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### Publication Date

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UNIVERSITY OF CALIFORNIA, SAN DIEGO

**A Method for Identification of Receptors for YopE in the Type III Secretion (T3S)  
System of *Yersinia pseudotuberculosis***

A Thesis submitted in partial satisfaction of the requirements  
for the degree Master of Science

in

Biology

by

Surina R. Hemani

Committee in charge:

Professor Partho Ghosh, Chair  
Professor Aroian Raffi, Co-Chair  
Professor Emily Troemel

2012

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The thesis of Surina R. Hemani is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California, San Diego

2012

## **DEDICATION**

I lovingly dedicate this thesis to my family:

To my mother, Nergis, for teaching me to balance both my spiritual and worldly aspects of my life. To my father, Rafiq, for his never ending support and confidence in my ability to educate myself far beyond any member of my family. To my brother, Ali, for his admiration and words of wisdom during my years away from home.

To my closest friends for their immense passion for knowledge and for making San Diego a home away from home. To my mentees for reminding me that life without voluntary service is incomplete.

## EPIGRAPH

“How can we appreciate life without enduring the struggles it has to bring.

So let life bring much pleasure, occasional pain, constant success and only the necessary failures. It's one thing to live life; it's another to live with humility and to find your own niche of gratitude, success and serenity.

The people in this world have made you the person you are today; give back tenfold what you have received – you've come so far, you must be needed.”

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## ACKNOWLEDGEMENTS

I would like to acknowledge Professor Partho Ghosh for his tireless efforts as an exceptional research advisor. His intellectual and professional influence has encouraged me to question my findings, expand my knowledge and strive for a strong work ethic. Writing this thesis would not have been possible without the multiple drafts and editing he so kindly devoted time to. I would also like to acknowledge Professor Raffi Aroian and Professor Emily Troemel for their support as members of my committee.

I would like to thank all members of the Partho Ghosh Lab for their relentless support in troubleshooting over the years; the goals of this project would not have been accomplished without their guidance. I've truly appreciated the environment they've created to enjoy learning, inundated with laughter, late night protein purifications and birthday celebrations. I would like to thank Brent for his mentorship since I first entered lab in 2009. I thank Cosmo for his high spirits and poster printing skills and I thank Alicia for helping me transition into this project. I thank Romila for her T3S queen bee skills and her shared "Gossip Girl" passions. I thank Chelsea for her continuous early morning guidance in unfamiliar research territory, and Maher for his generosity. I thank Sanghamitra for her office laughter and late night after-hours Q&A sessions. I thank my fellow Masters student, Matt, for his companionship through the entrance and exit of success and struggle in lab. Finally, I thank Henry, our young blossoming undergraduate of the lab for whom I will never stop forwarding pre-med emails.

It has been a privilege to work amongst such hard-working, inspiring, dedicated, and good-natured colleagues.

ABSTRACT OF THE THESIS

**A Method for Identification of Receptors for YopE in the Type III Secretion (T3S)  
System of *Yersinia pseudotuberculosis***

by

Surina R. Hemani

Master of Science in Biology

University of California, San Diego, 2012

Professor Partho Ghosh, Chair  
Professor Raffi Aroian, Co-Chair

Numerous Gram-negative bacterial pathogens have virulence mechanisms that modulate host targets. One such mechanism is the type III secretion (T3S) system. YopE is one of the six virulence proteins delivered by the T3S system of the bacterial pathogen *Yersinia* into host cells. The targeting signals that direct virulence factors into the host have been mapped, but the receptors for these signals are unknown. The *Y. pseudotuberculosis* effector YopE requires both an N-terminal region, which is termed the signal sequence (SS) and consists of amino acids 1-15, and a region just downstream, which is termed the chaperone-binding (Cb) region, for translocation into host cells. The focus of this project is to set up a system to identify the receptor, or receptors, that recognize the YopE SS.

Prior work has shown that interactions in the T3S system tend to be transient. Thus, I have taken an approach that utilizes formaldehyde as a crosslinking reagent. Formaldehyde is highly reactive, cell permeable, and forms short crosslinks (2.3-2.7 Å). Furthermore, its crosslinks may be reversed at boiling temperatures. Two additional crosslinking reagents, glutaraldehyde and disuccinimidyl tartrate (DST), are presented as alternatives for optimization of crosslinking efficiency. Glutaraldehyde forms a 5 Å irreversible crosslink and DST forms a 6.4 Å reversible crosslink. Reversibility is not strictly necessary, but allows for ease in isolation and identification of potential receptors through mass spectrometry. Recommendations are made for future experiments using the crosslinking parameters proposed for identification of receptors for YopE's signal sequence region.

## INTRODUCTION

A large number of bacterial species live in close proximity to host organisms. These bacteria often communicate with their hosts by secreting select bacterial proteins. In the case of pathogenic bacteria, these secreted proteins are often virulence factors that modulate host cell behavior and promote disease progression (Galán and Collmer 1999). A common mechanism is the type III secretion (T3S) system, which is responsible for the direct secretion of proteins from the bacterial cytosol into the host cell cytosol (Cornelis 2006). This process of direct secretion is termed translocation. The T3S system is found only in gram-negative bacteria, including numerous animal pathogens, such as *Salmonella*, *Escherichia coli*, *Yersinia*, *Bordetella* and *Chlamydia*, and certain plant pathogens.

*Yersinia*, the microorganism on which this study focuses, consists of three species: *Y. pestis*, *Y. enterocolitica* and *Y. pseudotuberculosis*. *Y. pestis* was responsible for killing one-third of Europe's population in the mid-14<sup>th</sup> century while the other two elicit serious but typically non-lethal infection within the mammalian gastrointestinal tract. *Y. enterocolitica* induces Yersiniosis, an infectious disease marked by enteritis, ileitis, and pseudoappendicitis, and *Y. pseudotuberculosis* triggers acute gastroenteritis and lymphadenitis. *Yersinia* can evade host defense mechanisms through the translocation of effectors, known as *Yersinia* outer proteins (Yops) into the host cytosol. Here they use the T3S system to inhibit phagocytosis by disrupting the host's actin cytoskeleton and inducing apoptosis through formation of novel signaling complexes (Aepfelbacher et al. 2007). Interference with cytokine production decreases the ability of

the inflammatory system to respond to infection (Bhavsar et al. 2007; Navarro et al. 2005).

Electron microscopy has indicated that the central apparatus of the T3S system, termed the injectisome, is visually similar to a syringe with a needle at the end (**Fig. 1A**). It is composed of 20-25 proteins and protrudes from the bacterial surface (Akeda and Galan 2005). Upon contact with the target cell, *Yersinia* use the T3S system to inject effectors directly into the cytosol of the host through a single step process of secretion and translocation (Viboud and Bliska 2005). However, recent studies also indicate the existence of a parallel two-step process in which effectors localize and form a translocation-competent intermediate on the cells' exterior prior to contact with the target cell (Akopyan et al. 2010; Ghosh 2004).

Specific dimeric chaperone proteins are often required to bind to effectors in the bacterial cytosol prior to translocation (Ghosh 2004) (**Fig. 1B**). The chaperone proteins, termed the "Specific Yop Chaperones" (Syc), generally bind to a single or only a few effectors, and remain in the bacterial cytosol (Birtalan and Ghosh 2001). Six effector proteins, YopE, YopH, YopM, YopO, YopP, and YopT, are translocated directly into the host cytosol. The detailed mechanisms of T3S effector protein translocation are still unknown. Conservation amongst *Yersinia*, *Salmonella* and *Shigella* indicates that insights gained on this mechanism from one species are likely to be applicable to the others.

YopE, the effector of interest for this study, contains an N-terminal region, termed the signal sequence (SS), and a region just downstream, termed the chaperone-binding (Cb) region. Similar to other effectors, YopE requires not only the Cb region for

translocation, but also the SS region (Sory et al. 1995). Previous research has indicated the SS region may be composed of the first 15 amino acids of YopE or the first 15 mRNA codons of YopE, or both (Lloyd et al. 2002; Ghosh 2004; Ramamurthi and Schneewind 2005) (**Fig. 2**). The YopE Cb region, which is composed of residues 23-78, is where the 15 kDa SycE chaperone binds to the 23 kDa YopE effector in a 2:1 stoichiometric fashion (**Fig 3A, 3B**). The binding of SycE produces a disorder-to-order transition in YopE (Rodgers et al. 2008). These modulations are specific to the Cb region and have no effect on the other residues of YopE. Following both the SS and Cb regions, residues 100-219 confer GTPase activating protein (GAP) activity within the host. The GAP activity stimulates down-regulation of the Rho pathway, resulting in the depolymerization of actin microfilament structure in eukaryotic cells (Von Pawel-Rammingen et al. 2000).

The primary aim of this project was to produce a system for the identification of the possible receptor, or receptors, involved in recognizing the N-terminal amino acid signal sequence region of the *Y. pseudotuberculosis* effector YopE. I took an approach that utilized crosslinking to freeze transient protein-protein interactions (Klockenbusch and Kast 2010). I explored the use of formaldehyde, which is highly reactive, cell permeable, and forms short crosslinks (2.3-2.7 Å). A benefit of using formaldehyde is that it forms crosslinks that can be reversed at temperatures above 99 °C. I also explored the use of glutaraldehyde and disuccinimidyl tartrate (DST) as crosslinking reagents, with similar potentials for isolation and identification of potential YopE SS receptors (**Fig 4**). Studies reported here establish the parameters for further experiments in *Y. pseudotuberculosis*.

## MATERIALS AND METHODS

**Protein Expression and Purification.** A previously cloned construct of SycE-YopE with an N-terminal His-tag was expressed from the pET28b expression vector (Novagen) in *Escherichia coli* BL21 (DE3). Bacteria were grown at 37 °C in LB media supplemented with 50 mg/L kanamycin until an OD<sub>600</sub> of 0.6. Protein expression was induced with 0.4 mM IPTG, and the culture was grown for 12 hours at room temperature and then harvested by centrifugation at 5,500 x g for 20 minutes at 4 °C. The pellet was resuspended in 1/100<sup>th</sup> of the original volume in lysis buffer (300 mM NaCl, 50 mM sodium phosphate buffer (NaPi), pH 8.0, 10 mM β-mercaptoethanol (βME) supplemented with 20 μg/mL DNase (Sigma) and an EDTA free protease inhibitor cocktail tablet © (one tablet per 2 L of bacterial culture, Roche)). Bacteria were lysed using an Emulsiflex C-5 homogenizer (Avestin) for three cycles at 15,000 psi. The cell lysate was then centrifuged at 14,000 x g for 15 minutes at 4 °C, and the supernatant was separated from the pellet and loaded on a Ni<sup>2+</sup> nitrilotriacetic acid (NTA) agarose column (Sigma) with a 15 mg/mL capacity. The column was equilibrated at 4 °C with 5 column volumes of deionized water and 10 column volumes of lysis buffer supplemented with 10 mM imidazole. Proteins were eluted with 5 column volumes of lysis buffer supplemented with 500 mM imidazole. The eluate was concentrated with an Amicon Ultra-15 30k centrifugal filter (Millipore) and further purified with a 16/60 Superdex 200 size-exclusion column (GE Healthcare) in 300 mM NaCl and 50 mM NaPi, pH 8.0. The concentration of the purified protein was determined with a Thermo-Scientific Nanodrop



1000 spectrophotometer using a molar extinction coefficient ( $\epsilon_{280}$ ) of 23,950 M<sup>-1</sup>cm<sup>-1</sup>. The purified protein was concentrated to 5 mg/mL.

**Crosslinking Conditions.** Twenty-five percent glutaraldehyde solution (Sigma-Aldrich) was added to 20  $\mu$ g of SycE-YopE with incubation at room temperature. Disuccinimidyl tartarate (DST) was prepared by dissolving the powder (Thermo Scientific) in 100% DMSO at room temperature to a stock concentration of 25 mM. Formaldehyde was prepared by dissolving paraformaldehyde in 1x PBS at 60 °C to yield a 4% paraformaldehyde stock solution. The pH of the formaldehyde was decreased to 6.9, and the solution was then filtered with a 0.22  $\mu$ m syringe filter. The formaldehyde solution was stored at 2-8 °C for up to four weeks in the dark. All crosslinking reactions were quenched with 0.5 M Tris, pH 7.5 for 15 minutes. All samples were heated to 65 °C prior to loading onto an SDS-PAGE, except for formaldehyde-treated samples in which crosslinks were to be reversed. These latter samples were heated slightly above 99 °C.

**Cloning of YopE Construct.** A method of overlapping PCR using two forward and two reverse primers as a DNA template was used to produce two novel constructs. The first was a "WT" construct containing amino acid residues 1-20 of YopE and the second was a "No-Secretion" construct containing the same initial 20 amino acids of YopE but with serine substitutions in residues 2-8 (**Fig. 5A**). Downstream of the coding sequence for amino acids 1-20 were placed a DNA sequence encoding an NcoI restriction site (CCATGG), a streptavidin binding peptide (SBP) tag of 38 amino acids (MDEKTTGWRGGHVVEGLAGELEQLRARLEHHPQGQREP), a linker region

(GSGS), and a His-tag (HHHHHH) (**Fig. 5B**). One  $\mu\text{l}$  of the forward and reverse primers (10  $\mu\text{M}$ ) was added to 5  $\mu\text{l}$  of 10x Pfu DNA polymerase reaction buffer, 8  $\mu\text{l}$  of 10 mM dNTPs, and 1  $\mu\text{l}$  of 1x Pfu Polymerase in a 50  $\mu\text{l}$  reaction volume. Samples were heated in a thermocycler for one cycle of initial denaturation at 95 °C for 2 minutes, 30 cycles of denaturation at 95 °C for 30 seconds, annealing at 55 °C for 30 seconds and extension at 72 °C for one minute, followed by one final extension cycle at 72 °C for 10 minutes. The PCR products (210 bp) were purified from a 1.3% agarose gel, concentrated with the Savant SpeedVac DNA 110 Concentrator to 100 ng/ $\mu\text{l}$ , and sequenced. This PCR product was used as the DNA template for a PCR reaction in which the primer encoded a 15 bp sequence complementary to the site of insertion into the vector pBAD. In contrast with the above preparation of the two constructs, addition of the 15 bp overhangs required an increase to 100 ng of DNA template and a decrease to 4  $\mu\text{l}$  of 10 mM dNTPs. The PCR annealing temperature was decreased from 55 °C to 46 °C. The resulting 240 bp PCR products were purified from a 1.3% agarose gel and concentrated to 65 ng/ $\mu\text{l}$ .

Inverse PCR of the 4.1 kb pBAD vector required similar PCR preparation as with the 15 bp extension; however, the annealing temperature was decreased to 53 °C and the elongation step was increased to 5 minutes. PCR products were treated overnight with 1  $\mu\text{l}$  of DpnI per 50  $\mu\text{l}$  total reaction volume at 37 °C to remove the template DNA. DpnI-treated DNA was concentrated to 65 ng/ $\mu\text{l}$ . SLIC (Sequence and Ligation Independent Cloning) was then used to insert the desired fragment into the vector (Li and Elledge 2007) (**Fig. 6**). Briefly, 1  $\mu\text{g}$  of insert and 1  $\mu\text{g}$  of the PCR product were incubated individually for 30 minutes at 30 °C with 0.5 U of T4 DNA Polymerase plus 2  $\mu\text{g}$  of 1

mg/ml 10X BSA and 1x NEBuffer2 (New England Biolabs) in a 20  $\mu$ l reaction. The reaction was quenched by adding 1/10<sup>th</sup> the volume of 10 mM dCTP on ice.

The final annealing step required a 1:1 ratio of insert to vector and was carried out for 30 minutes at 37 °C in a 10  $\mu$ l reaction volume with 100-150 ng of vector and insert and 1x T4 DNA Ligase Buffer (NEB). Transformation into *Escherichia coli* Top10 and selection with ampicillin were used to obtain colonies containing the pBAD vector with the YopE insert. DNA sequencing was used to verify the final 7 kDa WT and No-secretion sequences.

**Expression Assay.** Bacterial cultures of the WT and No-Secretion constructs were grown in *E. coli* Top10. Unlike other *E. coli* strains, Top10 cells do not metabolize L-arabinose, which is required to induce the pBAD promoter for expression of the pBAD vector. Bacteria were grown at 37 °C in LB media supplemented with 100 mg/L ampicillin until an OD600 of 0.6. Unlike Protein expression was induced with 2.0% arabinose, and the cultures were grown further for eight hours at room temperature, with the addition of arabinose every two hours. Cultures were harvested by centrifugation at 4,000 x g for 5 minutes at 4 °C. One mL of bacteria was resuspended in 30  $\mu$ l of deionized water and 30  $\mu$ l of 2X tricine sample buffer and lysed by boiling. Samples were loaded onto a 10% Tris-Tricine gel for one hour at 30 V and one hour at 110 V for separation. Proteins were transferred to a PVDF membrane through wet transfer at 100 V for 40 minutes. The PVDF membrane was washed for two minutes in 1x TBS (150 mM NaCl, 10 mM Tris, pH 8.0) then placed in blocking solution (1x TBS containing 5% w/v dry milk) for one hour at room temperature. The membrane was washed with 1x TBST

(TBS with 0.05% v/v Tween-20), and then incubated at room temperature for one hour with primary antibody. This consisted of a 1:2000 dilution of mouse anti-His monoclonal primary antibody diluted in TBS containing 5% w/v dry milk. The membrane was then washed three times in TBST for 5 minutes each. The membrane was then incubated for 30 minutes with an HRP-conjugated goat anti-mouse secondary antibody diluted 1:7500 in TBST containing 5% w/v dry milk. Three final washes of 5 minutes each in TBST were carried out. The SuperSignal West Pico Chemiluminescent Substrate (Pierce) was used for development, and the BioRad ChemiDoc Imaging System for visualization.

## RESULTS

### Optimization of crosslinking conditions with SycE-YopE

To study protein-protein interactions *in vivo*, transient interactions of native protein complexes can be stabilized by a crosslinking reagent. The protein complexes can then be purified and characterized by mass spectrometry (MS) analysis (Sutherland et al. 2008). However, prior to experimenting *in vivo*, *in vitro* studies can assist in establishing the parameters for crosslinking. Two areas of particular interest are incubation time and crosslinking reagent concentration. Formaldehyde was chosen as a primary reagent for multiple reasons. Formaldehyde consists of only four atoms, accounting for its small size. It forms a relatively short distance between conjugated molecules, known as the spacer arm length, in the range of 2.3 to 2.7 Å (**Fig. 7**). Furthermore, after purification, its protein-protein interactions can be reversed by boiling at temperatures above 99 °C. Although not necessary, reversal simplifies the process of determining the identity of individual proteins during MS analysis.

SycE and YopE are known to bind tightly *in vitro* and were thus used to determine optimal crosslinking parameters. In preparation for crosslinking optimization, the N-terminal His-tagged SycE-YopE complex was purified on a nickel column. The 12 kDa SycE band and 23 kDa YopE band were visible by SDS-PAGE (**Fig 8A**). Further purification was carried out on a size exclusion column, followed by concentration with an Amicon Ultra-15 30k centrifugal filter (**Fig. 8B**). A second round of purification was required once the original protein stock was diminished (**Fig. 8C**). For all experiments, visualization of crosslinking was done by 15% SDS-PAGE.

### Formaldehyde Crosslinking with SycE-YopE

A stock of formaldehyde solution (37 wt % in H<sub>2</sub>O) with 10-15% methanol stabilizer was added to purified SycE-YopE. Varying concentrations of formaldehyde from .4 – 1.8% were added at room temperature for 10 minutes. A sample of SycE-YopE containing similar conditions without formaldehyde was also prepared as a negative control. Aggregation was visible in all lanes at the top of the wells due to crosslinking, becoming most prominent at 0.8% formaldehyde. Samples with lower formaldehyde concentrations produced less aggregation, yet the 35 kDa crosslinked complex was also difficult to visualize in these lanes, indicating a lack of crosslinking (**Fig. 9A, 9B**). During this initial experiment, reactions were quenched with 0.5 M glycine, pH 7.5, which resulted in an unexpected yellow color prior to the addition of SDS buffer. Previous protocols indicated use of either glycine or Tris as a quenching agent; therefore, 0.5 M Tris, pH 7.5 was used from here onwards to avoid yellow coloring.

Since the formaldehyde solution initially used was present in the lab for an extended period of time, an alternate stock of formaldehyde was required. Sixteen percent methanol-free formaldehyde ampules were diluted to a 10% stock in 1x PBS. When formaldehyde was added to SycE-YopE over a concentration range of 0.1 – 5.0% during a 20 minute incubation, no crosslinking or aggregation was observed (**Fig. 10A, 10B**). A single concentration of 3% formaldehyde was then tested over incubation times ranging from 5 to 60 minutes. Once again, however, crosslinking or aggregation was not visible in any of the lanes (**Fig. 10C**). Hence, two additional crosslinkers, glutaraldehyde and disuccinimidyl tartrate were also tested.

### **Optimization of glutaraldehyde and DST crosslinking conditions with SycE-YopE**

Glutaraldehyde, with a 5 Å spacer arm length, has greater crosslinking potential than formaldehyde due to the presence of two aldehyde groups on either side of the three methylene bridges (**Fig. 11**). The ability to bond with protein nitrogens in its vicinity results in multiple crosslinks. Glutaraldehyde also differs from formaldehyde in that its crosslinks are irreversible. Glutaraldehyde was applied to SycE-YopE at 1% and 2% concentrations for 5 to 20 minutes. Although aggregation was present at the top of the wells, higher molecular weight bands were clearly visible in all the samples (**Fig. 12A**). The negative control, which did not have glutaraldehyde by comparison, lacked these higher molecular weight bands. To decrease aggregation, samples were tested for 10 and 20 minute incubation times with lower concentrations of glutaraldehyde, ranging from 0.05% to 0.5%. Aggregation was still visible; however, higher molecular bands were even more defined and clearly visible in all samples, indicating successful crosslinking (**Fig. 12B**).

The third reagent tested was disuccinimidyl tartrate (DST), a 6.4 Å arm length crosslinker with amine reactive N-hydroxysuccinimide (NHS) ester ends (**Fig. 13**). Preparation requires dissolving the solid in water or an organic solvent, such as dimethyl sulfoxide (DMSO) or dimethyl formamide (DMF). Although the reversibility of DST was not tested here, the central diol of DST is cleavable through periodate oxidation, such as with 0.015 M sodium meta-periodate (Smith et al. 1978). DST was prepared initially by dissolving the solid in water. A concentration range from 0.5 to 4.0 mM DST was incubated with SycE-YopE for 20 minutes, but no crosslinking was observed (**Fig. 14A**).

An increase in the concentration of DST to 10 mM with both a 10 and 20 minute incubation time also yielded no crosslinking (**Fig. 14B**).

DST was then prepared by dissolving the powder in 25 mM DMSO. This DMSO-solubilized DST was incubated at concentrations ranging from 0.5 to 3.0 mM at room temperature for 30 minutes and from 0.5 to 4.0 mM for 60 minutes with SycE-YopE. Once again, crosslinking was not detected (**Fig. 15A, 15B**). The DST powder was then dissolved in 100% DMSO to a final concentration of 50 mM. This stock was incubated with SycE-YopE at concentrations ranging from 1 to 20 mM DST at room temperature for 30 minutes (**Fig. 16**). A faint 37 kDa molecular weight band was visible starting at the 5 mM concentration. Although these data may not provide conclusive evidence for the ability of DST to serve as a successful crosslinking reagent, they do, however, indicate a potential with further optimization.

### **Formaldehyde and glutaraldehyde crosslinking with Avd**

It has been demonstrated previously that formaldehyde prepared from paraformaldehyde powder successfully resulted in protein crosslinking (Klockenbusch and Kast 2010). Formaldehyde was prepared by dissolving paraformaldehyde in 1x PBS pH 7.4 for 2 hours at 60 °C, followed by cooling and filter sterilizing. Alongside glutaraldehyde, this formaldehyde reagent was tested on Avd, a 60 kDa pentameric protein. Visualizing the 12 kDa Avd monomer as a dimer, trimer, tetramer or pentamer would confirm protein crosslinking and assist in determining optimal crosslinking parameters.



To test this, formaldehyde and glutaraldehyde were added to Avd at a 1% concentration for 30 minutes. Higher molecular weight bands were visualized with both formaldehyde and glutaraldehyde. **(Fig. 17)**. A large level of aggregation at the top of the well was observed in glutaraldehyde-treated samples; higher molecular weight bands that indicated crosslinking were clearly observed in formaldehyde-treated samples. Nevertheless, this experiment was repeated to decrease the aggregation visualized with glutaraldehyde. A lower glutaraldehyde concentration of 0.5% and shorter incubation times of 5 and 10 minutes were tested. Formaldehyde was also tested by addition to Avd at 1% and 2% concentrations for 30 minutes. In order to test the reversal of formaldehyde crosslinking, half of each sample was boiled past 99 °C **(Fig. 18A)**. SDS-PAGE still indicated excessive aggregation of glutaraldehyde-treated samples at the top of the well, indicating the need to decrease concentration and incubation time even further. Both 1% and 2% formaldehyde-treated samples successfully indicated crosslinking when heated to 65 °C and reversal of crosslinking when heated past 99 °C.

Since glutaraldehyde-treated samples still required further optimization, Avd was tested with an even lower concentration of glutaraldehyde and a shorter incubation time. Glutaraldehyde was added at a 0.25% concentration for 2 minutes while formaldehyde was added to Avd in the same manner as above, at 1% and 2% concentrations for 30 minutes. SDS-PAGE indicated the presence of crosslinked dimeric, trimeric, and tetrameric forms of Avd with both glutaraldehyde and formaldehyde **(Fig. 18B)**. Samples boiled past 99 °C indicated reversal of formaldehyde crosslinks. Thus, the optimal concentration and incubation time for glutaraldehyde was determined to be 0.5%

for 2-5 minutes; the optimal concentration and incubation time for formaldehyde was determined to be 1% for 30 minutes.

### **Expression Assay**

In order to determine whether the 7 kDa WT and No-secretion constructs were expressed, bacterial cultures of *E. coli* Top10 were grown. Expression could have also been determined in *Yersinia*. However, due to difficulty in transforming constructs into *Yersinia*, expression assays were conducted in Top10 cells, which do not metabolize arabinose required for induction of the pBAD promoter. Expression was visualized by western blot with an anti-His antibody. When WT and No-secretion constructs were induced with 0.15% arabinose, the western blot did not indicate expression of the WT and No-secretion constructs (**Fig. 19A**). The WT construct was then induced with a gradient of arabinose from 0.1 to 0.6%. A 7 kDa band was not visible in the un-induced or induced lanes (**Fig. 19B**). Since difficulty had previously been noticed in visualizing results with this stock of polyclonal primary antibody, experiments were conducted here onwards with a monoclonal primary antibody. Moreover, to ensure the anti-His antibody was working effectively, a 37 kDa His-tag M protein positive control was used. Expression was then attempted with an increase in arabinose concentration to 1%. The western blot indicated the presence of a his-tag protein for the positive control. However, the 7 kDa His-tag YopE WT construct was not visible, indicating it had not been expressed (**Fig. 20A**).

In the first three experiments, arabinose was added only once at an OD600 of 0.6. To ensure that the levels of arabinose were not depleted over the 10 hour period of the

experiment, arabinose was added again 4 hours after induction, and then again every 2 hours. Samples were taken at 4 hours, 6 hours, and 8 hours after induction. Western blot analysis indicated the presence of a faint 7 kDa band in the induced lanes, indicating possible expression of the WT and No-secretion constructs (**Fig. 20B**). However, the high level of background present in all lanes, including the positive control, indicates the need to repeat this experiment.

## DISCUSSION

Since the discovery of type III secretion over 20 years ago (Michiels et al. 1990; Rosqvist et al. 1990), the interaction between infectious bacteria using this apparatus and their target hosts has been under constant analysis. The World Health Organization reports an average of 1,000 to 3,000 cases of plague per year caused by the *Yersinia* microbe globally. Pathogenic *Yersinia* use the T3S system to inject certain toxic proteins into eukaryotic cells. *Yersinia* targets its six effectors into host cells by the signal sequence region, which serves as a mode of effector recognition; however, whether recognition occurs via signal peptide or mRNA is under scrutiny (Ghosh 2004). Lack of consensus amongst the N-terminus of different Yop proteins makes it difficult to identify the manner in which such diverse sequences may be recognized by the T3S apparatus.

Previous research has indicated that even when the amino acid sequence of the SS region of YopE is completely altered, secretion is unaffected *in vitro* (Anderson and Schneewind 1997). This supports the hypothesis of mRNA functioning as a signal for T3S of Yops. However, other studies have indicated that mutations in the SS region that alter codons without altering the sequence of the protein also do not affect secretion *in vitro* (Lloyd et al. 2001). These studies challenge the role of mRNA targeting signals and support the latter hypothesis of recognition via signal peptide sequence. Thus, although the location of the signal sequence is confirmed, its molecular composition of either mRNA, amino acid, or both, is controversial.

Two major goals were achieved from this study. First, *in vitro* crosslinking parameters were established for formaldehyde and glutaraldehyde. These data indicate

the need for formaldehyde to be prepared from paraformaldehyde powder, as shown by Klockenbusch (Klockenbusch and Kast 2010). SDS-PAGE of Avd crosslinking indicated that a 1% formaldehyde concentration and 30 minute incubation time resulted in the largest amount of crosslinking. The advantage of using formaldehyde is the ability to reverse linkages for ease of identification through mass spectrometry. Moreover, formaldehyde interacts over a short spacer arm length and will crosslink only the most proximal proteins.

Glutaraldehyde was shown to work effectively in crosslinking both SycE-YopE and Avd monomers. A concentration of 0.05% and an incubation of 2 to 5 minutes were sufficient to introduce a large number of crosslinks. However, with a larger spacer arm length, glutaraldehyde is less specific than formaldehyde and as an irreversible reagent it may not elicit similar ease in isolation of proteins in preparation for mass spectrometry.

The third protein crosslinker tested was disuccinimidyl tartrate, a 6.4 Å arm length crosslinker with amine reactive N-hydroxysuccinimide (NHS) ester ends. DST, prepared by dissolving in water, did not produce crosslinking as shown in Figure 12. Figure 13 indicated that DST prepared in 25 mM DMSO also did not produce crosslinking. The lack of successful crosslinking in the initial experiments was likely due to the need to dissolve DST in a 100% organic solvent rather than an aqueous solution. Figure 14 establishes the need to dissolve the DST powder in 100% DMSO for a final 25 mM stock to form the SycE-YopE complex. Crosslinking of SycE and YopE with DST prepared in 100% DMSO indicated the appearance of faint crosslinking bands at a 5 mM concentration and a 30 minute incubation. Although these data may not provide

conclusive evidence for the ability of DST to serve as a successful crosslinking reagent, they do indicate a potential with further optimization.

The second goal achieved was the formation of two novel DNA constructs. The first, termed wildtype, contained the first 20 amino acid residues of YopE. Replacement of the YopE N-terminus with hydrophilic serine residues or hydrophobic isoleucine residues was shown to drastically reduce the secretion of YopE (Lloyd et al. 2001). Thus, for the second construct, termed No-secretion, residues 2-8 of the first 20 amino acids of YopE were altered, in contrast with the WT sequence, to consist of serine substitutions.

A streptavidin binding peptide (SBP) tag was placed downstream of the first 20 amino acids of YopE for a one-step purification. This 38 amino acid sequence binds to streptavidin with a dissociation constant of 2.5 nM, allowing for greater purification than seen with the His-tag (Keefe et al. 2001). Similar to the role of imidazole in eluting protein from a nickel column, a wash buffer with high levels of biotin will elute the desired protein from streptavidin beads.

In order to determine expression of the 7 kDa WT and No-secretion constructs, expression assays were conducted in *E. coli* Top10 cells. Unlike other *E. coli* strains, such as DH5 $\alpha$ , arabinose is not metabolized in Top10 cells; arabinose induction is required to induce the *araC* promoter for expression from the pBAD vector. Previous studies have shown concentrations as low as 0.02% are sufficient to induce expression, although concentrations as high as 2% will also work (Khlebnikov et al. 2000). Arabinose concentrations ranging from 0.1 to 1.0%, as shown in Figures 17 and 18, were initially tested. These first two trials did not yield expression of the WT and No-secretion

constructs. However, the polyclonal antibody used here had previously been recently problematic in the hands of others. This was addressed by switching to a monoclonal anti-His antibody for the next two experiments. In the third expression experiment, induction from 0.2 to 1.0% arabinose was carried out (Figure 18A). The positive control, a 37 kDa His-tag M protein, was clearly visible, indicating that the monoclonal antibody was working. Expression of the 7 kDa construct was not visible at any arabinose concentration, however.

It was possible that the arabinose was being depleted over the 8 hour time course of bacterial growth, resulting in the lack of expression. To assess this, a fourth experiment, shown in Figure 18B, was conducted in which arabinose was added 4 hours after induction and again every 2 hours. When visualized on a western blot, the 7 kDa protein, although faint, could be visualized in the induced lanes and not in the un-induced lanes. However, the high level of background made it difficult to definitively conclude expression had occurred. The positive control at 37 kDa was present, but other unidentifiable bands were also visible in this lane. Thus, it will be necessary to repeat this last experiment and to further optimize arabinose concentrations. Testing an arabinose concentration range of up to 2% and adding arabinose every 2 hours from the point of induction may prove successful.

If higher concentrations of arabinose and 2 hour additions are incorporated and results still indicate the same background, it would be useful to test a few controls. First, visualizing Top10 cells without the transformed construct would indicate whether the high background might be due to unexpected binding of the antibody to proteins already present in the cell line in the absence of the pBAD vector. Another possible area to test

would be the pBAD vector itself. To confirm expression from the pBAD vector, a His-tagged protein known to express well from pBAD can be tried. If such results do not indicate the ability for detection, perhaps a different vector needs to be used.

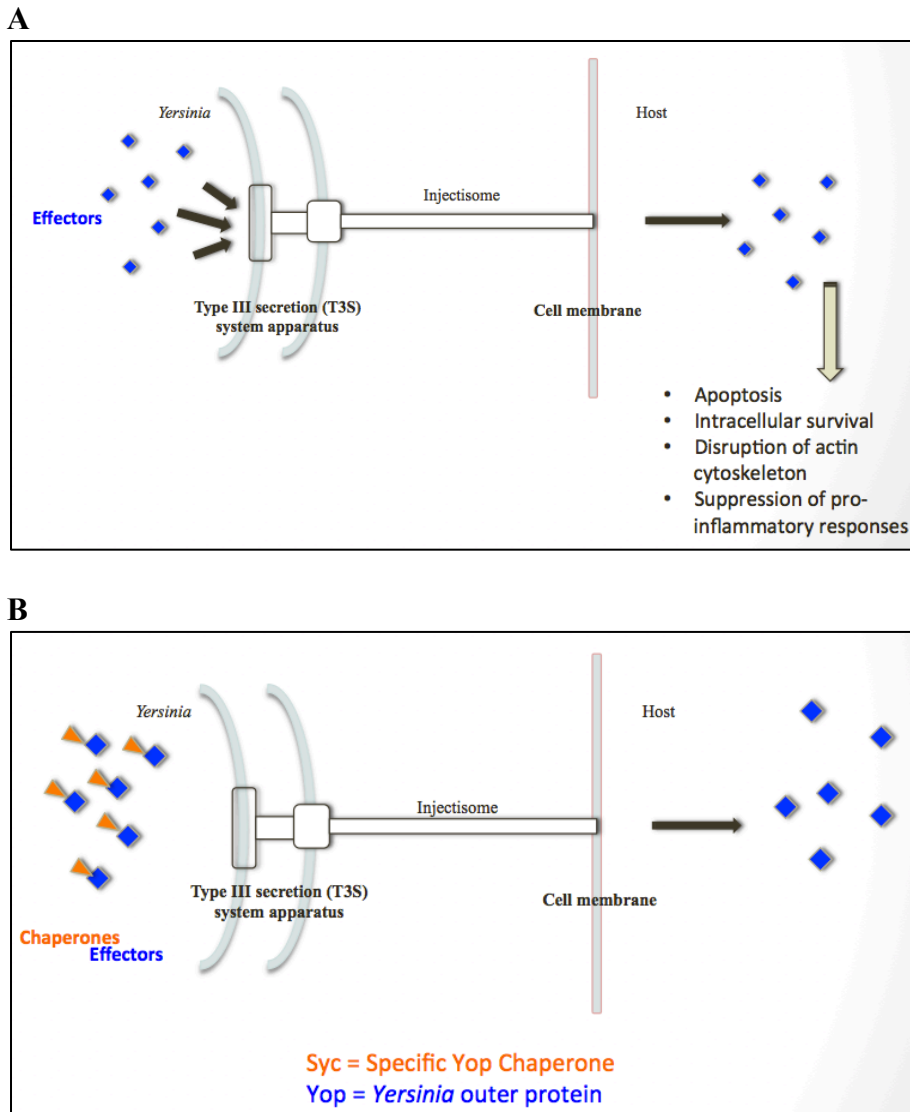
Simultaneously, applying the western blot to the empty pBAD vector alone without the construct can also serve as a negative control for the WT and No-secretion constructs.

Furthermore, detection of the 38-amino acid streptavidin binding peptide with an anti-SBP antibody can be tried instead of detection through the His-tag.

While further effort is required to isolate potential YopE SS receptors, these data clearly indicate that both formaldehyde and glutaraldehyde may serve as crosslinking reagents *in vivo*. The ability to disrupt protein linkages at temperatures past 99 °C is unique for formaldehyde. Glutaraldehyde is irreversible and DST requires the addition of a periodate oxidation reagent for reversal. These crosslinking parameters along with the two WT and No-secretion constructs provide a system in which crosslinking reagents can be added *in vivo* to stabilize transient reactions of potential receptors of the SS region of YopE. Future directions require confirmation of secretion, or lack of secretion, in WT and No-secretion constructs, respectively. The addition of crosslinker followed purification with streptavidin beads, reversal of crosslinks, and finally identification of proteins through mass spectrometry offer a way of identifying potential receptors that recognize and bind only to the N-terminal amino acid signal sequence of the WT-secretion construct of YopE.



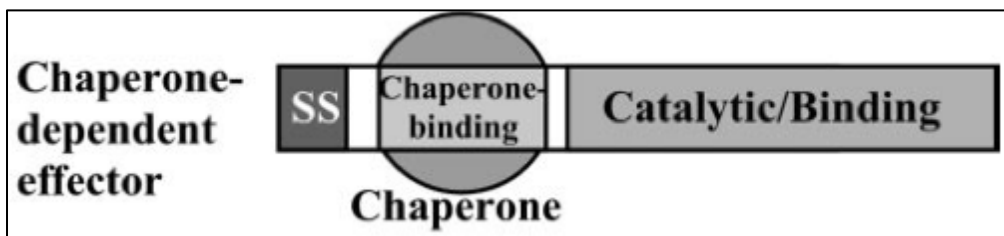
## FIGURES



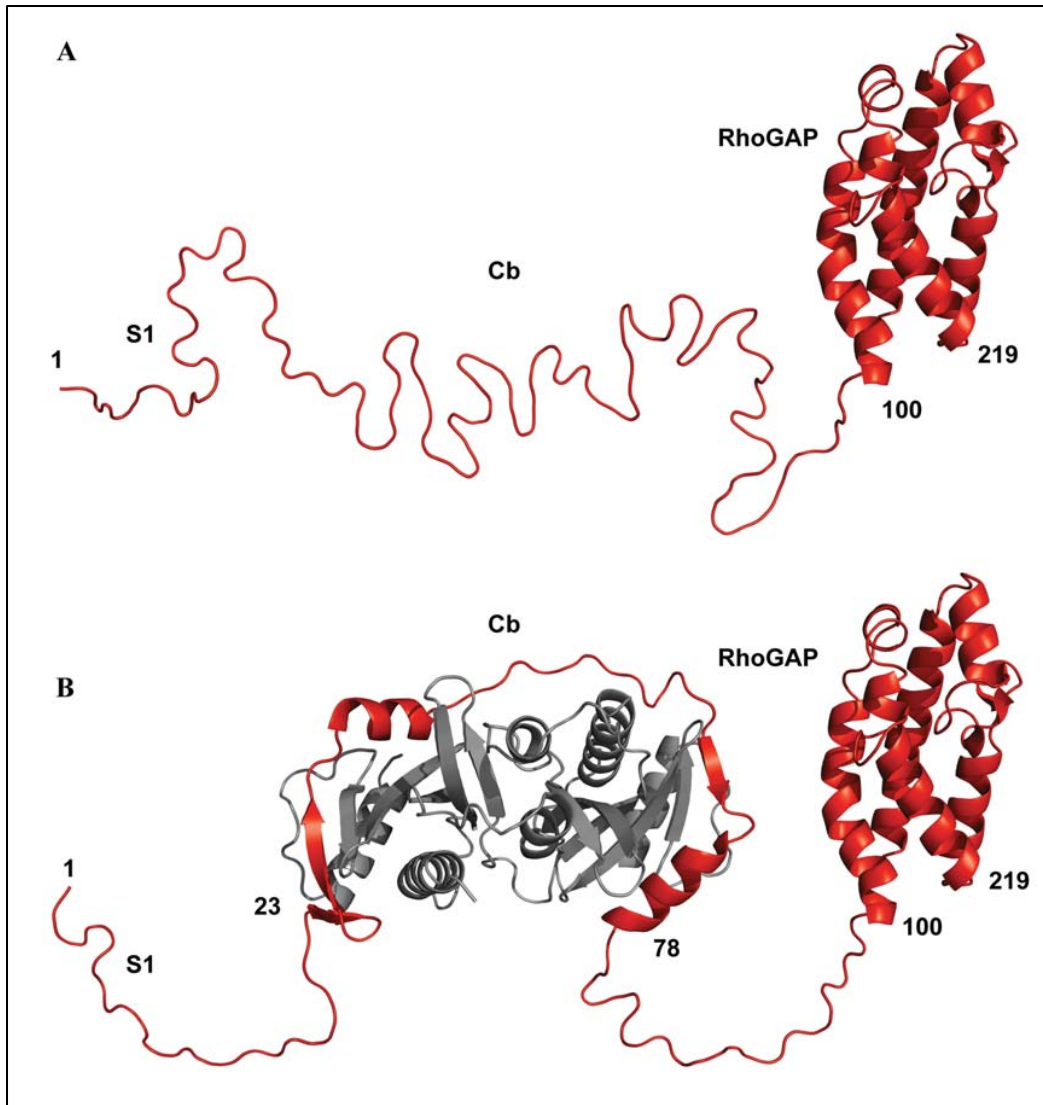
**Figure 1. Schematic of the type III secretion (T3S) system apparatus.**

**A.** Termed the injectisome, the T3S central apparatus is a syringe with a needle at the end. It is comprised of 20-25 proteins and protrudes from the bacterial surface (Akeda and Galan 2005). Upon contact with the target, *Yersinia* inject effectors directly into the host cell cytosol using the injectisome. Once inside the target cell, effectors are responsible for host cell modulation and disease progression.

**B.** Specific dimeric chaperone proteins are often required to bind to effectors in the bacterial cytosol prior to translocation (Ghosh 2004). The chaperones are not translocated and remain in the bacterial cytosol (Birtalan and Ghosh 2001).



**FIGURE 2. Schematic of the chaperone-dependent effectors of the type III secretion (T3S) system.** Effectors and secreted proteins of the T3S system have a signal sequence (SS) region consisting of the first 15 amino acid residues, 15 mRNA codons, or both. Chaperones often bind to a chaperone-binding region just downstream of the SS region, which is followed by the host cell binding or catalytic region that is responsible for modulation of activity within the target cell. Figure from Ghosh, *Microbiol Mol Biol Rev* (2004) 68(4): 771-95.



**FIGURE 3. Free YopE and SycE-bound YopE.**

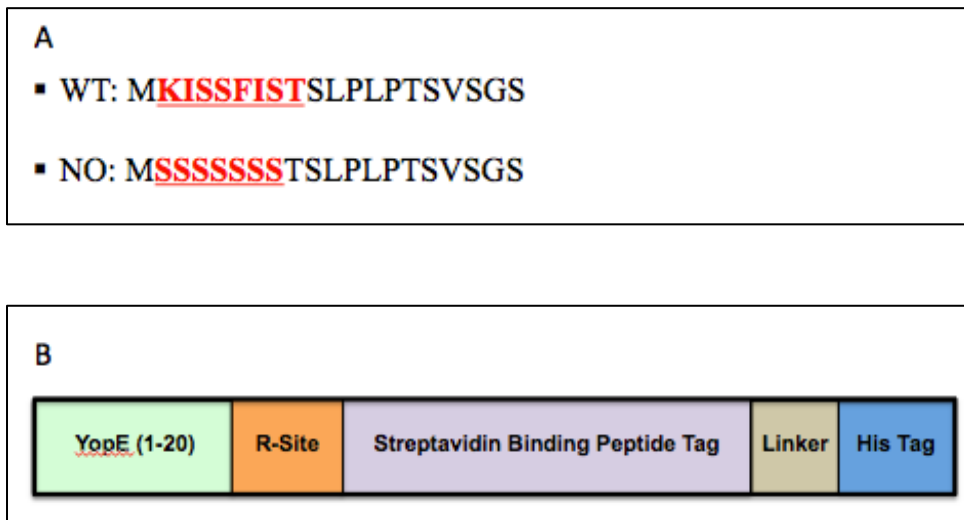
**A.** Ribbon representation of free YopE, with the first 100 residues in random coil followed by the crystal structure of the RhoGAP domain (residues 100-219) shown (Evdokimov et al. 2002).

**B.** Ribbon representation of SycE (grey) bound to YopE (red) with the crystal structure of the Cb region (residues 23-78) shown (Birtalan et al. 2002) and the crystal structure of the RhoGAP domain (residues 100-219) shown (Evdokimov et al. 2002); other portions are shown in random coil conformation.

Figure from Rodgers et al., *J Biol Chem* (2008) 283(30): 20857-63.

	Membrane Permeable?	Spacer Arm Length	Reversible?
Formaldehyde	Yes	2.4 – 2.7 Å	Yes
Glutaraldehyde	Yes	5.0 Å	No
DST	Yes	6.4 Å	Yes

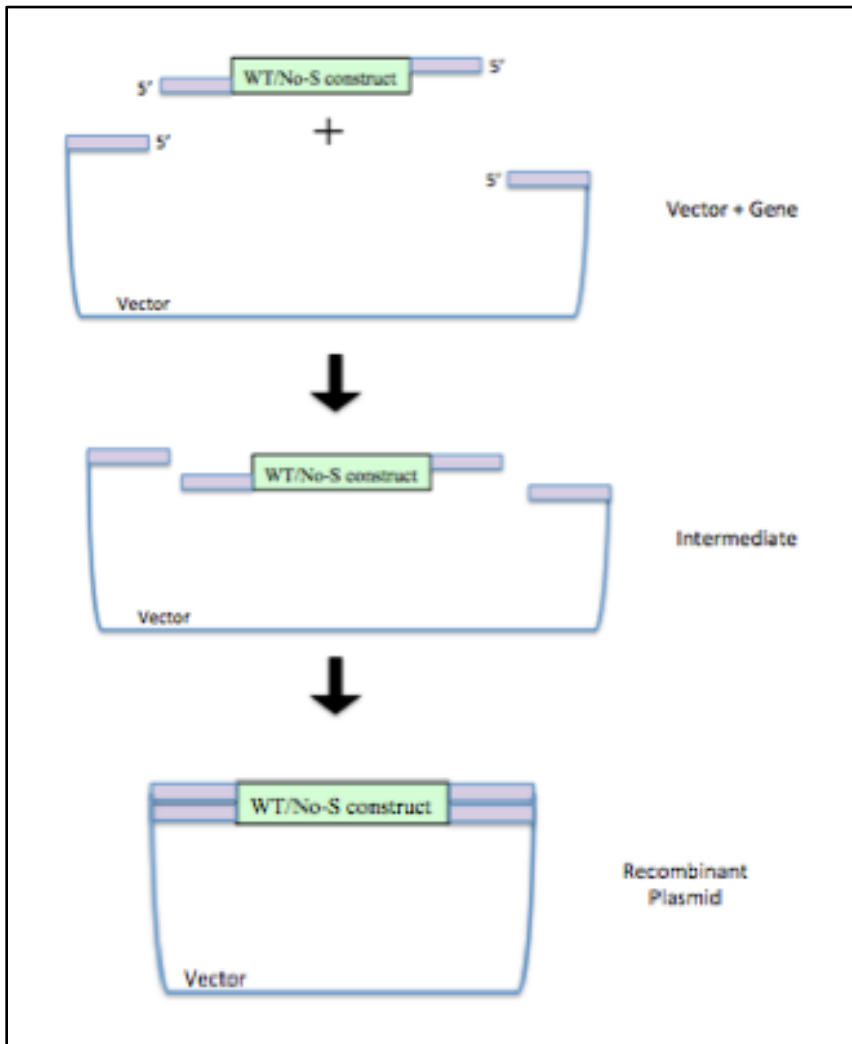
**FIGURE 4. Crosslinking reagents.** Three crosslinking reagents used *in vitro*. Three characteristics were of particular interest: membrane permeability, spacer arm length, and ability to reverse linkages.



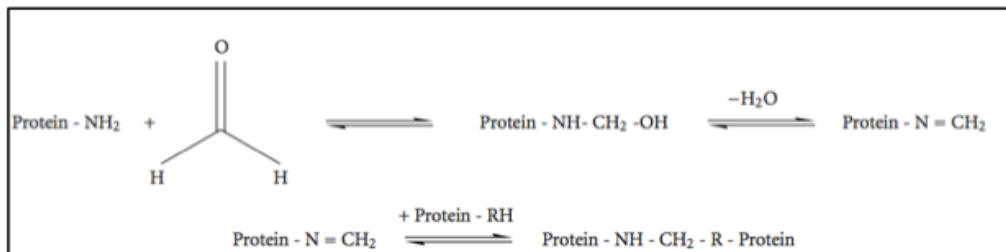
**FIGURE 5. WT and No-secretion constructs.**

**A.** Peptide sequences of the two recombinant DNA constructs containing the first 20 amino acids of YopE. The WT sequence differs from the No-secretion in residues 2-9 (red).

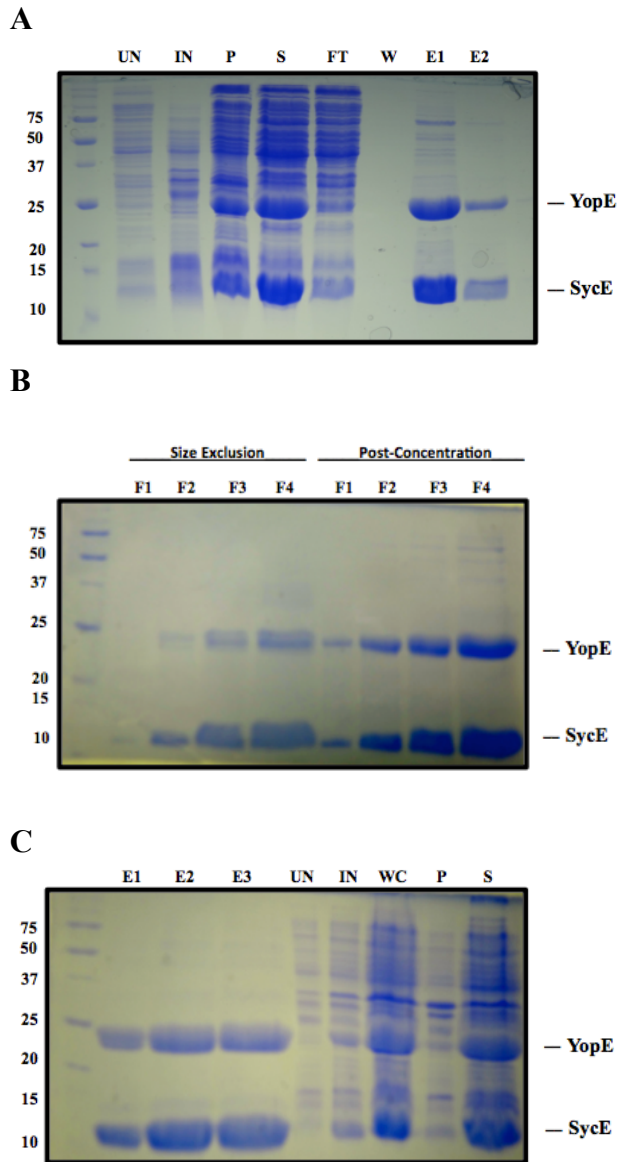
**B.** Diagram indicating the four regions downstream of the 20 amino acid WT and No-secretion YopE constructs: an NcoI restriction site, SBP tag, linker region and His tag. The final product resulted in a 7 kDa protein.



**FIGURE 6. Sequence and ligation independent cloning (SLIC).** A schematic for the production of recombinant DNA through *in vitro* homologous recombination and single-strand annealing (Li and Elledge, 2007). Inverse PCR amplification of the vector and PCR amplification of the gene product is required. The use of a 15 base pair overhang between gene and target vector allows for annealing and formation of the final recombinant DNA product.



**FIGURE 7. Formaldehyde crosslinker.** Reaction scheme of protein crosslinking in the presence of formaldehyde. The distance between conjugated molecules, or spacer arm length, is 2.3 to 2.7 Å. Reversibility of crosslinks is possible at temperatures above 99 °C. Temperatures around 65 °C retain linkages (Klockenbusch and Kast 2010). Figure from Klockenbusch and Kast, *J Biomed Biotechnology* (2010) 2010: 927585.



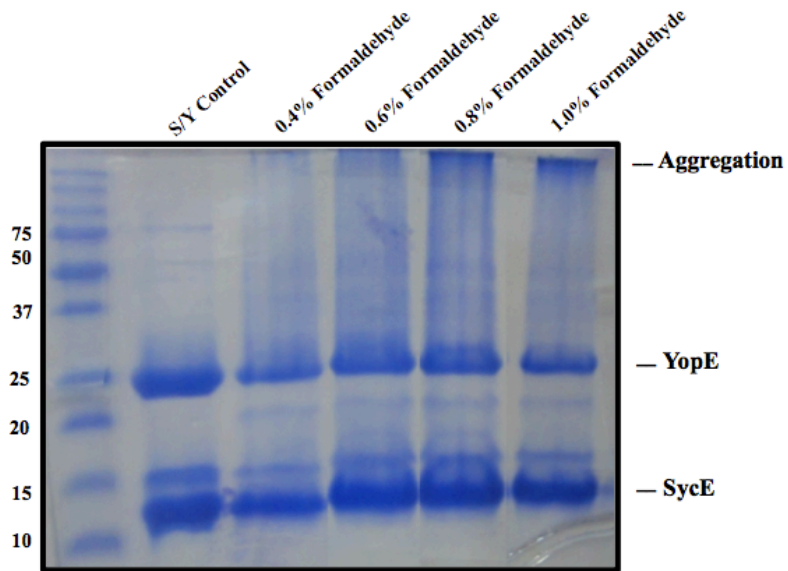
**FIGURE 8. SycE-YopE-His purification from a  $\text{Ni}^{2+}$  nitrilotriacetic acid (NTA) agarose column.**

**A.** Uninduced (UN), induced (IN), pellet (P), supernatant (S), wash (W), flow-through (FT) and two elutions (E1, E2) are shown to indicate purification of SycE-YopE-His from a nickel column.

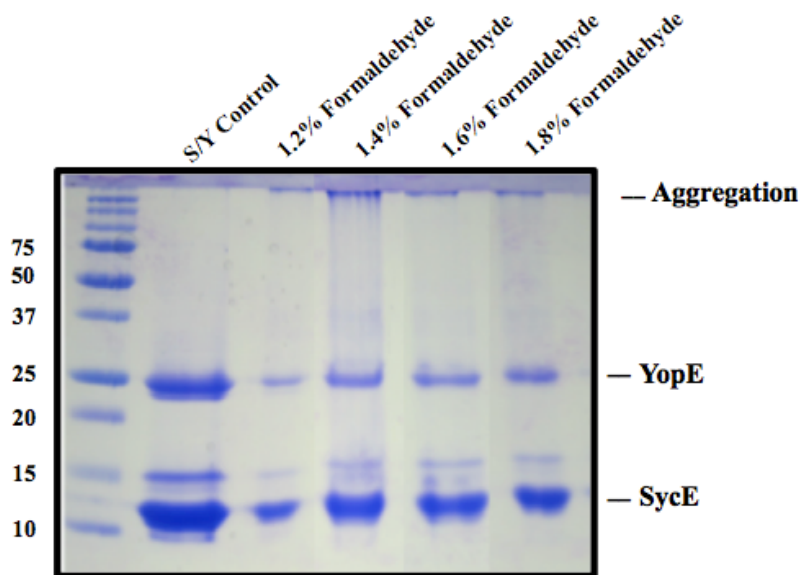
**B.** The nickel column eluate of SycE-YopE was applied to a 16/60 Superdex 200 size-exclusion column. Fractions 1 to 4 are shown both prior to concentration and after concentration.

**C.** A second round of purification was carried out. Uninduced (UN), induced (IN), whole cell lysate (WC), pellet (P), supernatant (S), and three elution (E1, E2, E3) are shown.

A



B

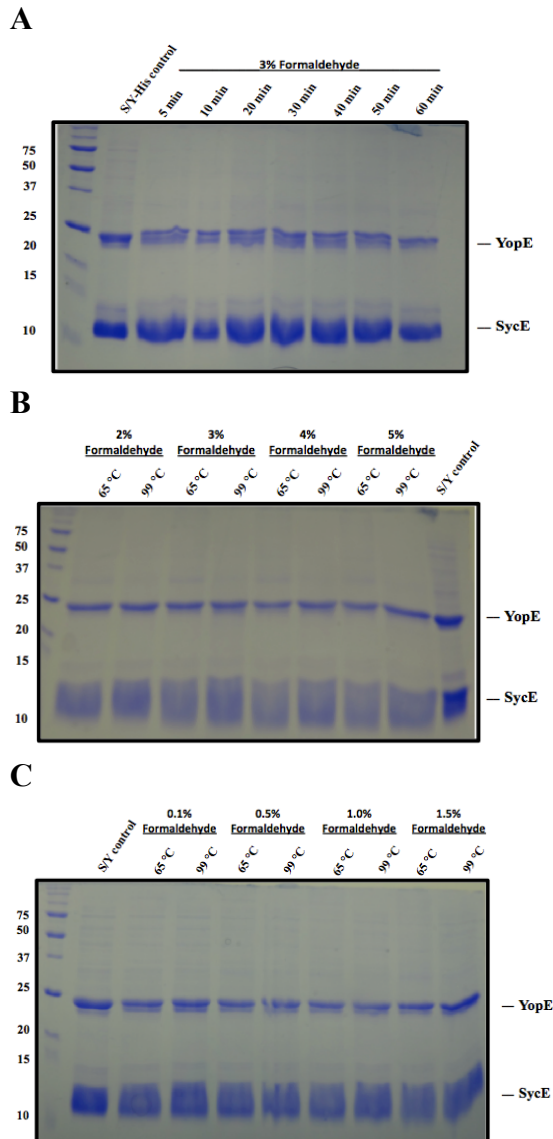


**FIGURE 9. Crosslinking of SycE-YopE with 0.4 to 1.8% formaldehyde.**

**A.** A stock of formaldehyde solution (37 wt % in H<sub>2</sub>O) with 10-15% methanol stabilizer was added from a range of 0.4 to 1.0% formaldehyde. Aggregation was visible at the top of the wells beginning at 0.8%.

**B.** A concentration range of 1.2 to 1.8% formaldehyde also resulted in aggregation at the top of the well in all lanes.



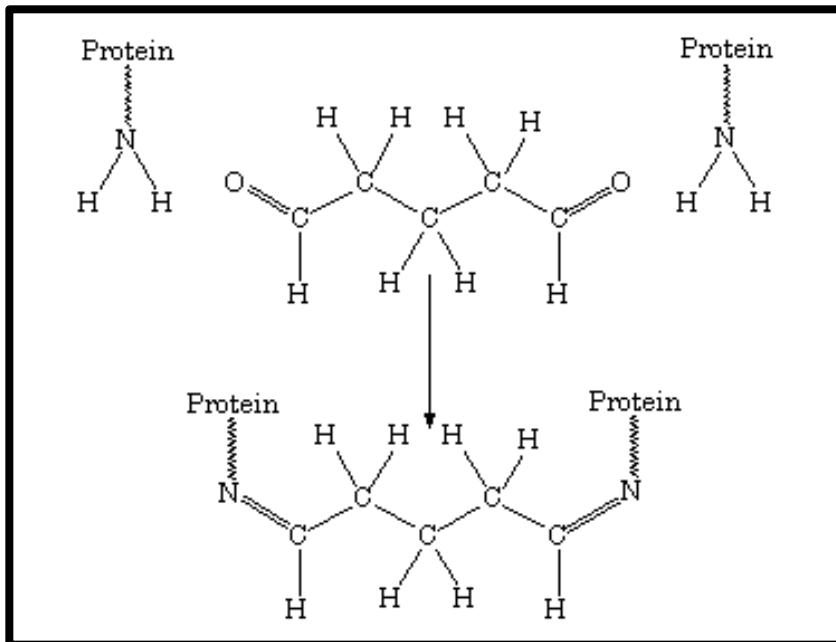


**FIGURE 10. Optimization of formaldehyde crosslinking with SycE-YopE.**

**A.** A stock of formaldehyde solution (16% methanol-free formaldehyde ampules) was added over a concentration range of 0.1 – 1.5% for 20 minutes. After addition of SDS-PAGE sample buffer, half of the sample was heated at 65 °C and half was boiled above 99 °C. The SycE-YopE complex was not visible and did not indicate crosslinking.

**B.** Formaldehyde was incubated over a concentration range of 2.0 – 5.0% with SycE-YopE for 20 minutes. There was no crosslinking in samples heated to 65 °C or boiled above 99 °C.

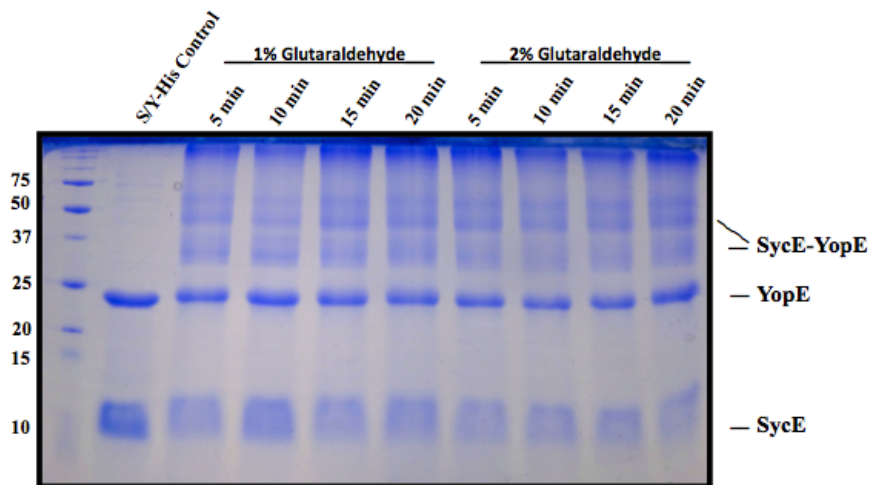
**C.** SycE-YopE was incubated with 3% formaldehyde over time points ranging from 5 minutes to 60 minutes. No crosslinking was observed.



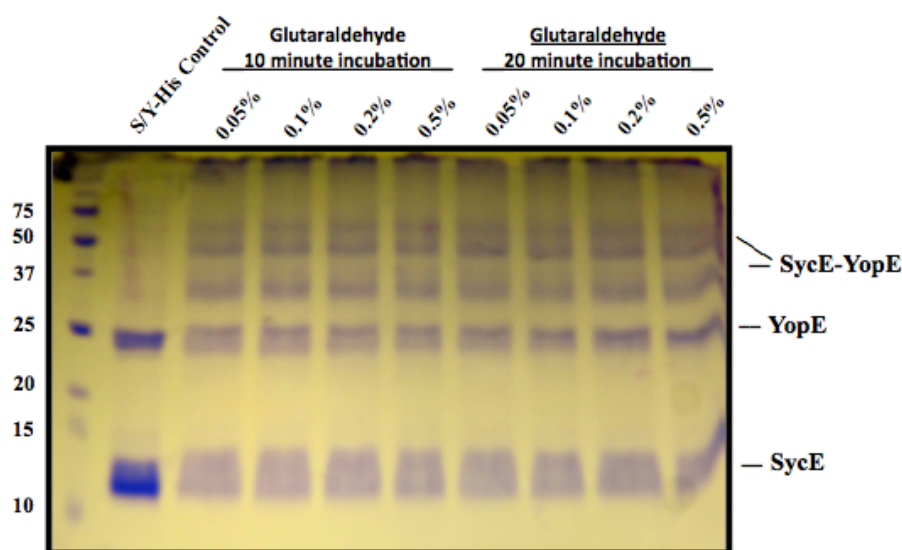
**FIGURE 11. Glutaraldehyde crosslinker.** Reaction scheme of protein crosslinking in the presence of glutaraldehyde on either side of the three methylene bridges. The distance between conjugated molecules, or spacer arm length, is 5 Å. Glutaraldehyde is an irreversible crosslinker.

Figure from <http://www.lsbu.ac.uk/biology/enzyme/practical5.html>.

A



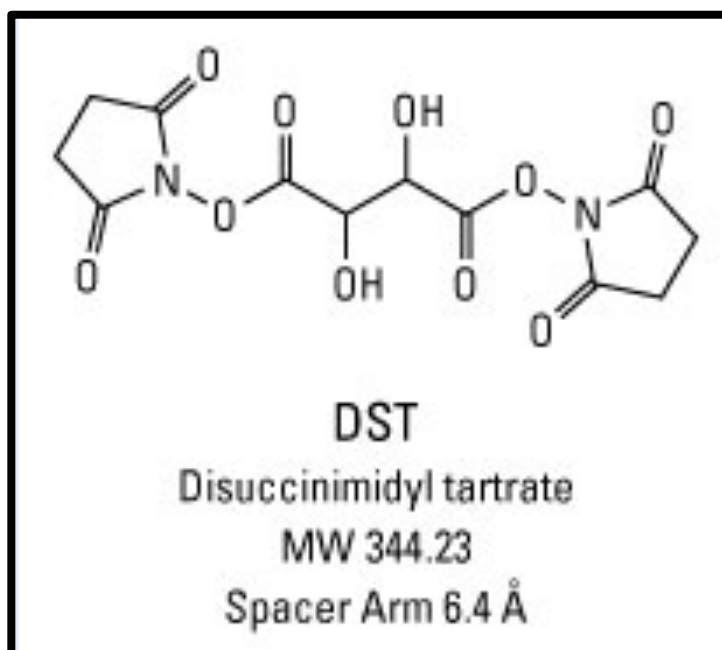
B



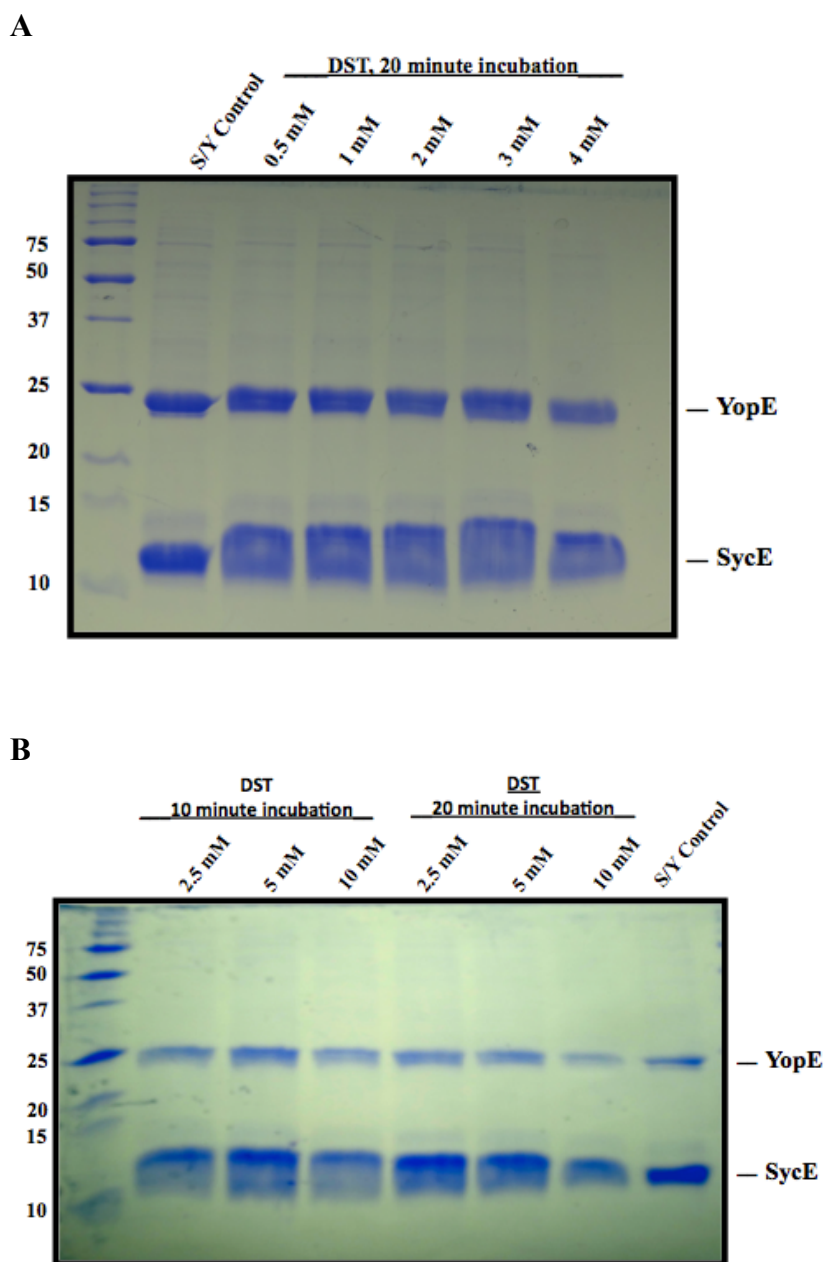
**FIGURE 12. Crosslinking of SycE-YopE with 0.05 to 0.50% glutaraldehyde**

**A.** Glutaraldehyde solution was incubated with SycE-YopE at 1% and 2% concentrations over incubation times ranging from 5 – 20 minutes. Aggregation at the top of the wells indicated excessive crosslinking and the need to decrease the glutaraldehyde concentration and possibly the incubation time

**B.** Glutaraldehyde was added at concentrations ranging between 0.05% and 0.50% for 10 or 20 minutes. A decrease in the amount of aggregation and the presence of more defined higher molecular weight bands of the SycE-YopE complex compared to Figure 10A indicated successful crosslinking.



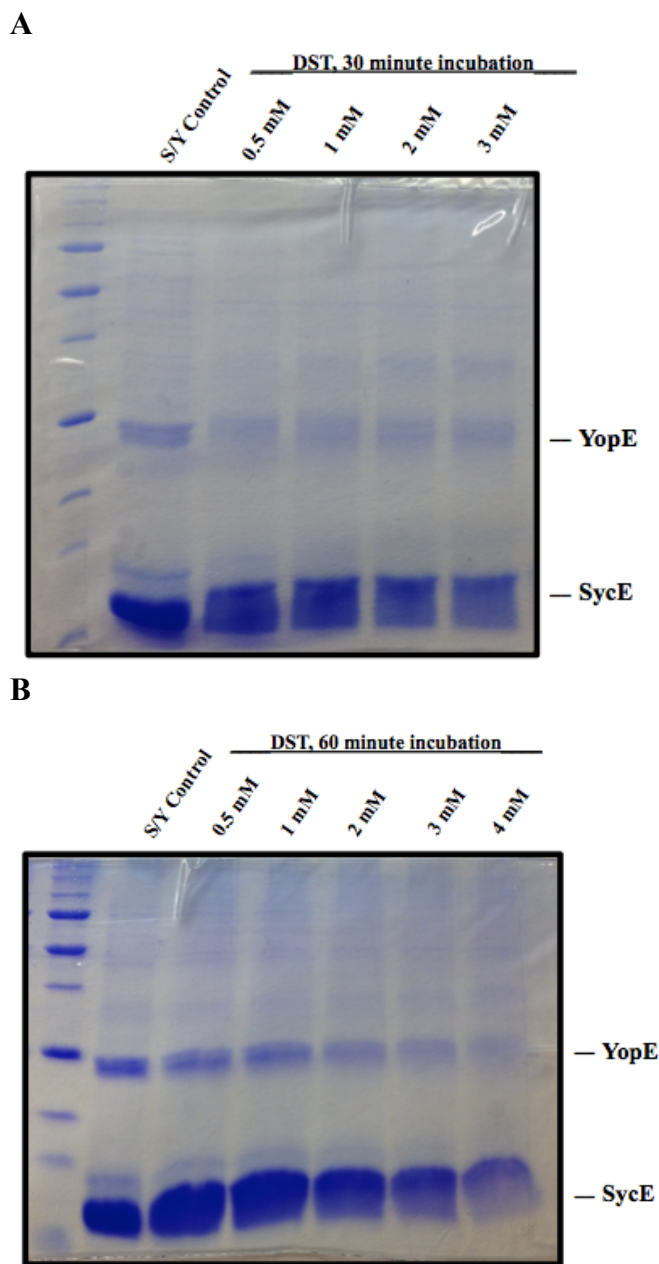
**FIGURE 13. DST crosslinker.** Molecular structure of disuccinimidyl tartrate (DST), a 6.4 Å protein crosslinker with amine reactive N-hydroxysuccinimide (NHS) ester ends. Although its reversibility was not tested, the central diol of DST is cleavable through periodate oxidation, such as with 0.015 M sodium meta-periodate (Smith et al. 1978). Figure from <http://www.piercenet.com/browse.cfm?fldID=02030238>.



**FIGURE 14. Crosslinking of SycE-YopE with 0.5 to 10 mM disuccinimidyl tartrate (DST).**

**A.** DST, prepared by dissolving in water, was incubated with SycE-YopE over a concentration range of 0.5 to 4.0 mM for 30 minutes. No crosslinking was observed.

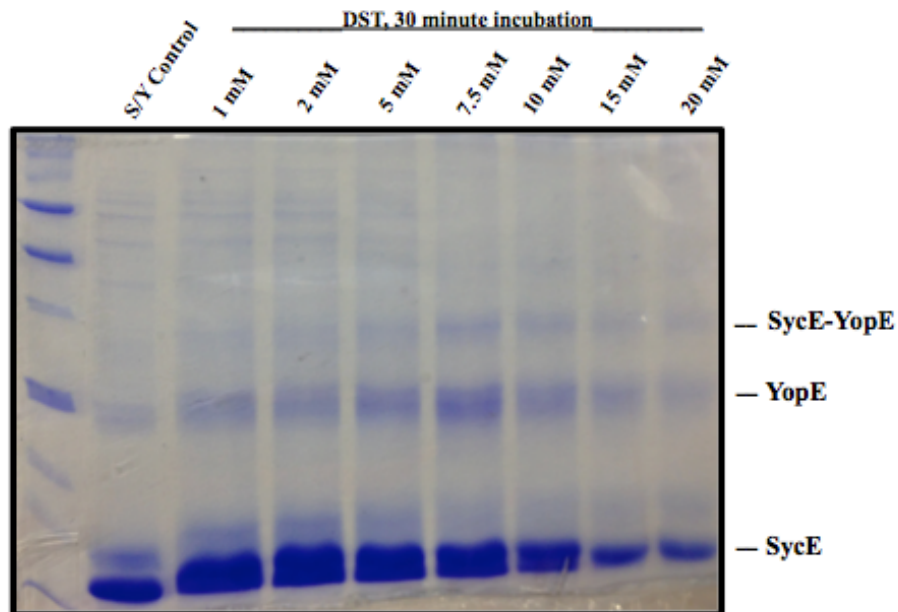
**B.** The amount of DST added was increased to a concentration range of 2.5 to 10 mM for 10 minute and 20 minute incubations. No crosslinking was observed.



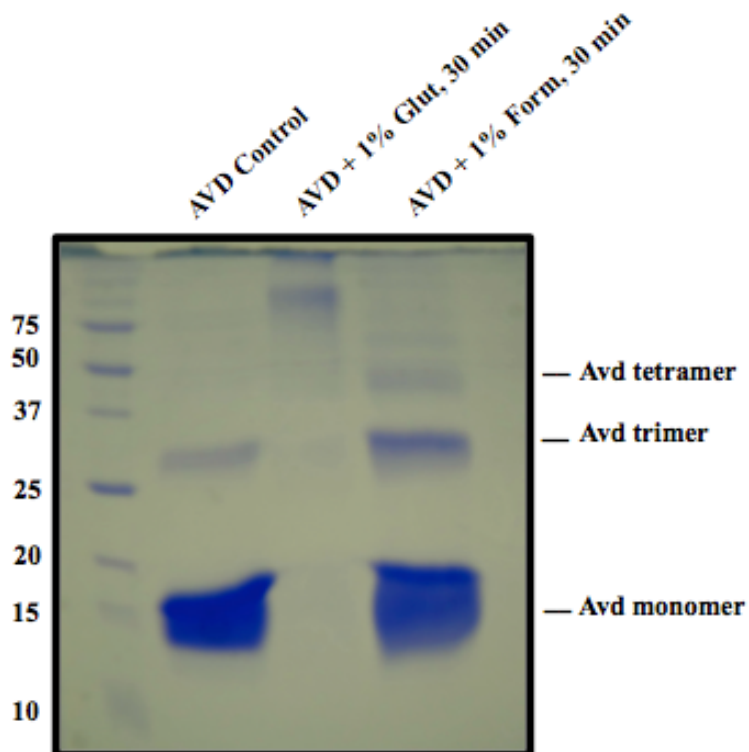
**FIGURE 15. Optimization of DST crosslinking at 30 minute and 60 minute incubation times.**

**A.** DST, prepared by dissolving in 25 mM dimethyl sulfoxide (DMSO), was incubated with SycE-YopE over a concentration range of 0.5 to 3.0 mM for 30 minutes at room temperature. No crosslinking was observed.

**B.** DST was added to SycE-YopE at over a concentration range of 0.5 to 4.0 mM for 60 minutes at room temperature. No crosslinking was observed.

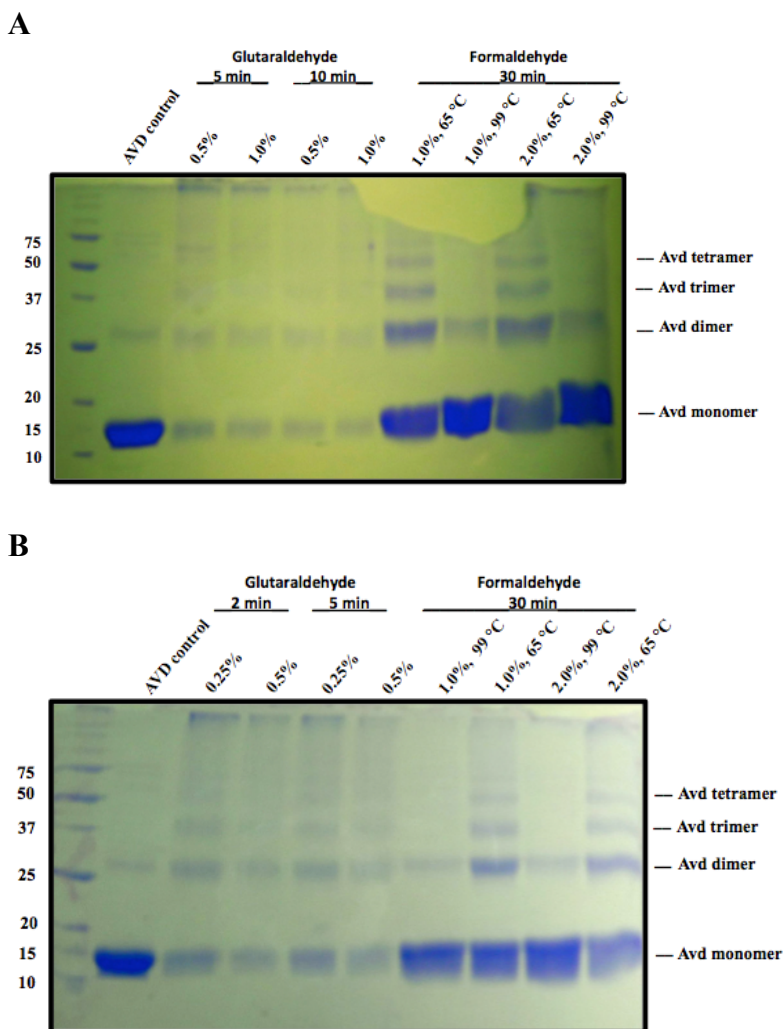


**FIGURE 16. Crosslinking of SycE-YopE with 1 to 20 mM DST.** The DST stock solution was prepared by dissolving in 100% DMSO. SycE-YopE was incubated with DST at concentrations ranging from 1 to 20 mM for 30 minutes. A slight amount of crosslinking not present in the negative control can be visualized beginning at the 5 mM concentration.



**FIGURE 17. Glutaraldehyde and formaldehyde crosslinking with Avd.** 1% glutaraldehyde and 1% formaldehyde were incubated with Avd, a 60 kDa pentameric protein, for 30 minutes at room temperature. Excessive crosslinking of Avd in the presence of glutaraldehyde was visible, indicated by the aggregation at the top of the well. Distinct higher molecular weight bands of crosslinked Avd were visible in the presence formaldehyde.

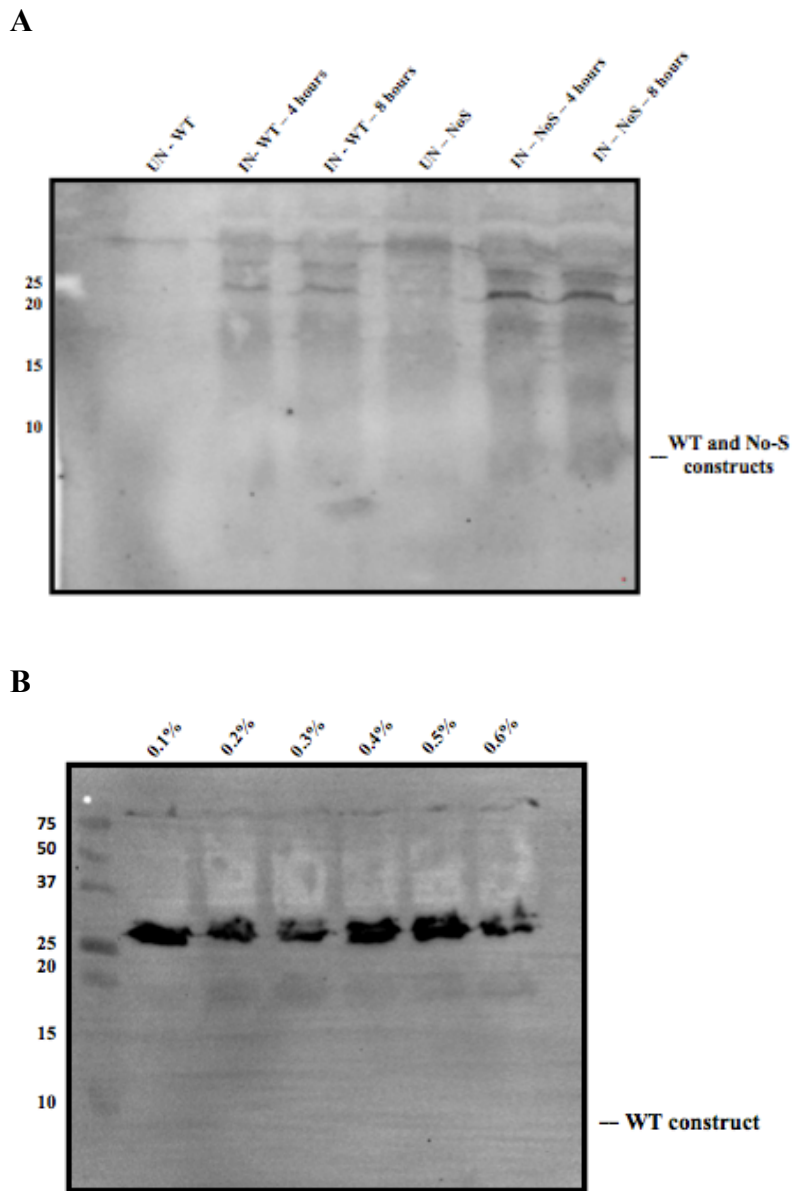




**FIGURE 18. Optimization of glutaraldehyde and formaldehyde crosslinking with Avd.**

**A.** Glutaraldehyde concentrations of 0.5 and 1% were added to Avd for 5 min and 10 min. Aggregation was observed at the top of the wells, indicating the need to decrease concentration and incubation time. Formaldehyde was added to Avd at 1 and 2% concentration for 30 minutes. Half of the sample was heated to 65 °C and the other half was boiled above 99 °C. Samples heated to 65 °C indicate distinct crosslinking. Boiled samples do not contain the same higher molecular weight bands, indicating reversal of linkages.

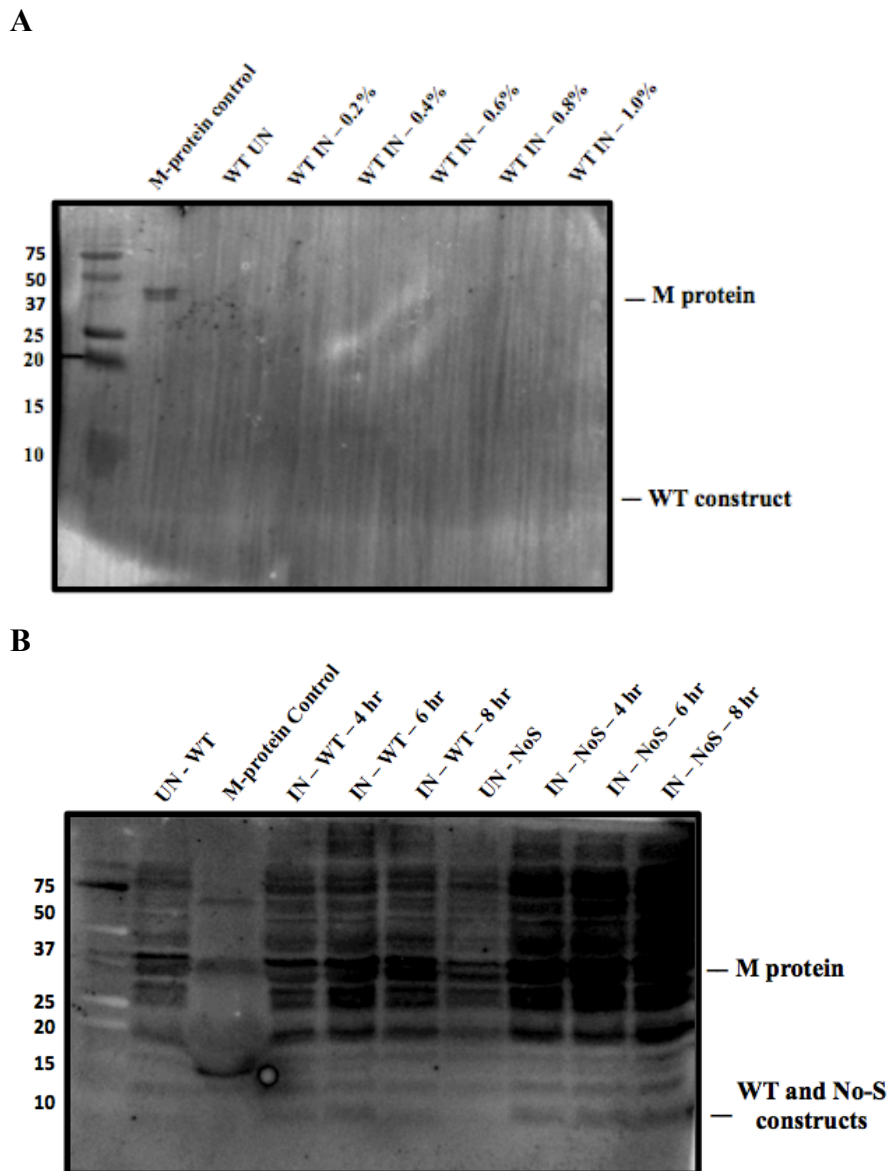
**B.** Decreased concentrations of glutaraldehyde were used to minimize the aggregation present at the top of the wells. Clear bands from addition of 0.25 and 0.5% glutaraldehyde concentrations are clearly visible from incubation times of 2 and 5 minutes. Formaldehyde concentrations and incubation times were replicated as in Figure 17A. Successful crosslinking was observed from both glutaraldehyde and formaldehyde.



**FIGURE 19. Western blot of WT and No-secretion expression in *E. coli* Top10**

**A.** WT and No-secretion constructs were induced with 0.15% arabinose for 8 hours. Samples were taken at 4 hours and 8 hours. Western blot with an anti-His antibody did not indicate expression of the 7 kDa constructs in any of the induced samples.

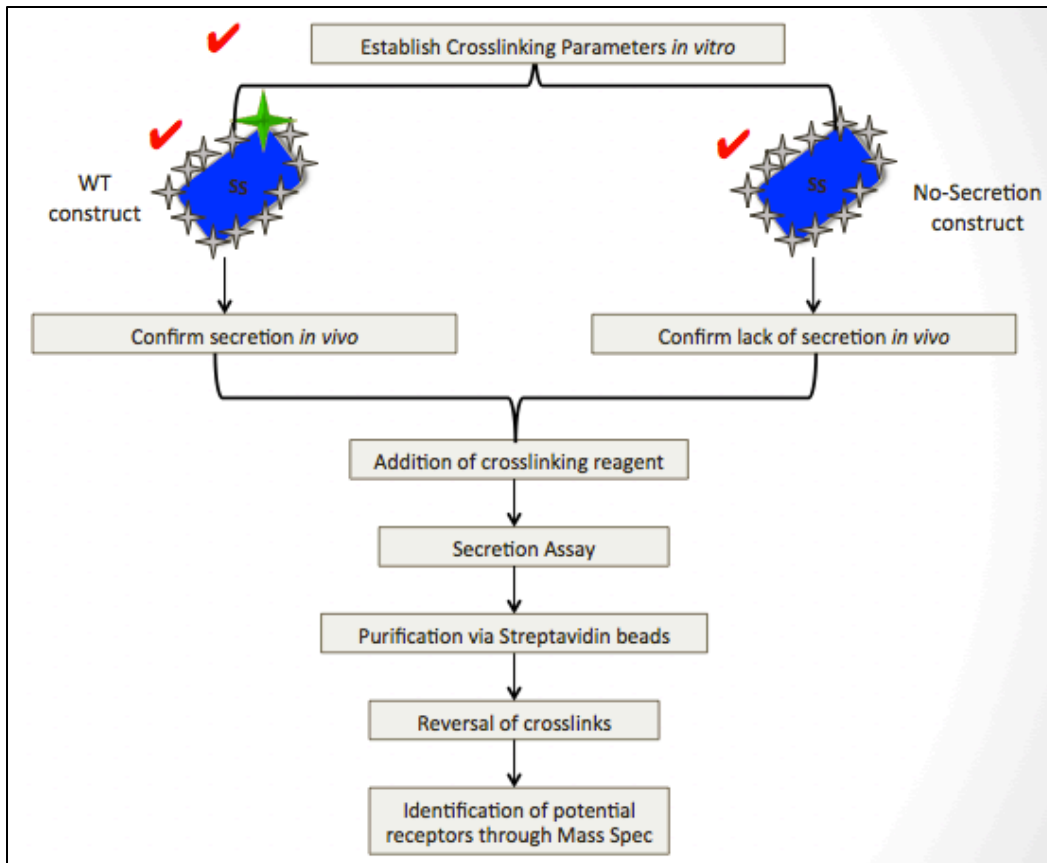
**B.** WT constructs were induced over a concentration range of 0.1 to 0.6% arabinose. Western blot did not indicate expression of the 7 kDa construct.



**FIGURE 20. Optimization of WT and No-secretion expression in *E. coli* Top10**

**A.** WT constructs were induced over a concentration range of 0.2 to 1.0% arabinose. A positive control His-tagged “M-protein” is visible at 37 kDa. The 7 kDa WT construct is not visible on the western blot, indicating a lack of expression.

**B.** WT and No-secretion constructs were induced with 1% arabinose. Samples were taken 4 hours, 6 hours and 8 hours after induction. The positive control His-tagged protein is visible at 37 kDa. A 7 kDa band is present in the induced lanes indicating possible expression of the WT and No-secretion constructs. However, due to the high level of background present on this western blot, it is difficult to conclusively state that the constructs are being expressed.



**Figure 21. Flowchart of project overview and future directions.** The data in this study indicate the establishment of crosslinking parameters *in vitro* of the WT and No-secretion constructs (blue). The stars indicate proteins that are hypothesized to interact with the SS region. *In vivo* confirmation of secretion, or lack of secretion, will be required prior to beginning experiments in *Y. pseudotuberculosis*. Crosslinking reagent will then be added followed by a secretion assay, purification via streptavidin beads, reversal of crosslinking, and finally, mass spectrometry. The identification of a potential receptor for the YopE SS region (shown in green) is hypothesized through these future experiments.

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