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Quantification of YopE protein of *Yersinia pseudotuberculosis* reveals differential secretion rates in other Yops deleted strains

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Gram negative pathogenic bacteria *Yersinia pseudotuberculosis* use a type III secretion system (T3SS) to translocate toxic proteins into the eukaryotic cell. Once these proteins are inside the host cell, they interfere with the cell signalling pathways and alter the cellular response. The genes for the bacterial T3SS are located on the 70-kbp virulence plasmid that is common in all pathogenic species of *Yersinia*. A tetracysteine tag (FLNCCPGCCMEP) has been introduced in the C terminal end of Yersinia outer membrane protein E (YopE) as a translational fusion to observe secretion of these proteins into host cell that is seen in naturally occurring proteins which allows the expressed fusion protein to be specifically recognised by a biarsenical compound. The Lumio/tetracysteine and FLAsH/tetracysteine labelling system was used to fluorescently label YopE-Tc tag in *Y. pseudotuberculosis* to observe the secretion and quantification of these specific proteins. In this experiment, different bacterial strains (YPIIiplB102, YPIIiplB102(ETC12), YPIIiplB155(ETC12), YPIIiplB526, YPIIiplB529(ETC12), YPIIiplB604 (ETC12), YPIIiplB621(ETC12), YPIIiplB625(ETC12) and YPIII) with different proteins deletion (YopK, YopB, YopE, YopDΔ4-303, YopDΔ4-20 and YopEDΔ54-79), with or without tetracysteine tag were used to quantify the expression of YopE. Here, we demonstrated that the proteins YopE exhibit different secretion pattern with the deletion of other proteins. This may allow the possible role of these particular deleted proteins to activate or suppress the secretion of YopE. Overall, data from this experiment suggest that the total YopE expression depends on the conformation of regulator.

**Key words:** *Yersinia pseudotuberculosis*, T3SS, tetracysteine tag, YopE quantification.

INTRODUCTION

Proteomics studies on *Yersinia pseudotuberculosis* - a Gram negative gastrointestinal pathogen has given insight on how bacteria invade and survive in mammalian cells. *Yersinia* species rarely enters the bloodstream and exhibit high affinity to lymphatic system via interactions between adhesins and integrin receptors on the surface of M cells of the Peyer’s patches. Successful colonisation of the Peyer’s patches causes local inflammation, enterocolitis (Salyers et al., 2002; Bacterial pathogenesis- A Molecular approach; Salyers and Whitt, 2nd edition). When further spread from the Peyer’s patches to mesenteric lymph node an inflammation that mimic appendicitis result. To survive inside the host and escape the human immune system, the bacteria utilises a set of proteins denoted Yops that are secreted by T3SS.

All pathogenic *Yersinia* species carry a 70 kd virulence plasmid (Costa et al., 2012; Lindler, 2004) pYV (pIB in *Y. pseudotuberculosis*) which is encoded by the T3SS and

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deletion of this 70 kd pYV causes avirulence of all *Yersinia* species (Brubaker, 1983).

The Type III secretion/translocation system of *Yersinia* is made of about 27 Ysc proteins that form a needle-like structure (Edgren et al., 2012). The prevailing model is that the effectors proteins are secreted/translocated through this needle-like structure, which then will act as a “molecular syringe”. The type III secretion/translocation of *Yersinia* is in vivo induced by host cell contact whereas during in vitro condition, secretion can be triggered by depletion of Ca²⁺ in the culture medium. Maximal level of Yop secretion occurs at 37°C in a rich media deprived of Ca²⁺ whereas at a concentration of 2.5 mM Ca²⁺ the Yops remains associated to the bacterial envelope and low levels of secretion occurs (Dewoody et al., 2013). The Type III secretion/translocation of Yops involves two events, the crossing of the bacterial envelope (secretion) and the passage through the eukaryotic cell membrane (translocation). This procedure has been done in a single step when both (host-pathogen) are in close contact (Akopyan et al., 2011). The type III secreted proteins can accordingly be divided into two functionally distinct groups. One group constitutes the virulence effectors having their respective targets inside the host cells. The other group of proteins are either directly involved in translocation of effectors proteins across the target cell membrane or controlling this process. Three of the secreted proteins in *Yersinia* (YopB, YopD, and LcrV) are required for delivery of effectors proteins into the eukaryotic cells (Rosqvist et al., 1990; Bölin and Wolf-Watz, 1984; Olsson et al., 2004). These proteins are required for pore formation in infected cells and it has been suggested that translocation occurs via this pore into the host cell membrane (Cornelis, 2006; Galan and Wolf-Watz, 2006).

YopE is a GTPase activating protein (GAP) that targets and down regulate small Rho GTPases within the target cell (Wölke et al., 2012; Von Pawel-Rammingen et al., 2000). The translocated YopE interferes with the phagocytosis process and cause a cytotoxic effect on the target cell (Rosqvist et al., 1990). YopE has also been shown to have a regulatory role and auto regulate Yop expression during infection of Hela cells (Ali et al., 2008). Translocated YopE localises to the perinuclear region of the target cell. A bacterial localisation domain (MLD) has been identified in YopE consisting of amino acids 54-75. A YopE MLD mutant (YopEΔ54-75) is secreted and translocated to the same level as wild-type YopE but induces a delayed cytotoxic response in Hela cells. The MLD-mutant showed a changed intracellular localisation of YopE after infection of Hela cells and was showed to be dispersed in the whole cell including the nucleus (Isaksson et al., 2009).

Since YopE is an essential virulence determinant and also involved in regulatory events, we wanted to establish a new tools to study whether other proteins impair the secretion of YopE that is likely to occur on the bacterial membrane prior translocation. A tetracysteine motif (cys-cys-pro-gly-cys-cys) was therefore introduced in the C-terminal of YopE (denoted YopE-TC) in different mutants strains (yopK, yopB, yopD, yopDΔ4-20 and yopEΔ54-75). Proteins containing the tetracysteine motif form a high affinity complex with the small bisarsenic, organometallic FIAsh reagent, rendering the FIAsh molecule fluorescent (Adams et al., 2002; Enninga et al., 2005). The fluorescent signal is dependent on the amount reagent bound and on the conformation of the target protein. Thus, the use of this reagent allows in situ labelling of the pre-made pool of YopE hence making it a suitable tool for analysing the micro-environment of YopE. TC tags were introduced in different mutants of *Yersinia* and analysed the amount of YopE-TC expressed and the fluorescent signal after FIAsh binding to the TC-tag. Our result shows that the fluorescent signal of FIAsh bound to YopEΔ54-75 relative to the amount of expressed YopE was significantly higher compared to wild-type YopE, indicating that deletion of the amino acid region 54-75 results in a changed conformation of YopE. This emphasises that this technique can be used to investigate the possible role of the deleted Yops to maintain the regulation of YopE.

**MATERIALS AND METHODS**

**Bacterial strains, Growth medium and condition**

Bacterial strains with different deleted Yops used in this experiment are described in Table 1. The small tetracysteine tag FLNCCPGCGMEP was previously incorporated in the C terminal end of YopE (YopE-TC; YopE fused with the tag FLNCCPGCGMEP at the C terminal end at amino acid position 219) (Rosqvist R, unpublished work). Introduction of a tetracysteine tag did not interfere with the YopE function. The FIAsh reagent is readily membrane permeable which allow specific detection of TC-tagged YopE. When the thiol group of the cysteine residue binds to the "molecular syringe". The Type III secretion/translocation system of *Yersinia* is made of about 27 Ysc proteins that form a needle-like structure (Wölke et al., 2012; Von Pawel-Rammingen et al., 2000). The translocated YopE interferes with the phagocytosis process and cause a cytotoxic effect on the target cell (Rosqvist et al., 1990). YopE has also been shown to have a regulatory role and auto regulate Yop expression during infection of Hela cells (Ali et al., 2008). Translocated YopE localises to the perinuclear region of the target cell. A bacterial localisation domain (MLD) has been identified in YopE consisting of amino acids 54-75. A YopE MLD mutant (YopEΔ54-75) is secreted and translocated to the same level as wild-type YopE but induces a delayed cytotoxic response in Hela cells. The MLD-mutant showed a changed intracellular localisation of YopE after infection of Hela cells and was showed to be dispersed in the whole cell including the nucleus (Isaksson et al., 2009).

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**FIASh staining and analysis of the fluorescence**

After incubation at 37°C for 2 has described above, 1.5 ml of the bacterial suspensions were centrifugated at 12000 g for 3 min. The pellets were resuspended in 500 µl PBS containing 0.5 mM CaCl₂ and used for labelling with the FIAsh reagent. The FIAsh labelling procedure was in principle performed according to the manufacturer’s instructions (Invitrogen). Briefly, to 100 µl bacterial suspension 75 µl reagent (final concentration 8.6 µM) was added and incubated at 26°C for 1 h in the dark. The bacteria were washed three times with 100 µl BAL wash solution (Invitrogen). Finally, the pellets were resuspended in PBS-containing 0.5 mM CaCl₂ for analysis of the fluorescence, 75 µl of the bacterial suspension were added to a
96-well plates and analyzed in the TECAN Infinite M200 PRO fluorescent plate reader using excitation filter 505 nm and emission filter above 530 nm. As a background, control bacteria were treated as described above except that no FlAsH reagent was added. As an internal plate, control (75 μl) of fluorescent beads (15μm Focal check™ microspheres, Molecular probe, Eugena, Oregon, U.S.A) was used.

### Quantification of the FlAsH fluorescent signal

For quantification of the FlAsH fluorescent signal, western anti-YopE (Nutella) internal control signal was used as a loading control. In all cases, two different bacterial suspensions (10x and 2x) were used to calculate the final value. First, the strain with FlAsH reagent (for 10x and 2x bacterial suspensions) was subtracted from the strain without FlAsH reagent and then multiply with loading control (internal ctrl (10x) + internal ctrl (2x))/2= mean loading control. This value was used to compensate for different loading because in most experiment, it seems to be the same value, so it will not affect the calculations so much.

### Quantification of the fluorescent signal of the TC-tagged YopE

After incubation at 37°C for 2 h, 1.5 ml of the bacterial suspensions were centrifuged at 4000 rpm for 10 min. The pellets were resuspended in 200 μl PBS containing 0.5 mM CaCl₂ and used for labelling with the Lumio reagent in two different bacterial suspensions (10x and 2x).

The Lumio green reagent (Invitrogen) were used to label the TC-motif in poly acryl amide gels according to the manufacturer’s instructions. Briefly, to 15 μl bacterial suspension 5 μl Lumio gel sample buffer, 0.2 μl Lumio green detection reagent (final concentration 2 mM) was added and incubated at 70°C for 10 min. Then, the bacteria were kept on ice 1-2 min and pelleted by short centrifugation. The pellet was resuspended and 2 μl Lumio gel detection enhancer were added and incubated for 5 min at room temperature. Bacterial samples were loaded on SDS-PAGE gels and run approximately 1-2 h at 250 V. 2 μl Fluorescent protein standards (Bench Mark™) and 2 μl Western blot standard (Magic Mark™ XP) were also loaded as a marker for protein size estimation. Recording and documenting the results of fluorescence gel was done through cooled CDC camera (Fujifilm, Luminescent image analyzer, LAS 4000). The gel was then subsequently subjected to Western blot analysis using anti-YopE antibodies to quantify the total YopE expressed. For quantification of the fluorescent signal western anti-YopE (Nutella), internal control signal was used as a loading control. In all cases, only two different bacterial suspensions (10x and 2x) was used to calculate the final value. Mean YopE was compensated by loading the control.

### RESULTS

To analyze YopE and its possible role with other Yops/translocators on the bacterial cell surface we used a YopE variant (YopE-TC) containing a tetracycstylene (TC) motif inserted in the C-terminal end. This TC-motif can be fluorescently labelled using a biarsenical reagent (FlAsH) which upon binding to the TC-motif gives a high fluorescent signal.

The different bacterial strains were grown under conditions that induce YopE expression (2 h at 37°C in TMH media), divided in two parts and in parallel analyzed for YopE expression and the accessibility of the TC-motif was labelled with the FlAsH reagent.

The level of YopE expression was determined after separation on SDS-PAGE followed by analysis using two different detection reagents; the Lumio reagent that binds to the TC-motif and gives a fluorescent signal in the gel that was recorded by a cooled CCD camera, thereafter the gel was subjected to Western blotting analysis using YopE antibodies to detect and quantify the total YopE expressed. No significant difference in total YopE expression was found between the wt-strain and the yopK, yopB, yopD₄₄-20, yopE₅₄-75 mutants containing the YopE-TC12 (Figures 1 and 2). Whereas yopD₄₄-303 mutants containing the YopE-TC12 (Figures 1 and 2) expressed higher level of YopE. The Lumio reagent used to detect the fluorescent signal from the TC-motif (Figure 1) gave similar results as Western blotting using YopE antibodies (Figure 2). This study tries to find out whether other proteins impair YopE secretion by specifically detected TC-tagged YopE. From Figures 1 and 2, it was obvious that other factor can tightly control the secretion of YopE as yopD₄₄-303 mutants containing the YopE-TC12

### Table 1. Bacterial strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant characteristic</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>YPIIIpIB102</td>
<td>Kmᵣ (wild type)</td>
<td>(Bölin and Wolf-Watz, 1984)¹</td>
</tr>
<tr>
<td>YPIIIpIB102(ETC12)*</td>
<td>Kmᵣ (wild type YopETC12) Ca²⁺ Dependent</td>
<td>This study*</td>
</tr>
<tr>
<td>YPIIIpIB155(ETC12)*</td>
<td>Kmᵣ (YopETC12 ΔyopK) Ca²⁺ Dependent</td>
<td>This study*/(Olsson et al., 2004)²</td>
</tr>
<tr>
<td>YPIIIpIB526</td>
<td>Kmᵣ (ΔyopE) Ca²⁺ Dependent</td>
<td>This study*/(Ali et al., 2008)³</td>
</tr>
<tr>
<td>YPIIIpIB529(ETC12)*</td>
<td>Kmᵣ (YopETC12 ΔyopD₅₄-₇₅) MLD Ca²⁺ dependent</td>
<td>This study*/(Isaksson et al., 2009)⁴</td>
</tr>
<tr>
<td>YPIIIpIB604(ETC12)*</td>
<td>Kmᵣ (YopETC12 ΔyopB) Ca²⁺ Dependent</td>
<td>This study*/(Håkansson et al., 1996)⁵</td>
</tr>
<tr>
<td>YPIIIpIB621(ETC12)*</td>
<td>Kmᵣ (YopETC12 ΔyopD₄₄-303) Temp. Sensitive</td>
<td>This study*/(Olsson et al., 2004)⁶</td>
</tr>
<tr>
<td>YPIIIpIB625(ETC12)*</td>
<td>Kmᵣ (YopETC12 ΔyopD₄₄-20) Ca²⁺ Dependent</td>
<td>This study*/(Olsson et al., 2004)⁶</td>
</tr>
<tr>
<td>YPIII</td>
<td>Cured from the virulence Plasmid</td>
<td>Bölin and Wolf-Watz, 1984</td>
</tr>
</tbody>
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*YopETC12, YopE fused with the tag FLNCCPGCCMEP at the C terminal end at amino acid position 219. 1, 2, 3, 4, 5 and 6: Mutant strains were obtained from them.
Figure 1. Quantification of YopE (B) in different Yersinia strains using YopE labelled with Lumio followed by western blotting (A) and analysed using a cooled camera. After shifting the overnight bacterial cultures from 26 to 37°C, the bacterial suspension were centrifuged. The pellets were resuspended with appropriate buffer and labelled with Lumio reagent (two different dilution) followed by adding Lumio gel enhancer. In order to finding the results of fluorescence gel, samples were loaded on SDS-PAGE for better separation (A) with Fluorescent protein standards. The signal of fluorescence gel was prepared by CDC camera (Fujifilm, Luminescent image analyzer, LAS 4000). In figure A, there were no bands observed for the strains YPIIIpIB102 (wild type) and YPIIIpIB526 (YopE deleted). As the reagent only detect TC-tagged YopE, the strains without TC-tag or deleted YopE did not recognize by the methods. In fig B, Bar charts represents the quantization of TC-tagged YopE in different strains.

Figure 2. Quantification of YopE (B) in different Yersinia Strains using YopE followed by western blotting (A) and analysed using a cooled camera. Samples labelled with Lumio reagent (described above) loaded on SDS-PAGE gels were analyzed through western blotting using anti-YopE antibodies for the quantification of expressed YopE. Loading control (internal control) was used to compensate the final mean (value from two different diluted sample) YopE value. In Fig A, YopE deleted strains and non-virulent one did not show any signal although internal control exist. The intensity of the YopE signal in different strains depends on the YopE secretion level by the specific deleted other Yops and bound tag molecules. In figure B, the bar charts showed the quantification of the YopE in different bacterial strains.

expressed higher level of YopE. It means that in the absence of these proteins, YopE did not have to encounter any hindrance to secrete. The other part of the samples were in parallel subjected to in situ labelling using the FLAsh reagent to determine whether the respective mutation had altered the
A

Figure 3. Quantification (A) and ratio (B) of YopE in different Yersinia Strains using YopE labelled with FLAsH followed by absorbance measurement and analysed using Tecan. The bacterial suspension (prepared the same way described above) were centrifuged. The pellets were resuspended with appropriate buffer and incubate with FLAsH reagent at 26°C for 1h in the dark (two different dilution) followed by three times washing with BAL solution. Thereafter 96-well plates were used to detect the fluorescent signal using TECAN Infinite M200 PRO. The bar charts in fig A explain the level of YopE detected by FLAsH bound TC-tag. To compensate the results, FLAsH reagent (from two different dilution) was substracted from the strain without FLAsH reagent followed by multiplication with western blotting loading control as this value for different loading were the same and not alter the calculation too much. The bar charts in fig B showed the normalized value of secreted TC-tag YopE.

availability of the YopE-TC12 to be labelled. The fluorescent signals were subsequently analyzed in a 96-well fluorescent plate reader.

A significant increase in the mean FLAsH fluorescent signal intensity was detected in those strains [YPIIIpIB102 (ETC12), YPIIIpIB155 (ETC12), YPIIIpIB529 (ETC12), YPIIIpIB604 (ETC12), YPIIIpIB621 (ETC12) and YPIIIpIB625 (ETC12) expressing the tetracysteine tag compare to the wild type strain (YPIIIpIB102)] and the YPIIIpIB526 where the yopE gene is deleted (Figure 3). And YPIIIpIB621 (ETC12) strain showed higher signal among all other strains. As FLAsH labelling is specific to detect TC-tagged YopE (Figure 3), it emphasize on the relation of deleted protein to quantitate the TC-tagged YopE.

DISCUSSION

YopE plays a significant role in Y. pseudotuberculosis pathogenesis (Black and Bliska, 2000; Von Pawel-Rammingen, 2000). The expression and conformation of native structure of YopE is important to understand the virulence mechanism of Y. pseudotuberculosis. In this study, we tried to understand the microenvironment of YopE interms of secretion by using two methods. Genomically, incorporated tetracysteine motif (cys-cys-cys-pro-gly-cys-cys) to the C-terminal end of YopE helps to detect the total label of protein easily. This small tag rarely occurs with the native structure of protein and this binding is specific with fluorescence compound. One FLAsH molecule binds to one TC-motif. The binding of the reagent to the TC-motif is thus dependent on the amount of YopE-TC expressed and the accessibility of the TC-motif. The fluorescent signal is also dependent on the conformation of the target protein (Ignatova et al., 2004). In this project, we analyzed the ratio between the measured fluorescence signal of bound FLAsH and the amount of expressed YopE in different yop mutants. We predict that if YopE is in a complex or associated with other Yops, the TC-motif will be less or more accessible for binding to the FLAsH reagent which will result in a changed ratio between fluorescent signal and the amount of expressed YopE. Alternatively, the conformation of the target protein will be changed resulting in a changed ratio.

In this study, experiment was done to find out the level of YopE signal between wild type (with or without TC-tag) and different mutant strains. To determine the YopE level, the coordination among FLAsH/TC-tag, Lumio/TC-tag and Western blotting method has been established. The FLAsH/TC-tag method showed a number of advantages than other experiment as it provides direct monitoring in the translocation system. Our result shows that in FLAsH/TC-tag experiment, YPIIIpIB102 (wt), YPIIIpIB526 and YPIII (NonVirulent) strains reveals more or less same lower signal compare to other strains; YPIIIpIB102 (wt) strain do not contain any tag with YopE and unable to bind with FLAsH reagent to raise highly fluorescence compound. The same is true for Lumio experiment. YPIIIpIB526 strain is yopE deletion and YPIII (non-virulent) is plasmid cured; usually give raise to lower
signal in FlAsH/TC-tag and no signal in Lumio/TC-tag or western blot analysis. On the other hand, in FlAsH/TC-tag experiment YPlllPlB621 strain showed the higher amount of YopE level than other tag containing strains. The TC-tag containing strains YPlllPlB155, YPlllPlB604 and YPlllPlB529 showed similar signal but lower compared to YPlllPlB621 strain. The YPlllPlB621 strain is temperature sensitive YopD mutant which is responsible to maintain coiled coil domain and deletion of which causes the interruption of effectors translocation (Pallen et al., 1997). As this coiled-coil domain is responsible for protein - protein interaction (Lupas, 1998), this mutant reveals that Yersinia translocons composed of multiprotein complex with YopD, YopB and LcrV. YopD has several functions that leads to efficient effectors translocation including regulation, pore formation and secretion (Olsson et al., 2004). All of this data support that YopD may have a role to guide the translocation and also interact with YopE in vitro (Hartland and Robins-Browne, 1998; Francis et al., 1998). As it is deleted, no control has been found and showed a higher YopE signal than any other strain in this study. Though more studies is necessary, it is recommended from our results that the secretion and expression of YopE is modulated by the conformation of regulator of the multiprotein complex.

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REFERENCES


