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Identification of TyeA Residues Required to Interact with YopN and to Regulate Yop Secretion

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Abstract. The secretion of Yops via the *Yersinia* type III secretion system (T3SS) is controlled, in part, by a cytoplasmic YopN/TyeA complex. This complex is required to prevent Yop secretion in the presence of extracellular calcium and prior to contact between the bacterium and a eukaryotic cell. In this study we utilized site-directed mutagenesis to analyze the role of specific TyeA regions and residues in the regulation of Yop secretion. We identified two spatially distinct, surface-exposed regions of the TyeA molecule that were required to regulate Yop secretion. One region, identified by residues M51, F55 and P56, was required for TyeA to interact with YopN. A second region, identified by residues R19, W20 and D25 was not involved in the interaction of TyeA with YopN, but may be required for the YopN/TyeA complex to interact with the T3S apparatus in a manner that blocks Yop secretion.

21.1 Introduction

Yersinia pestis is the etiologic agent of plague, one of the most devastating diseases known (Perry and Fetherston 1997). The ability of *Y. pestis* to produce disease primarily results from its capacity to avoid or disrupt the innate defenses of its host (Cornelis 2000). This capability enables the bacterium to grow and multiply essentially unhindered. *Y. pestis* actively blocks bacterial phagocytosis and prevents the early production of proinflammatory cytokines (Fallman and Gustavsson 2005; Navarro et al. 2005). These abilities are strictly dependent upon the presence of a functional plasmid pCD1-encoded type III secretion system (T3SS). The *Yersinia* T3SS functions to inject effector proteins, termed *Yersinia* outer proteins (Yops), directly into host cells where they function to undermine cellular processes that normally prevent bacterial growth and survival.

The *Yersinia* T3SS is a complex protein secretion system that functions to transport Yops from the cytoplasm of the bacterial cell to the cytoplasm of a eukaryotic cell (Mota and Cornelis 2005). The T3S apparatus consists of a base structure that spans the bacterial inner and outer membranes and of an external needle-like structure composed of the secreted YscF protein that extends 40 to 60 nm from the bacterial surface. The injection process occurs in two distinct steps: (i) secretion of Yops across the bacterial membranes and (ii) translocation of Yops across a eukaryotic membrane. The second step in this process is dependent upon three secreted pore-forming proteins (LcrV, YopB and YopD) that form a translocation complex, or translocon, at the eukaryotic membrane (Marenne et al. 2003; Neyt and Cornelis 1999; Nilles et al. 1998; Pettersson et al. 1999). The LcrV protein forms a needle-tip

complex that facilitates assembly of the needle-translocon structure at the eukaryotic membrane (Mueller et al. 2005).

The secretion of Yops is normally triggered by contact between a bacterium and a eukaryotic cell. *In vitro*, Yop secretion is blocked in the presence of millimolar levels of extracellular calcium and is triggered by the removal of extracellular calcium (Michiels et al. 1990). The regulation of Yop secretion is dependent upon at least six *Yersinia* pCD1-encoded proteins: LcrG (Matson and Nilles 2001; Skryzpek and Straley 1993), YopN (Forsberg et al. 1991; Yother and Goguen 1985), SycN (Iriarte and Cornelis 1999), YscB (Jackson et al. 1998), TyeA (Cheng and Schneewind 2000; Iriarte et al. 1998) and YscF (Torruellas et al. 2005). These proteins function to block Yop secretion in the presence of calcium *in vitro* and prior to contact with a eukaryotic cell *in vivo*. A deletion in any one of these genes results in constitutive secretion in the presence and absence of calcium and prior to contact with a eukaryotic cell (constitutive secretion [CS] phenotype). The LcrG protein forms a 1:1 complex with LcrV within the cell and assists in blocking Yop secretion when present in an excess amount over LcrV (Matson and Nilles 2001). YopN is a secreted protein that directly interacts with the cytosolic SycN/YscB chaperone and TyeA prior to its export from the bacterial cell (Cheng et al. 2001; Day et al. 2003). The SycN/YscB chaperone binds to an N-terminal region of YopN and is required for efficient YopN secretion and translocation. The TyeA protein binds to a C-terminal region of YopN and functions with YopN to block Yop secretion. Although YopN and TyeA are normally expressed as two separate proteins, an engineered YopN-TyeA fusion protein is secreted, translocated and regulates Yop secretion (Ferracci et al. 2004). Interestingly, most bacterial pathogens that employ T3SSs express and secrete a YopN/TyeA-like fusion protein, not separate YopN-like and TyeA-like proteins (Pallen et al. 2005). YscF is a secreted protein that assembles to form the extracellular needle-like structure (Hoiczyk and Blobel 2001). YscF point mutants that secrete Yops constitutively have been identified, indicating that the YscF needle, which is required for Yop secretion, also plays a role in the regulation of Yop secretion (Torruellas et al. 2005). The mechanism by which LcrG, the YopN/SycN/YscB/TyeA complex and YscF regulate Yop secretion is not understood.

Recently, several YopN mutants were identified that block Yop secretion constitutively (no secretion [NS] phenotype) (Ferracci et al. 2005). These mutants required TyeA, but did not require LcrG, the SycN/YscB chaperone, a functional YopN N-terminal secretion signal or chaperone-binding domain to block Yop secretion. These results suggest that the YopN/TyeA complex blocks Yop secretion from a cytosolic location. We hypothesize that the YscF needle/LcrV tip complex functions as a signal sensor/transmission device that controls the activity of the YopN/TyeA molecular plug.

The mechanism by which the cytosolic YopN/TyeA complex blocks Yop secretion is unknown. We hypothesize that the YopN/TyeA complex interacts with an unidentified cytosolic T3S component. If correct, this would indicate that the YopN/TyeA complex contains an essential surface-exposed binding site that could potentially be identified through genetic analyses. In this study we investigated the role of specific TyeA regions and residues in the regulation of Yop secretion using

alanine-scanning mutagenesis. These studies identified a surface-exposed region of TyeA that is required to block Yop secretion, but is not required for TyeA to interact with YopN.

21.2 Materials and Methods

21.2.1 Bacterial Strains and Growth Conditions

Escherichia coli DH5α was used for routine cloning experiments (Cambau et al. 1993). *Y. pestis* and *E. coli* strains were grown in heart infusion broth (HIB) or on tryptose blood agar (TBA) plates (Difco) at 27°C and 37°C, respectively. For growth/secretion assays *Y. pestis* strains were grown with or without 2.5 mM $CaCl_2$ in TMH medium (Goguen et al. 1984) inoculated from cultures grown overnight at 27°C. Strains were grown for 1 h at 27°C and then shifted to 37°C for 5 h of growth. Bacteria carrying resistance markers were grown in the presence of the appropriate antibiotic at a final concentration of 25 μg/ml (kanamycin) or 50 μg/ml (ampicillin or streptomycin).

21.2.2 Generation of TyeA Point Mutants

The DNA fragment encoding TyeA was generated by PCR amplification of *tyeA* encoded on pCD1 using primers (TyeA-*Eco*RI; 5'- TTTGAATTCGGCAATTTTTT TCAGAGGGTAAAAC-3') and (TyeA-*Hin*dIII; 5'-TTTAAGCTTTCAATCCAAC TCACTCAATTCTTC-3'). The resulting 310 base pair PCR fragment was digested with *Eco*RI and *Hin*dIII and inserted into *Eco*RI- and *Hin*dIII-digested pBAD18, generating plasmid pTyeA. Alanine-scanning, site-directed mutagenesis of pTyeA was performed by the PCR-ligation-PCR procedure as previously described (Ali and Steinkasserer 1995; Torruellas et al. 2005). The DNA sequence of the entire *tyeA* gene present in each pTyeA derivative was confirmed by DNA sequence analysis.

21.2.3 SDS-PAGE and Immunoblotting

Bacterial cell pellets and culture supernatants were separated by centrifugation at 12,000 x g for 10 min at 4ºC. Culture supernatant proteins were precipitated on ice overnight with 10% (v/v) trichloroacetic acid (TCA) and collected by centrifugation at 12,000 x g for 10 min at 4ºC. Volumes of cellular fractions corresponding to equal numbers of bacteria were analyzed by SDS-PAGE and immunoblotting as described (Ferracci et al. 2005). The YopM and YopN proteins were detected with polyclonal antisera raised against purified 6X-histidine-tagged YopM or YopN proteins.

21.2.4 GST-Pulldown Experiments

Plasmid pGST-YopN$^{78-293}$ encodes a GST-YopN$^{78-293}$ fusion protein. A 648-base pair DNA fragment encoding *yopN* codons 78 to 293 was PCR amplified with primers pGST-YopN-78 (5'-ATGGCTCGAGTTAGCGACGTTGAGGAG-3') and

pGST-YopN-293 (5'-TTTGAATTCTCAGAAAGGTCGTACGCCATTAGTTTT-3'), digested with *Eco*RI and inserted into *Eco*RI- and *Psh*AI-digested pET42b, generating pGST-YopN$^{78\text{-}293}$. Plasmid pGST-YopN$^{78\text{-}293}$ and pTyeA, pTyeA (R19A W20A), pTyeA (W20A D25A), pTyeA (M51A F55A) or pTyeA (S6A G10A V13A) were transformed into *E. coli* BL21. The resultant strains were grown for 3 h at 37^{o}C after which time expression of GST-YopN$^{78\text{-}293}$ and the wild-type or mutant TyeA protein were induced by addition of IPTG (1 mM) and L-arabinose (0.2%), respectively. The bacterial cells were harvested, lysed using a French pressure cell and centrifuged at 8,000 x g for 10 min to remove unlysed cells and large debris. The resulting lysates were further centrifuged at 35,000 x g for 30 min and the supernatant fractions containing the soluble proteins were applied to glutathione sepharose columns. The soluble fractions were incubated with the glutathione sepharose beads overnight at 4^{o}C with shaking to allow efficient binding. The columns were subsequently washed three times with Tris-HCl (20 mM), NaCl (150 mM), pH 8.0. The bound GST-YopN$^{78\text{-}293}$ and TyeA proteins were eluted with glutathione (10 mM). The soluble samples, wash fractions and elutions were collected and analyzed by SDS-PAGE and immunoblotting with antibodies specific for the GST moiety and TyeA.

21.3 Results

21.3.1 Alanine-Scanning Mutagenesis of *tyeA*

TyeA is a small 92 amino-acid residue protein that directly interacts with the C-terminus of YopN (Iriarte et al. 1998). TyeA is composed of two sets of parallel alpha helices (Schubot et al. 2005). In the YopN/TyeA complex, the C-terminal helix of YopN is found intercalated between the first and third helices of TyeA. The N-terminal helix of TyeA also makes several contacts with YopN helix nine, composed of YopN residues 212 to 222. To investigate the role of specific TyeA residues in the interaction of TyeA with YopN and in the regulation of Yop secretion, we replaced selected TyeA residues with alanine and characterized the resulting mutant TyeA proteins.

Oligonucleotide site-directed mutagenesis of plasmid pTyeA was performed to replace select TyeA amino-acid residues with alanine (Table 1). The resultant pTyeA derivatives were electroporated into a *tyeA* deletion strain and the Yop expression and secretion phenotypes of the resultant strains were determined. Thirty-one mutants with single alanine substitutions in TyeA were analyzed for Yop expression and secretion following growth in the presence or absence of 2.5 mM calcium in TMH medium for 5 h at 37°C. All of the TyeA mutants expressed a stable TyeA protein (data not shown). The parent strain *Y. pestis* KIM5-3001.P39 (Day et al. 2003), the *tyeA* deletion strain complemented with pTyeA and 25 of the 31 TyeA mutants exhibited normal calcium-regulated secretion (RS) of Yops (Table 1; data not shown). The *tyeA* deletion strain and four TyeA alanine missense mutants (W20A, D25A, F55A and P56A) showed constitutive secretion (CS) of Yops in both the presence and absence of calcium (Fig. 1A). In addition, two TyeA mutants (R19A and M51A)

Table 1. Yop Secretion Phenotypes of *Y. pestis* TyeA Point Mutants

Strain + plasmid	Yop secretion	Strain + plasmid	Yop secretion
KIM5-3001.P39 (parent)	RS	Δ*tyeA* + pTyeA (L48A)	RS
KIM-3001.P63 (Δ*tyeA*)	CS	Δ*tyeA* + pTyeA (M51A)	RS/CS
Δ*tyeA* + pTyeA	RS	Δ*tyeA* + pTyeA (R53A)	RS
Δ*tyeA* + pTyeA (Y3A)	RS	Δ*tyeA* + pTyeA (F55A)	CS
Δ*tyeA* + pTyeA (L5A)	RS	Δ*tyeA* + pTyeA (P56A)	CS
Δ*tyeA* + pTyeA (S6A)	RS	Δ*tyeA* + pTyeA (G58A)	RS
Δ*tyeA* + pTyeA (M9A)	RS	Δ*tyeA* + pTyeA (D62A)	RS
Δ*tyeA* + pTyeA (G10A)	RS	Δ*tyeA* + pTyeA (E63A)	RS
Δ*tyeA* + pTyeA (D11A)	RS	Δ*tyeA* + pTyeA (E64A)	RS
Δ*tyeA* + pTyeA (V16A)	RS	Δ*tyeA* + pTyeA (Q65A)	RS
Δ*tyeA* + pTyeA (D17A)	RS	Δ*tyeA* + pTyeA (C73A)	RS
Δ*tyeA* + pTyeA (K18A)	RS	Δ*tyeA* + pTyeA (Q74A)	RS
Δ*tyeA* + pTyeA (R19A)	RS/CS	Δ*tyeA* + pTyeA (I81A)	RS
Δ*tyeA* + pTyeA (W20A)	CS	Δ*tyeA* + pTyeA (E84A)	RS
Δ*tyeA* + pTyeA (I23A)	RS	Δ*tyeA* + pTyeA (R19A W20A)	CS
Δ*tyeA* + pTyeA (D25A)	CS	Δ*tyeA* + pTyeA (W20A D25A)	CS
Δ*tyeA* + pTyeA (E27A)	RS	Δ*tyeA* + pTyeA (M51A F55A)	CS
Δ*tyeA* + pTyeA (F33A)	RS	Δ*tyeA* + pTyeA (F55A P56A)	CS
Δ*tyeA* + pTyeA (L35A)	RS	Δ*tyeA* + pTyeA (S6A G10A V13A)	RS/CS
Δ*tyeA* + pTyeA (F44A)	RS		

RS, calcium regulated secretion; CS, constitutive secretion irrespective of calcium
NS, no secretion; RS/CS, partial secretion in the presence of calcium

had intermediate phenotypes and secreted low amounts of Yops in the presence of calcium (RS/CS phenotype). These studies identified six TyeA amino-acid residues that were required to block Yop secretion in the presence of millimolar levels of extracellular calcium. Interestingly, the identified residues mapped to two distinct regions of the TyeA molecule (Fig. 1B). TyeA residues M51, F55 and P56 are predicted to be located at the interface between TyeA and YopN (Schubot et al. 2005). In fact, the crystal structure of the YopN-TyeA complex predicts that each of the identified residues forms a direct contact point between TyeA and the C-terminal helix of YopN. These results suggest that the role of TyeA residues M51, F55 and P56 in the regulation of Yop secretion is to mediate the interaction of TyeA with YopN. On the other hand, TyeA residues R19, W20 and D25 mapped to a surface-exposed region of the TyeA molecule that was not predicted to be involved in the interaction of TyeA with YopN. Thus, this region of TyeA may be involved in interactions between the YopN-TyeA complex and the T3S apparatus that are required for the YopN/TyeA-dependent block in Yop secretion.

21.3.2 Construction and Analysis of TyeA Double and Triple Alanine-Substitution Mutants

To further analyze the role of the two identified regions of TyeA required to regulate Yop secretion (Fig. 1B), we generated a series of double alanine substitution mutants (Table 1). Oligonucleotide site-directed mutagenesis of pTyeA (W20A) and pTyeA (F55A) was performed to generate plasmids pTyeA (R19A W20A), pTyeA (W20A

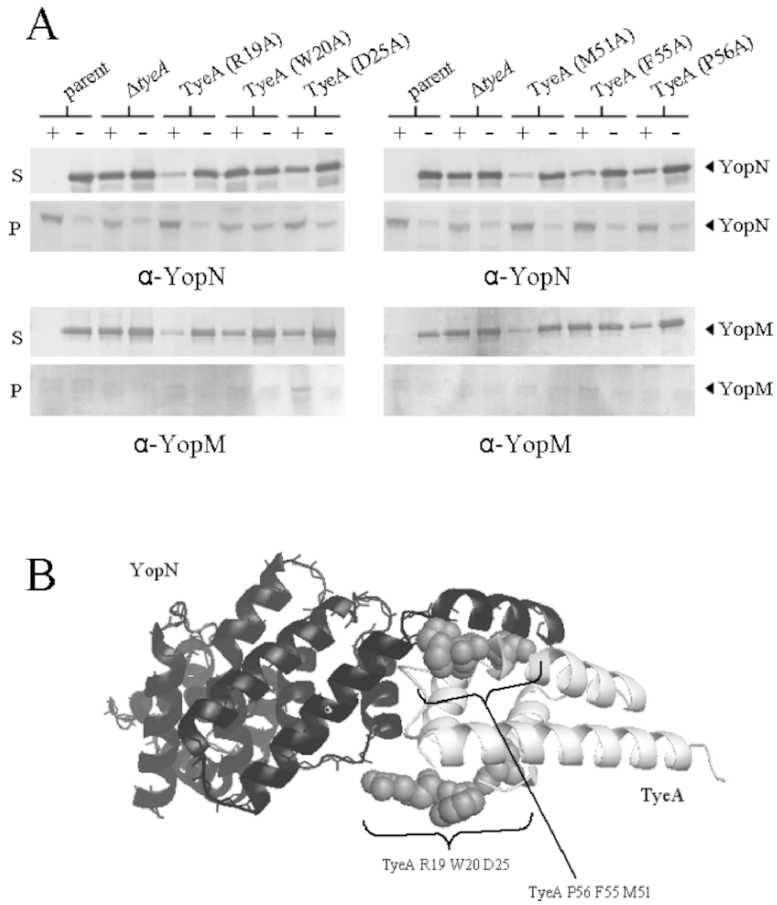

Fig. 1. Analysis of TyeA single alanine missense mutants. (A) Expression and secretion of YopN and YopM by TyeA mutants. *Y. pestis* KIM5-3001.P39 (parent), KIM-3001.P63 (Δ*tyeA*) and six TyeA alanine missense mutants were analyzed by SDS-PAGE and immunoblot analysis of cell pellet (P) and culture supernatant (S) fractions derived from bacteria grown for 5 h at 37°C in the presence and absence of 2.5 mM $CaCl_2$. Blots were probed with α-YopN and α-YopM antisera. The location of YopM and YopN are shown by arrowheads. (B) Ribbon model of the structure of the $YopN^{76\text{-}293}$–TyeA complex highlighting the two identified regions of TyeA required for regulating Yop secretion. YopN and TyeA are shown in black and light grey, respectively. The location of TyeA residues M51, F55 and P56, which form contacts with residues on helix 12 of YopN, as well as TyeA residues R19, W20 and D25 are shown (dark grey space-filled representation). This model was generated using PyMOL (DeLano 2001).

D25A), pTyeA (M51A F55A) and pTyeA (F55A P56A). Previous analyses of the YopN-TyeA crystal structure indicated that, in addition to the TyeA residues that directly interact with the C-terminal helix of YopN, TyeA amino-acid residues S6,

G10 and V13 form contacts with YopN helix number nine that may play a direct role in mediating the interaction of TyeA with YopN (Schubot et al. 2005). To investigate the role of these contact residues in the interaction of TyeA with YopN and in the regulation of Yop secretion, we used oligonucleotide site-directed mutagenesis of pTyeA to generate plasmid pTyeA (S6A G10A V13A).

The resultant pTyeA derivatives encoding TyeA double and triple alanine-substitution mutants were electroporated into the *tyeA* deletion mutant and analyzed for Yop expression and secretion, following growth in the presence or absence of 2.5 mM calcium in TMH medium for 5 h at 37°C. The parent strain showed typical calcium regulated Yop secretion; however, the *tyeA* deletion strain expressing TyeA (R19A W20A), TyeA (W20A D25A), TyeA (M51A F55A) or TyeA (F55A P56A) secreted Yops constitutively in both the presence and absence of calcium (Fig. 2A). In addition, the *tyeA* deletion strain expressing TyeA (S6A G10A V13A) exhibited a partial phenotype, secreting low amounts of Yops in the presence of calcium. These results confirm a role for the two identified TyeA regions in the regulation of Yop secretion and suggest that TyeA residues S6, G10 and V13 may also play a role in TyeA function.

21.3.3 Interaction of TyeA Mutants with YopN

The ability of TyeA (R19A W20A), TyeA (W20A D25A), TyeA (M51A F55A) and TyeA (S6A G10A V13A) to interact with YopN was evaluated using a GST-pulldown procedure and the GST-YopN$^{78\text{-}293}$ protein. The GST-YopN$^{78\text{-}293}$ protein, encoded by plasmid pGST-YopN$^{78\text{-}293}$, carries an intact TyeA-binding domain, but lacks the YopN N-terminal secretion signal and SycN/YscB chaperone-binding domain (Schubot et al. 2005). *E. coli* BL21 was transformed with plasmid pGST-YopN$^{78\text{-}293}$ and plasmid pTyeA, pTyeA (R19A W20A), pTyeA (W20A D25A), pTyeA (M51A F55A) or TyeA (S6A G10A V13A). The resultant strains were initially grown for 3 h at 37ºC, at which point IPTG (1 mM) and L-arabinose (0.2%) were added to induce expression of the GST-YopN$^{78\text{-}293}$ and TyeA proteins and the cultures were incubated an additional 2 h at 37ºC. Bacteria expressing the GST-YopN$^{78\text{-}293}$ protein and one of the TyeA derivatives were harvested, lysed, and the lysate centrifuged. The cleared lysates were added to glutathione sepharose columns, washed, and the bound GST-YopN$^{78\text{-}293}$ proteins (and YopN-bound TyeA proteins) eluted. The amount of GST-YopN$^{78\text{-}293}$ and TyeA in the initial lysates, the wash fractions and the elutions were determined by SDS-PAGE and immunoblot analysis (Fig. 2B). Approximately equal amounts of the GST-YopN$^{78\text{-}293}$ protein bound to, and were eluted from, each of the columns. The TyeA, TyeA (R19A W20A) and TyeA (W20A D25A) proteins efficiently bound to, and co-eluted with, the GST-YopN$^{78\text{-}293}$ protein. In contrast, the TyeA (M51A F55A) and TyeA (S6A G10A V13A) proteins did not interact or bound poorly to the GST-YopN$^{78\text{-}293}$ protein. These results indicate that the regions of TyeA defined by amino-acid residues M51, F55 and P56 and by amino-acid residues S6, G10 and V13 are required for TyeA to efficiently interact with YopN. On the contrary, the region of TyeA defined by amino-acid residues R19, W20 and D25 is required for TyeA to regulate Yop secretion, but is not required for TyeA to interact with YopN.

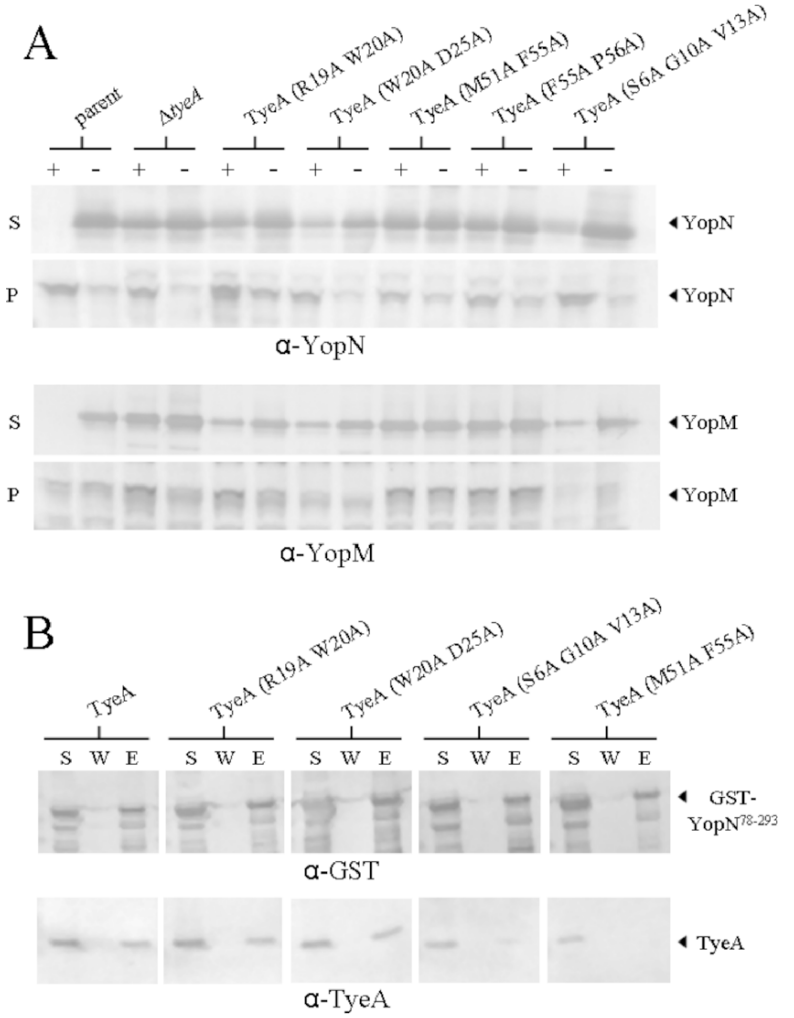

Fig. 2. Analysis of TyeA double and triple alanine missense mutants. (A) Expression and secretion of YopN and YopM by TyeA mutants. *Y. pestis* KIM5-3001.P39 (parent), KIM-3001.P63 (*ΔtyeA*), four TyeA double alanine missense mutants and one TyeA triple alanine missense mutant were analyzed by SDS-PAGE and immunoblot analysis of cell pellet (P) and culture supernatant (S) fractions using antisera specific for YopM and YopN (arrowheads). (B) Analysis of the interaction of YopN with TyeA proteins. The GST-YopN$^{78\text{-}293}$ protein and interacting (co-purifying) TyeA proteins expressed in *E. coli* BL21 were purified using glutathione sepharose. Soluble (S), wash (W) and elution (E) fractions were analyzed by immunoblotting with antisera specific for GST and TyeA.

21.4 Discussion

A cytoplasmic YopN/SycN/YscB/TyeA complex is required to block Yop secretion in the presence of millimolar amounts of extracellular calcium and prior to contact with a eukaryotic cell. Previous studies have identified YopN missense mutants that constitutevely block Yop secretion (YopN NS mutants) (Ferracci et al. 2005). Interestingly, these mutants required TyeA, but did not require LcrG or the SycN/YscB chaperone to block secretion, suggesting that the YopN/TyeA complex is the minimal complex required to prevent Yop secretion. In addition, several non-secretable, truncated YopN NS mutants that lack an N-terminal secretion signal and/or chaperone-binding domain, the $YopN^{85-293}$ (F234S) protein for example, still blocked Yop secretion constitutively (Ferracci et al. 2005). Together, these findings suggest that the YopN/TyeA complex has two independent means of interacting with the *Yersinia* T3S apparatus: (i) via its N-terminal secretion signal and chaperone-binding domain; and (ii) via the C-terminal domain of YopN complexed with the TyeA protein. This later interaction is hypothesized to be essential for the YopN/TyeA complex to block Yop secretion. The mechanism by which the cytosolic YopN/TyeA complex blocks Yop secretion is not understood; however, we hypothesize that the C-terminus of YopN complexed with TyeA must interact with a component of the T3S apparatus to block Yop secretion. Mutations that disrupt the interaction of the YopN/TyeA complex with the T3S apparatus would be expected to secrete Yops constitutively. The identification of a region of TyeA, defined by amino-acid residues R19, W20 and D25, that is required to block Yop secretion but is not required to interact with YopN, suggests that TyeA plays a direct role in blocking secretion independent of its binding to YopN. In fact, the R19, W20 and D25 amino-acid residues may be required for interactions that directly disrupt the T3S process. Conformation of this hypothesis awaits the identification of the target of the YopN/TyeA complex.

Analysis of the YopN-TyeA crystal structure revealed that the primary contacts between these proteins are mediated by hydrophobic interactions between amino-acid residues in the YopN C-terminal helix and helices one and three of TyeA (Schubot et al. 2005) (Fig. 1B). The TyeA (M51A/F55A) protein possesses alanine substitutions that disrupt hydrophobic contacts between TyeA M51 and YopN F278, as well as TyeA F55 and YopN V271. This protein did not co-purify with the GST-$YopN^{78-293}$ protein, confirming that TyeA M51 and TyeA F55 are required to form critical contacts between these two proteins. Similarly, the TyeA (S6A G10A V13A) protein carries alanine substitutions that disrupt hydrophobic contacts between TyeA S6 and YopN W216, TyeA G10 and YopN Y213 and TyeA V13 and YopN I212 (Schubot et al. 2005). The TyeA (S6A G10A V13A) protein, like the Tye (M51A F55A) protein, failed to efficiently co-purify with the GST-$YopN^{78-293}$ protein, indicating that hydrophobic contacts between the first helix of TyeA and helix nine of YopN also play an important role in the interaction of TyeA with YopN. The fact that both of these mutant TyeA proteins cannot properly regulate Yop secre tion, indicates that the interaction of TyeA with YopN is required to regulate Yop secretion.

21.5 Acknowledgements

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