all detectable cancer cells as shown by Fig. 5B, 3, but completely lost cytotoxicity with trypsin pretreatment, as shown by Fig. 5B, 4 ($p < 0.001$). However, as shown by Fig. 5B, 5, the OTG-PE38K RBS YebF-LK-SFTI pretreated with trypsin was also able to completely eliminate all detectable cancer cells and was statistically significant compared to trypsin pretreated OTG-PE38K ($p < 0.001$). This demonstrates the ability of the protease inhibitor to protect the cytotoxic protein from losing tumor cell killing activity in the presence of a protease.

Discussion

Production of functional proteins by microbial cells has long been met with challenges due to proteases, which can interfere with normal protein processing or degrade proteins of interest. The most common approach to solving this problem from the production standpoint is the use of cells that are deficient in one or more proteases. For example, the commonly used *E. coli* strain BL21 is deficient in both the *lon* protease, which is located in the cytoplasm, and the *ompT* protease, which is located in the periplasm, as reviewed by Schmidt [66]. However, this approach only addresses the production of the protein, and not the conditions under which it is used in biotechnology or as a therapeutic agent where the ability of a bacterial vector to colonize the targeted tissue could be compromised by mutational deletion of proteases. One such example is the HtrA family of proteases that play roles in colonization of the host [67]. Another approach in the context of therapeutic proteins has been to engineer the proteins to eliminate specific protease cleavage sites, such as was done with a therapeutic immunotoxin [2]. This latter approach has been successful in increasing resistance to cellular proteases, but as a general approach may have limitations in its applications due to constraints of protein structure-function relationships that would limit altering certain amino acids. In addition, the therapeutic targets of live bacterial vectors encounter a wide range of proteases that may require multiple protease inhibitors and/or mechanisms to effectively overcome. Multiple protease inhibitors could be engineered in concert to meet the requirements of inhibiting multiple proteases.

The secreted protease inhibitors we describe here are the first chimeric protease inhibitors completely secreted by a tumor-targeted bacterium such as VNP20009. The ability to effectively secrete a protease inhibitor by a gram-negative bacterium facilitates additional possibilities for further development of *Salmonella* and other gram-negative bacteria. As we have shown here, a polycistronic construct consisting of an effector gene that selectively kills tumor cells and a protease inhibitor is effective at protecting in a proteolytic challenge against the effector component. This combination has the potential to overcome the lack of antitumor activity of VNP20009 in humans.

YebF is a protein widely distributed among members of the Enterobacteriaceae, and has been previously demonstrated as an effective carrier protein for heterologous secretion [52]. YebF contains a typical N-terminal hydrophobic leader signal, but the mechanism of YebF secretion is also believed to include involvement with the porin proteins OmpF and OmpC [68]. We compared the ability of SFTI fusions with typical hydrophobic signal sequences derived from *E. coli* OmpA and *P. aeruginosa* ToxA (Fig. 1) and fusions with YebF (Fig. 2B) and found that YebF was highly suited for this purpose. We also found that the addition of a trypsin cleavage sequence increased the inhibition zone (Figs. 2C and 2D) and resulted in a higher degree of activity even in the absence of added trypsin (Fig. 3). In the absence of trypsin, the apparent molecular weight of the product was also smaller, and ran at the same apparent size as the synthetic SFTI peptide (Fig. 4, top panel). The active peptides were obtained by affinity purification using resin-bound trypsin, which both confirms their protease-binding activity and facilitates purification of the peptides. Analysis of the peptides by mass spectrometry shows multiple SFTI products in the absence of a trypsin cleavage sequence, with precise cleavage of the peptide from the carrier protein when a trypsin

cleavage sequence was added, resulting in the 14-amino-acid, disulfide-bonded peptide free in the media (Fig. 4; bottom panel). The presence of a wider inhibition zone (Fig. 2) and lower molecular weight of the active product produced by the construct containing the trypsin cleavage site (Fig. 4; bottom panel) indicates that the peptide is cleaved in the absence of added trypsin. We had envisioned that in the presence of its target, trypsin and trypsin-like proteases, cleavage from the carrier protein would occur and activate or enhance activity of the peptide. Therefore, we were surprised when we found that inclusion of the cleavage signal resulted in generation of the free peptide in the absence of the target protease. It is likely that an endogenous trypsin-like protease cleaves the sequence during the secretion process without themselves being inhibited. Additional studies will be required in order to identify this protease. In these studies, the tumor-selective ToxA derivative, OTG-PE38K, consistently killed all detectable EGFR-expressing MDA-MB-468 tumor cells (Fig. 5), which is consistent with earlier studies [41], but was rendered inactive by pre-incubation with trypsin. However, when co-expressed with the YebF^{LK}SFTI, the ability to kill cancer cells was completely retained in the presence of trypsin. These results suggest that expression of protease inhibitors such as SFTI have the potential to enhance the tumor cell killing ability of therapeutic proteins such as OTG-PE38K within the proteolytic environment of the tumor. In vivo assays to assess this potential are expected to be undertaken in the near future that will assess both the safety and efficacy of the system.

Proteases are components of all living cells and are necessary for life. However, overexpression of proteases by cancer cells is one of the primary factors that contributes to cancer metastasis because tumor proteases contribute to the breakdown of the extracellular matrix (ECM) and allows aberrant cells to spread and proliferate [69]. Inhibition of tumor-associated proteases may have multiple functions that could be beneficial. One influence could be to exert an anti-metastatic effect by inhibiting tumor-associated proteolysis of the ECM, limiting degradation of collagen and other proteins and thereby limiting escape of cancer cells into the bloodstream. Some protease inhibitors have been shown to induce apoptosis in cancer cells [70, 71] as well as sensitizing them to TNF- α [70], which could be an added effect of delivery of certain protease inhibitors to the tumor by a tumor-targeted bacterium. Another effect of limiting tumor-associated proteolysis could be to increase the effectiveness of protein-based therapeutics, such as antibodies, through inhibiting their destruction within the tumor. In the context of a tumor-targeting bacterium that releases one or more effector molecules outside of the bacterium into the tumor environment in order to kill cancer cells, protease inhibitors could have the ability to stabilize the effector proteins by limiting their degradation. Therefore, bacterially secreted effector molecules such as the EGFR-targeted ToxA (OTG-PE38K) could have greater stability, and thereby increase the overall antitumor activity that would be exerted within the intratumoral location. It is important to note that this is a substantially different approach than using a systemically administered protease inhibitor. In the case of systemic administration, side effects are more likely to occur and were documented in clinical trials of matrix metalloproteinase inhibitors [72]. Similarly, systemically administered aprotinin has been used as an antifibrinolytic during procedures such as cardiopulmonary bypass surgery [73], but it was later discontinued due to increased risk of complications. As with many drugs, localized delivery

may offer the potential to reduce systemic toxicities. Thus, one aspect of this work is aimed toward the goal of improving antitumor therapy without increasing systemic toxicity.

Matriptases are a major family of tumor proteases associated with cancers, including aggressive breast cancers [74,75]. Matriptase over-expression or its under-modulation by its cognate inhibitor hepatocyte activator inhibitor-1 (HAI-1) [76] leads to activation of several proenzymes involved in tumor cell proliferation such as the urokinase-type plasminogen activator (uPA), which is known to cause tissue remodeling, tumor cell invasiveness and angiogenesis in cancer. HAI-1 also inhibits hepatocyte growth factor (HGF), a mitogen that leads to a motile phenotype, morphogenic changes and also induces tumor angiogenesis [77]. As matriptase controls other key tumor proteases, inhibiting matriptase thereby downregulates other proteases, such as the urokinase protease (uPA) and the matriptase activated receptor-2 (PAR-2), a G-protein coupled receptor involved in cellular adhesion and inflammatory processes [78]. Inhibition of PAR-2 activation decreases the motility of highly metastatic cell lines such as MDA-MB-231 breast cancers that secrete proteases [79]. Thus matriptase represents a promising target for protease inhibition in cancer therapy. Matriptase is often coexpressed with other proteases such as prostasin [80] and further correlates with tumor progression [81]. The approach we have taken here offers the potential to have multiple protease inhibitors expressed simultaneously in order to meet a range of proteases that might be required.

Other pathologies that involve matriptase might also be treated by protease inhibitors [82]. Matriptase is associated with inflammatory bowel disease, and as we have shown here, bacterially secreted SFTI is an effective protease inhibitor that is also known to inhibit matriptase, and therefore may also be useful in the treatment of inflammatory bowel disease.

SFTI is remarkable among protease inhibitors for several reasons. It is the smallest known naturally occurring protease inhibitor; it remains functional in its non-end cyclized form (disulfide bonded only), and it retains activity in its linear form (no end cyclization and no disulfide bonding [83], although disulfide bonding does contribute to its stability) [84]. SFTI has also been shown to be malleable, and multiple forms have been generated with varying target specificities and improved K_i values [85]. SFTI-1 sequences have been permuted to achieve increased inhibitory activity against matriptase and variants have been produced with a 64-fold to a 350-fold increase over wild type [86]. These SFTI-1 mutants may improve the inhibitory activity demonstrated in this study with YebF-^{LK}SFTI. The finding that activity remains high even though they contain only the single disulfide bond (mono-cyclic; *i.e.*, not head to tail cyclized) is also of importance as that structure represents the structure of the SFTI peptide produced by expression of the YebF-^{LK}SFTI construct. Future studies may investigate the expression of other forms of SFTI in order to achieve more potent effects. Other secretion systems might also be used, such as the autodisplay system which can result in either surface display or complete secretion into the medium [87].

Finally, it is also interesting to note that this approach may have applications in other areas, such as the production of biofuels or other fermentations where contamination with proteolytic microorganisms limits the production of the intended products. For example, Schell *et al.* [88] identified *Lactobacillus sp.* in lignocellulosic fermentations producing

ethanol from corn fiber. As the *Lactobacillus sp.* identified included extracellular protease positive species such as *L. plantarum* [89, 90], proteolysis of the extracellular fermentative enzymes could limit production. Incorporation of protease inhibitors into the fermentation process may have the potential to increase product output.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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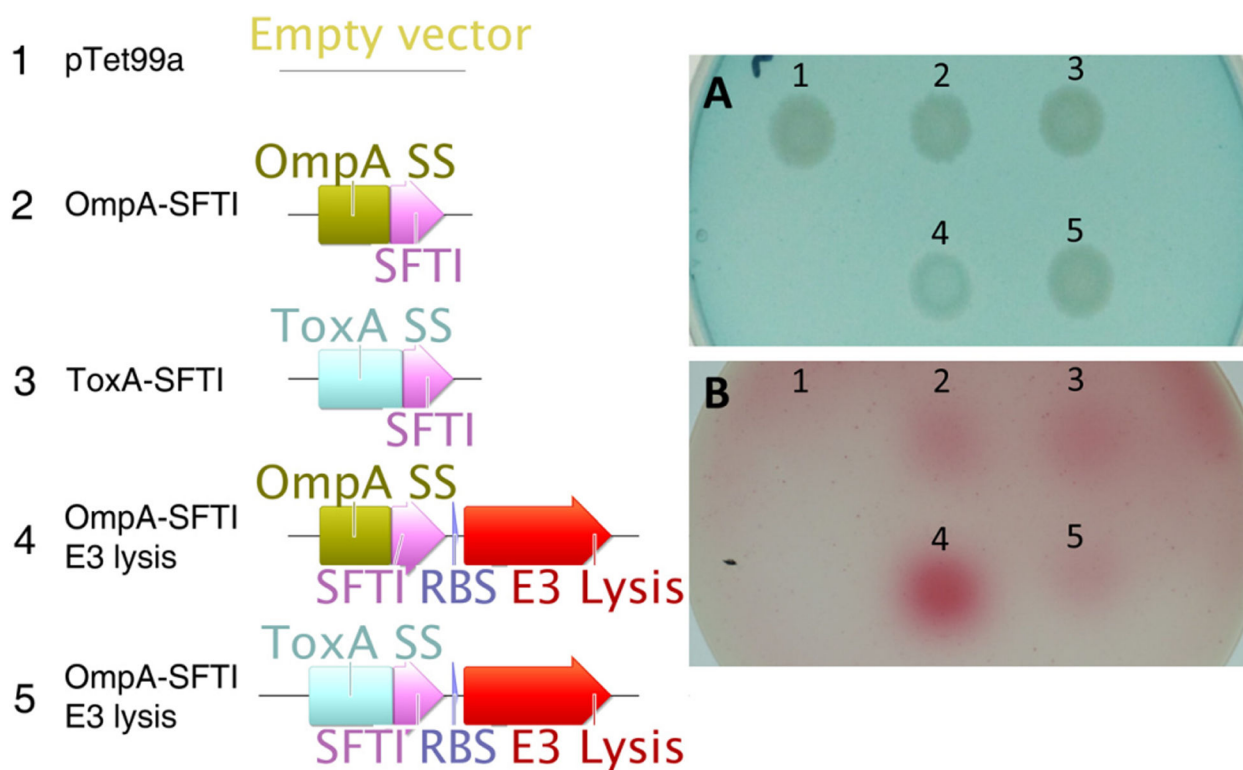


Fig. 1. Comparison of N-terminal hydrophobic secretion signal fusions with sunflower trypsin inhibitor (SFTI).

SFTI +/- the colicin E3 (ColE3) lysis protein was expressed as fusions with N-terminal hydrophobic secretion signals and as a polycistronic construct with a separate ribosomal binding site (RBS) in the pTet99a plasmid, expressed in *E. coli* strain JW2197-1. Left panel showing the DNA constructs. (1) The empty vector; no insert cloned into the pTet99a expression site (pDB221). (2) The SFTI DNA sequence fused to the signal sequence (SS) peptide derived from OmpA; OmpA-SFTI (pDQ71). (3) SFTI fused with the ToxA SS; ToxA-SFTI (pDQ70). (4) OmpA-SFTI and the ColE3 lysis protein; OmpA-SFTI E3 lysis (pDQ75). (5) ToxA-SFTI and the ColE3 lysis protein; ToxA-SFTI E3 lysis (pDQ76). Right panel showing the protease inhibition assay (A) Colonies of *E. coli* harboring 1–5 from panel A growing on a casein petri plate with Tet induction. (B) The protease inhibition staining pattern of 1–5.

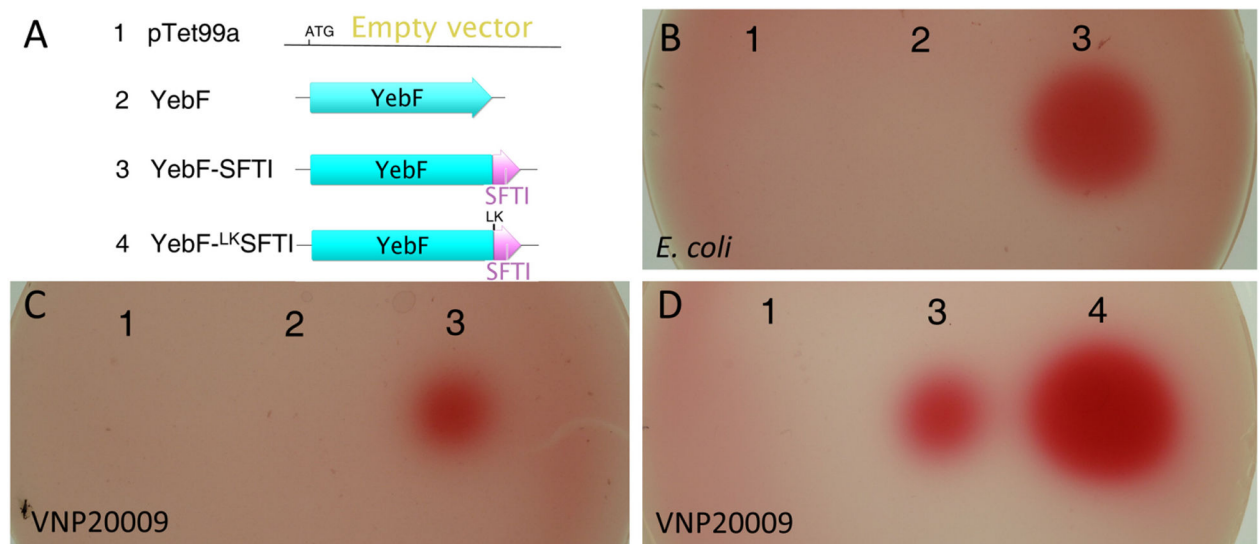


Fig. 2. Detection of secreted protease inhibitory activity by YebF chimeric with SFTI in *E. coli* strain JW2197-1 and *Salmonella* strain VNP20009.

(A) The DNA constructs showing (1) the pTet99a empty cloning vector (pDB221), (2) the YebF construct (pDQ84) and (3) the YebF-SFTI fusion construct engineered without (YebF-SFTI (pDQ221)) and (4) with the trypsin cleavage sequence LK (YebF-LK-SFTI (pDQ44)). (B) A petri plate protease inhibition assay of induced *E. coli* colonies carrying the pTet99a plasmid with (1) pTet99a empty vector, (2) YebF, and (3) YebF-SFTI. (C) A petri plate protease inhibition assay of induced *Salmonella* strain VNP20009 colonies carrying (1) pTet99a empty vector, (2) YebF, and (3) YebF-SFTI. (D) A petri plate protease inhibition assay of induced *Salmonella* strain VNP20009 colonies carrying (1) pTet99a empty vector, (3) YebF-SFTI and (4) YebF-LK-SFTI.

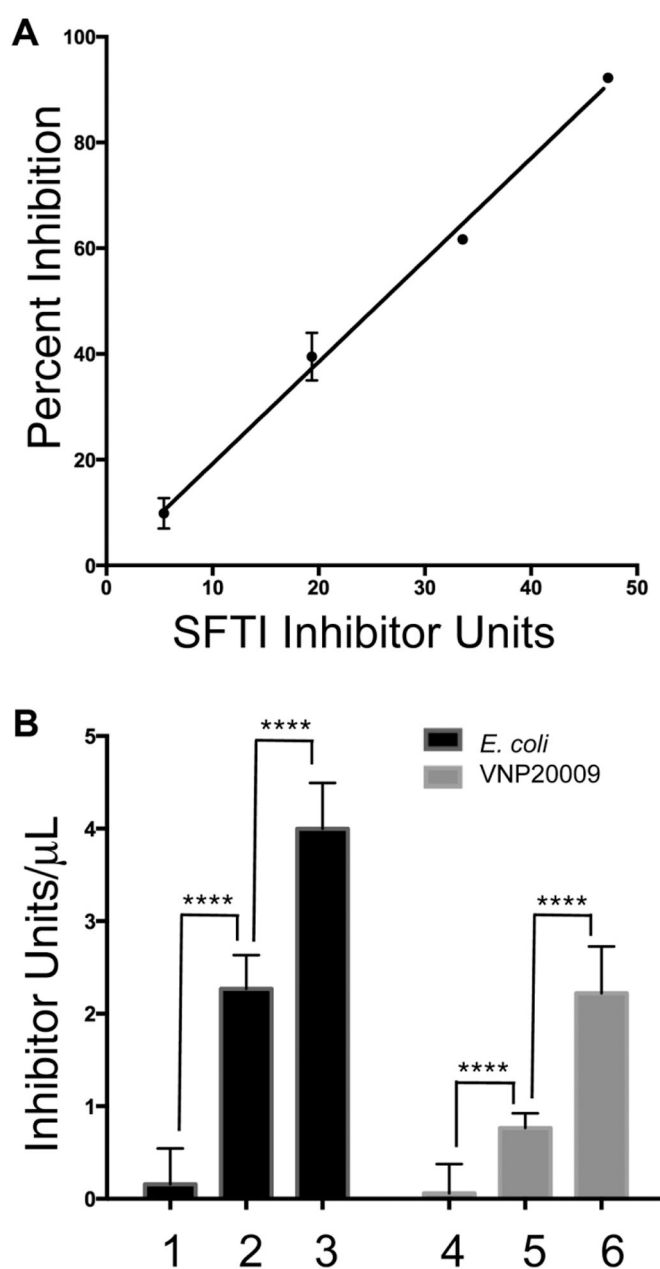


Fig. 3. Protease inhibition of *E. coli* JW2197–1 and VNP20009 strains assessed by the BApNA hydrolysis inhibition assay.

(A) A standard curve constructed using the synthetic SFTI peptide. **(B)** The inhibitory activity of (1) *E. coli* YebF, (2) *E. coli* YebF-SFTI, (3) *E. coli* YebF^{LK}SFTI, (4) VNP20009 YebF, (5) VNP20009 YebF-SFTI, and (6) VNP20009 YebF^{LK}SFTI, each of which had a $p < 0.0001$ (****).

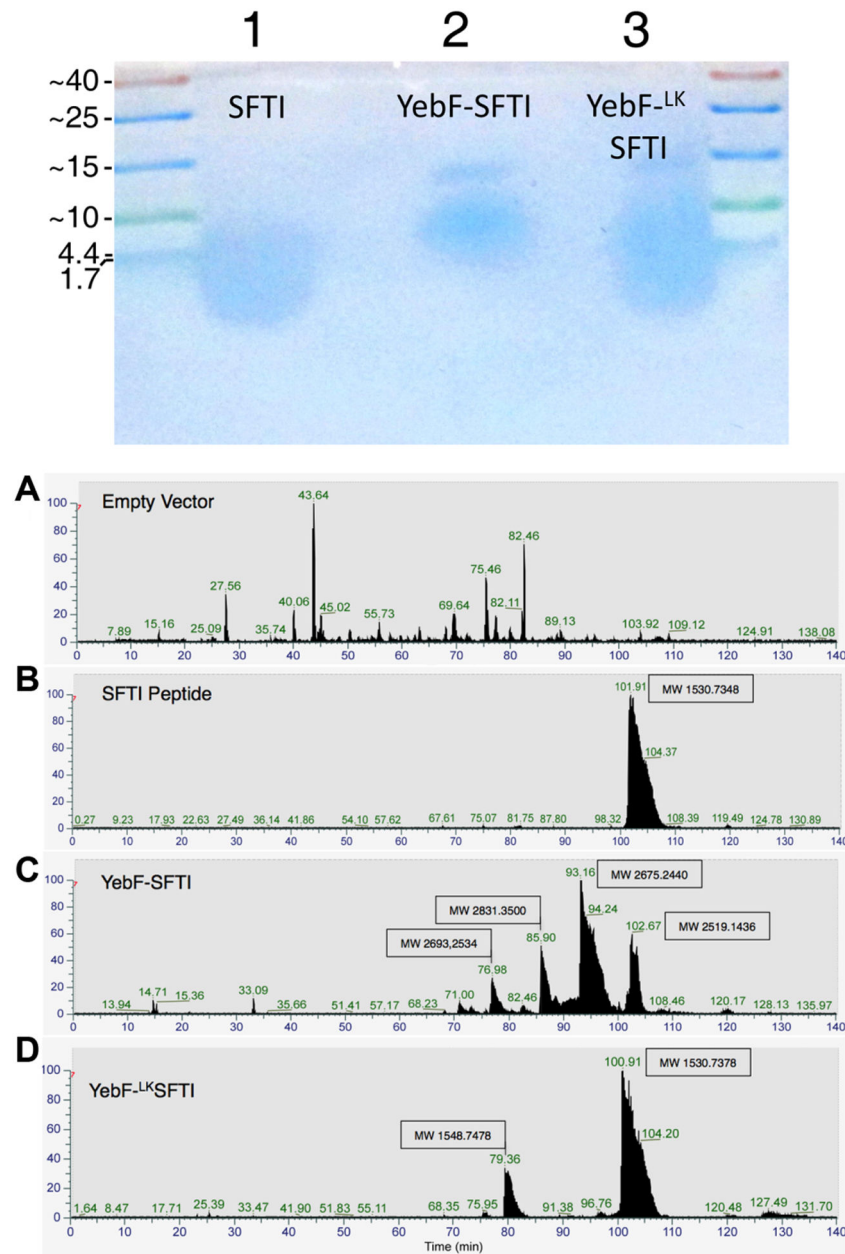


Fig. 4. Protease inhibition of *E. coli* JW2197–1 strains assessed by reverse zymography, and liquid chromatography and mass spectrometry analysis of trypsin-affinity purified peptides produced by *E. coli* strain JW2197–1.

Top panel: Protease inhibition was assessed by non-denaturing polyacrylamide gel electrophoresis containing the protein substrate gelatin of (1) the SFTI peptide, (2) YebF-SFTI, and (3) YebF^{LK}-SFTI, followed by incubation with trypsin and staining with Coomassie brilliant blue. Bottom panel: Purified peptides were fractionated online using reversed phase chromatography followed by ESI-MS determination of molecular weights (enclosed boxes). (A) The pTet99a empty vector strain. (B) The synthetic SFTI peptide. (C) The peptides produced in the absence of an LK trypsin cleavage site through induction of

YebF-SFTI. **(D)** The peptides produced with the LK trypsin cleavage site through induction of YebF-^{LK}SFTI.

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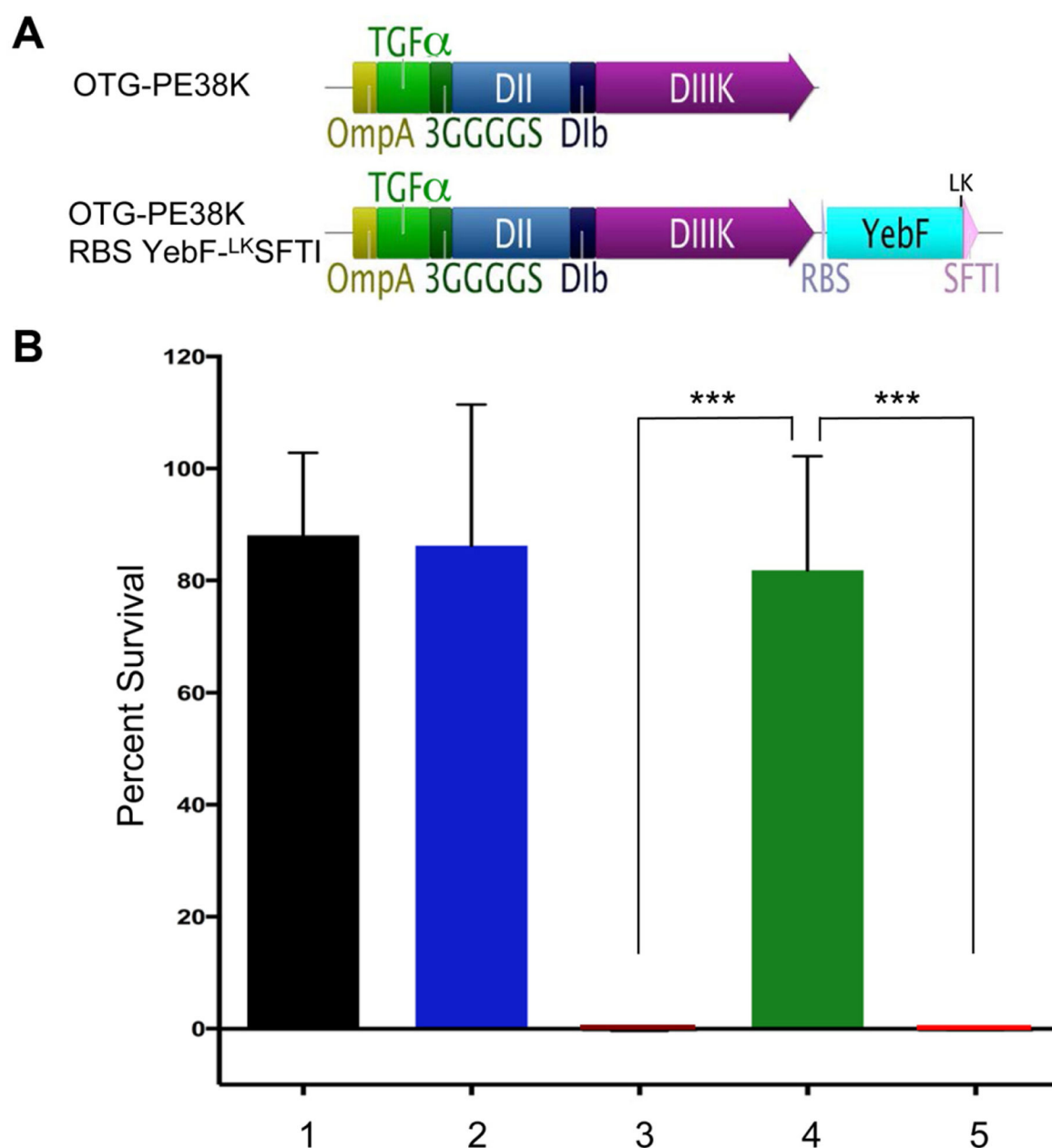


Fig. 5. Protection of a therapeutic protein from trypsin degradation by *Salmonella* VNP20009. In vitro cytotoxicity assays were performed against MDA-MB-468 tumor cells and displayed as the percentage of surviving cells compared with untreated following treatment with cell culture supernatants from *Salmonella* strain VNP20009 containing various DNA constructs. **(A)** The arabinose inducible chimeric *Pseudomonas* exotoxin A OTG-PE38K (upper construct) and the OTG-PE38K RBS YebF^{LK}SFTI (lower construct). **(B)** The percentage of surviving cancer cells treated with (1) YebF, (2) YebF^{LK}SFTI, (3) the OTG-PE38K chimeric *Pseudomonas* ToxA, (4) the OTG-PE38K pretreated with trypsin and (5) the OTG-PE38K RBS YebF^{LK}SFTI pretreated with trypsin (bar for small negative value not drawn to scale). Statistically significant comparisons shown as **** ($p < 0.001$).

PCR, gene assembly, oligonucleotide primers and constructs utilized in this study (n/a; not applicable).

Table 1.

#	Structural component or function (construct name)	DNA/primer sequence (5'-3') or construct description	Anneal temp for PCR	Primer enzyme site (s)	Plasmid construct designation
1	Tetracycline inducible expression plasmid (pTet99a)	Bases 225 to 928 of GenBank AY528506.1 modified with A618G and T627A, flanked by NdeI and NotI upstream and NcoI downstream, cloned into the NdeI and NcoI sites of pTet99a (GenBank U13872.1).	n/a	NdeI and NcoI	pDB221
2	OmpA signal sequence-SFTI fusion (OmpA-SFTI) in pTet99a	1 st Round 2a. GATCCCATGGCTAAATAAGACAGCTATCG 2b. GCTACGGTAGCAACACGACGAGTGGCCACTGCAATC GGATAGCTGCTTTTAGCCATG 2c. CTGGTTTGGTACCGTAGCGCAGGCCGCTCCGAAAG ATGCCGCTGCACCAAAAGCAATC 2d. GATCTCTAGACTAATCCGAAAGCAAAATCGCGGA ATGCTTTTGTGTGCAGCG 2 nd Round 2E. GATCCCATGGCTAAATAAGACAGC 2F. GATCTCTAGACTAATCCGAAAGCAA	62°C	NcoI and XbaI	pDQ71
3	ToxA signal sequence-SFTI fusion (ToxA-SFTI) in pTet99a	1 st Round 3a. GATCCCATGGCTCACCTGAT 3b. CGAGACGCCGAGCGTGGCGACGAGGGGATCCAA TGGGGTATCAGGTGAGCCATGGGAT 3c. GCCTCGCTGCTGCGCGCGGCTCTGTCGCGTCCG CCGCCGAGAAAGCGCTGCACCA 3d. GATCTCTAGACTAATCGGAAAGCAAAATCGCGGA ATGCTTTTGTGTGCAGGGCCCTT 2 nd Round 3E. GATCCCATGGCTCACCTGATA 3R. GATCTCTAGACTAATCCGAAAGCAA	62°C	NcoI and XbaI	pDQ70
4	Addition of an RBS and the ColE3 lysis protein to OmpA-SFTI (OmpA-SFTI E3 lysis) in pTet99a	n/a (Subclone of the E3 lysis protein from the pAra99a-RBS-ColE3- lysis construct; Quintero <i>et al.</i> , 2016.)	n/a	XbaI and PstI	pDQ75
5	Addition of an RBS and the ColE3 lysis protein to pTet-ToxA SS SFTI (ToxA-SFTI E3 lysis)	n/a (Subclone of the E3 lysis protein from the pAra99a-RBS-ColE3- lysis construct; Quintero <i>et al.</i> , 2016.)	n/a	XbaI and PstI	pDQ76
6	YebF in pTet expression vector (YebF)	6F. GATCCCATGGCTAAATAAGAGGGGCGTTTITAG 6R. GATCTCTAGACTAGGTACCCCTTATCGTCATCGTC	58°C	NcoI and XbaI	pDQ84
7	SFTI self-annealing primers for cloning into pAES40 (YebF-LKSFTI)	7F. GATCGGTACCCCTGAAAGGCGCGCTGCACCAAAAGCA TTCCGCGGATT 7R. GATCTCTAGACTAATCCGAAAGCAAAATCGCGGA ATGCTTTTGG	55°C	KpnI and XbaI	pDQ38
8	Cloning the YebF-LKSFTI from pAES40 into pTet99a	8F (6F). GATCCCATGGCTAAATAAGAGGGGCGTTTITAG 8R. CGACTCTAGACTAATCCGGAAG	58°C	NcoI and XbaI	pDQ44
9	Addition of hexahistidine with LK (YebF-LKSFTI-6H) in pTet99a	9F. GATCGGTACCCCTGAAAGGCGCGCTGCACCAAAAGCAATCCGCGGATT 9R. aactagactaATGGTGTGATGATGGTGGGAACCGCCAC CATCCGGAAAGCAAAATCGGC	58°C	KpnI and XbaI	pDQ92
10	Addition of hexahistidine without LK (YebF-SFTI-6H) in pTet99a	10F. GATCGGTACCGCGCGCTGCACCAAAAGCAATCCGCGGATT 10R. aactagactaATGGTGTGATGATGTTGGGAACCGCCAC CATCCGGAAAGCAAAATCGGC	58°C	KpnI and XbaI	pDQ93

#	Structural component or function (construct name)	DNA/primer sequence (5'-3') or construct description	Anneal temp for PCR	Primer enzyme site (s)	Plasmid construct designation
11	Cloning YebF-SFTI without LK (YebF-SFTI) in pTet99a	11a. GATCGGTACCGCGCGCTGCACCAAAAGCAATTCGCGCGAATT 11b. CGACTCTAGACTAATCCGGAAAG	60°C	KpnI and XbaI	pDQ221
12	Chimeric ToxA (OTG-PE38K) in pAra99a	Quintero et al., 2016	n/a	n/a	pDQ121
13	Wild-type ToxA in pAra99a (pAra-ToxA)	Quintero et al., 2016	n/a	n/a	pDQ14
14	OTG-PE38K polycistronic with YebF-L^KSFTI generated by PCR from DQ38 and cloned into DQ121 (pAra-OTG-PE38K-YebF-SFTI)	12F. CTAGGGATCGGTGTTATCGATTAGGACATAGAAATTCAT GAAAAAAGAG 12E. GATCTCTAGACTAATCCGGAAAGCAATTCGCGGAAAT GCTTTTGG	58°C	BamHI (BglII in vector) and XbaI	pDQ169
15	YebF in pAra99a	n/a (Subclone of the YebF from DQ84)	n/a	NcoI and XbaI	pDQ115
16	YebF-L^KSFTI in pAra99a	n/a (Subclone of the YebF-L^KSFTI from DQ44)	n/a	NcoI and XbaI	pDB573
Sequencing Primers					
17	pTet-F1 (external)	TGGGCTGTTAATCACTTTTACTTTT	n/a	n/a	n/a
18	pTet (pTet99a) seq R2	CGGCCAGGCAAAATCTGT	n/a	n/a	n/a
19	YebF-F1	CTTGGGTCAGTTTGCAGGAC	n/a	n/a	n/a
20	YebF-R2	GTCTGTGCAAACTGACCCAAG	n/a	n/a	n/a
21	pAra-F1	ACCTGACGCTTTTATCGCA	n/a	n/a	n/a
22	pAra-R2	CGGCCAGGCAAAATCTGT	n/a	n/a	n/a

Table 2.

A brief summary of the constructs and their components.

Construct name	Description
pTet99a	A tetracycline-inducible expression plasmid
pAra99a	An arabinose-inducible expression plasmid
OmpA-SFTI	A type II signal sequence (derived from OmpA) fused to the sunflower trypsin inhibitor (SFTI)
ToxA-SFTI	A type II signal sequence (derived from the <i>Pseudomonas</i> ToxA) fused to the sunflower trypsin inhibitor (SFTI)
ToxA-SFTI E3 lysis	A type II signal sequence (derived from the <i>Pseudomonas</i> ToxA) fused to the sunflower trypsin inhibitor (SFTI) polycistronic with the lysis protein from colicin E3
YebF	A protein from <i>E. coli</i> used as a secreted fusion carrier protein
YebF-SFTI	The YebF secreted carrier protein fused to the sunflower trypsin inhibitor (SFTI)
YebF-LK-SFTI	The YebF secreted carrier protein fused to the sunflower trypsin inhibitor (SFTI) with the intervening leucine and lysine (LK) amino acids that serve as a trypsin cleavage site
YebF-SFTI-6H	The YebF secreted carrier protein fused to the sunflower trypsin inhibitor (SFTI) followed by a hexahistidine tag (6H)
YebF-LK-SFTI-6H	The YebF secreted carrier protein fused to the sunflower trypsin inhibitor (SFTI) with the intervening amino acids (LK) that serve as a trypsin cleavage site, followed by a hexahistidine tag (6H)
OTG-PE38K	The secreted ToxA fusion with the targeting ligand TGF α .
pAra-ToxA	The wild-type <i>Pseudomonas</i> ToxA expression plasmid under arabinose-inducible control
OTG-PE38K RBS	The secreted ToxA fusion with the targeting ligand TGF α polycistronic, including an independent ribosomal binding site (RBS), with the YebF secreted carrier protein fused to the sunflower trypsin inhibitor (SFTI) with the intervening amino acids LK that serve as a trypsin cleavage site
YebF-LK-SFTI	