ADP-ribosylation by exoenzyme T of *Pseudomonas aeruginosa* induces an irreversible effect on the host cell cytoskeleton in vivo

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Abstract

*Pseudomonas aeruginosa* utilises a type III secretion system (TTSS) to introduce exoenzyme S and exoenzyme T into host cells to subvert host cell signalling and thereby promote infection. In this study, we have employed the heterologous TTSS of *Yersinia* to deliver different mutants of ExoT into HeLa cells. Wild-type ExoT and ExoT variants expressing either GAP (GTPase activating protein) or ADP-ribosyltransferase activity mediated changes in cell morphology, which correlated to disruption of the actin microfilaments of the infected cells. ExoT expressing ADP-ribosylating activity gave an irreversible effect on HeLa cell morphology, while ExoT expressing only GAP activity displayed a reversible effect where the cells regained normal cell morphology after killing of the infecting bacteria. This shows that ExoT can modify and inactivate host cell proteins involved in maintaining the actin cytoskeleton in vivo by two independent mechanisms.  

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Keywords: Type III secretion; *Pseudomonas aeruginosa*; Exoenzyme T; ADP-ribosylation

1. Introduction

*Pseudomonas aeruginosa* is an opportunistic pathogen that readily takes advantage of defects in host defences to cause severe human infections in patients with cystic fibrosis, leukaemia, burn injuries and serious eye infections related to the use of contact lenses [1,2]. *P. aeruginosa* expresses several virulence factors like toxins, adhesins, flagella and secretion systems, including the type II and type III secretion systems [3,4]. The type III secretion system (TTSS) is a key virulence mechanism shared by several Gram-negative animal and plant pathogens. The TTSS mediates translocation of virulence effectors directly into the cytosol of the eukaryotic cells by extracellular adhering bacteria [5]. The effector systems of *P. aeruginosa* are believed to be secreted across the two bacterial membranes via a secretion apparatus composed of around 20 proteins, Psc-proteins [6]. These proteins show high homology to the Ysc-proteins of *Yersinia* that constitute the well-characterised TTSS of pathogenic *Yersinia* species. The high level of similarity between the TTSSs of *P. aeruginosa* and *Yersinia* together with conservation of the overall mechanism of the TTSSs makes it possible to express, secrete and translocate effectors of *P. aeruginosa* in the heterologous host *Y. pseudotuberculosis* [7].

Most type III effector proteins interfere with host cell signal transduction and different cellular processes to promote infection. Four exoenzymes, ExoS, ExoT, ExoU and ExoY, have so far been identified as effectors of the TTSS of *P. aeruginosa*. Clinical isolates most often harbour *exoT*, *exoY* and either *exoS* or *exoU* [8,9 Sundin et al., unpublished results]. ExoS and ExoT are closely related and show about 75% identity at amino acid level [10]. ExoS is a bifunctional toxin where the N-terminal domain shows GAP activity and the C-terminal domain has...
ADP-ribosylating activity which targets several members of the Ras protein family in vivo [12–14]. Similar to ExoS, ExoT is bifunctional with an N-terminal located GAP domain which inactivates Rho GTPases [15–17] and a C-terminal ADP-riboyltransferase domain [10]. However, the enzymatic activity of ExoT is less than 1% compared to ExoS in vitro [18] and unlike ExoS no detectable modification of proteins belonging to the Ras family could be detected in vivo [13]. Additionally, while intracellular delivery of ExoS affects HeLa cell viability, ExoT has no significant effect on cell viability [16]. The GAP-activity of both ExoS and ExoT as well as the ADP-riboyltransferase activity of ExoS all result in disruption of actin microfilaments of the affected cell [13,16,19]. Interestingly, when HeLa cells were infected with a *Yersinia* strain expressing ExoT devoid for GAP-activity, ExoT(R149K), cell rounding was still observed [16]. This suggested that the C-terminal domain of ExoT could interfere with cell signalling by a mechanism that resulted in disruption of actin microfilaments. Similar results have been obtained with a *P. aeruginosa* strain expressing ExoT (strain expressing ExoT devoid for GAP-activity, ExoT(R149K)), cell rounding was still observed [16]. This suggested that the C-terminal domain of ExoT could interfere with cell signalling by a mechanism that resulted in disruption of actin microfilaments. Similar results have been obtained with a *P. aeruginosa* strain expressing ExoT (strain expressing ExoT devoid for GAP-activity, ExoT(R149K)), cell rounding was still observed [16].

2. Materials and methods

2.1. Cultivation and infection of HeLa cells

The bacterial strains were cultivated overnight (ON) in Luria-Broth (LB) on a rotary shaker at 26 °C. Prior to infection, the overnight cultures were diluted in RPMI 1640 with GlutamaxI (Gibco BRL, Life Technologies) to OD600 = 0.2 and incubated for 30 min at 26 °C followed by 1 h at 37 °C. The HeLa cells were seeded (1 × 105 cells per well) in a 24-well tissue culture plate (Sarstedt) in RPMI 1640 with GlutamaxI medium containing 10% heat inactivated fetal calf serum and 3 γ gentamicin. Prior to infection, the HeLa cells were washed and 500 μl fresh gentamicin-free medium was added. Infections were initiated by adding the bacteria to the wells at a final OD600 = 0.03. The cytotoxicity of the HeLa cells was monitored by light microscopy and photographs were taken 2 h after infection, where after the infected cells were washed once and the bacteria were killed by adding fresh medium containing 20 μg gentamicin. Finally the cells were incubated for another 20 h and cytotoxicity was monitored as described above. For actin staining, the HeLa infections were carried out as above with the exception that the cells were grown on collagen I coated 8-well culture slides (Becton-Dickinson) (1 × 103 cells per well). After 2 h, the cells were fixed with 2% paraformaldehyde (PFA) for 5 min, washed twice with phosphate-buffered saline (PBS) and permeabilized with 0.15% saponin for 10 min. The actin microfilaments were stained with FITC-conjugated phalloidin for 30 min at 37 °C. The coverslips were mounted with fluorescent mounting medium (Dako) as an anti-fading agent. In a parallel experiment, the bacteria were killed after 2 h of infection by gentamicin as detailed above. The HeLa cells were incubated for an additional 20 h and the actin microfilaments were stained as described above. The actin microfilaments were analysed using fluorescence microscopy. To study the in vitro production and secretion of the different forms of ExoT, the bacteria were treated as above with the exception that the bacteria were grown in BHI (Brain heart infusion, Difco Kebo Lab) containing 5 mM EGTA and 20 mM MgCl2 for 30 min at 26 °C followed by 3 h at 37 °C. The amount of ExoT in the total samples and in the supernatants were analysed by ECL-Western blot using anti-ExoS antibodies which due to the high levels of homology cross-react with ExoT [16].

2.2. Construction of ExoT(R149K, E385A) and ExoT(E385A)

Site-directed mutagenesis of the glutamic acid residue at position 385 was used as strategy to target the ADP-riboyltransferase activity of ExoT and ExoT(R149K). Primers were designed that substituted glutamic acid 385 to alanine and two PCRs were run. For PCR1, the forward primer Tt24b (5’-AGGTGATGGCGCTCGG TCTC-3’) and the reverse primer M13/pUC-forward (5’-GTAAAACGACGGCCAG TCTC-3’) were used, and for PCR2 the forward primer Tt25 (5’-AGGGCGAT GAGCAGCGATCTC-3’) and the reverse primer M13/pUC-forward (5’-GTAAAACGACGGCCAG-3’) were used. An overlapping PCR was then performed using the fragments from PCR1 and PCR2 and the Tt24b and the M13/pUC-forward primers to create a PCR product containing the mutated ExoT fragment. For all PCR reactions, the Pfx PCR cloning kit (Life Technologies) were used. The fragment was cleaved with EcoRV and EcoRI, and ligated into the pCS100 and pCS101 plasmids similarly cleaved with EcoRV and EcoRI to remove the part of the *exoT* gene containing glutamic acid at position 385. The resulting plasmids, pCS102 and pCS103, were transformed into *Escherichia coli* by selecting for ampicillin resistance. The mutations
were verified by DNA sequencing and the plasmids were then electroporated into *Y. pseudotuberculosis* strain YPIII (pIB251), which is deleted for the genes encoding the effector proteins YopE and YopH.

3. Results and discussion

3.1. Construction and expression of mutant forms of ExoT

In previous studies, we have employed *Y. pseudotuberculosis* as a heterologous host to deliver the *P. aeruginosa* effector exoenzymes, ExoS and ExoT, into host cells via the well-characterised plasmid encoded TTSS of *Yersinia* [7,16]. In this study, the same strategy was chosen to elucidate the effects of the two domains of ExoT on host cell function. Since no chaperone has been identified for ExoT the different constructs of ExoT were expressed under the control of the *exoS* promoter on a plasmid which also encoded the divergently transcribed ExoS chaperone Orf1 [16] (Materials and methods; Table 1). Due to the high sequence homology between ExoS and ExoT it is highly likely that Orf1 functions as a chaperone also for ExoT. The different ExoT expressing plasmids were introduced into the *Y. pseudotuberculosis* strain YPIII(pIB251), which lacks the genes encoding two major effector Yops, YopE and YopH [20], and analysed for expression and secretion of ExoT during growth in calcium-depleted medium at 37 °C. All four variants of ExoT expressed and secreted similar levels of ExoT as revealed by Western blot analysis using polyclonal ExoS antiserum (Fig. 1).

3.2. The ADP-ribosylating activity of ExoT induces an irreversible disruption of actin microfilaments of infected HeLa cells

In order to monitor the activity induced on infected host cells by the different forms of ExoT, HeLa cells were infected with strain YPIII(pIB251) expressing the different forms of ExoT for 2 h. ExoT, ExoT(R149K) and ExoT(E385A) all induced a morphological change of the HeLa cells revealed as rounding of the cells (Fig. 2A). All three strains caused morphological changes of the infected HeLa cells after 2 h of infection which shows that the three forms of ExoT were expressed and translocated at similar levels. Thus, both the GAP domain and the ADP-ribosyltransferase domain appeared to have an effect on the HeLa cell cytoskeleton. The disruption of the microfilaments was confirmed by staining the filaments with FITC-conjugated phalloidin (Fig. 3A). No change in cell morphology or any signs of disruption of actin microfilaments were observed for cells infected with the strain expressing ExoT(R149K/E385A) which is devoid for both enzymatic activities (Figs. 2A and 3A).

If ExoT mediated ADP-ribosylation was responsible for the observed effects on host cell morphology in the GAP negative variant of ExoT, then the putative target for ADP-ribosylation should be irreversibly inactivated and the effect persist even if the infecting bacteria are killed after the initial infection. In contrast, the effect mediated by the GAP-activity of ExoT does not induce a covalent modification of the target GTPases and the infected cells should recover to normal morphology after the infecting bacteria have been killed. In order to investigate this hypothesis, HeLa cells were infected with the different strains for 2 h where after the adhering bacteria promoting ExoT translocation were killed by gentamicin. Then the HeLa cells were incubated for an additional 20 h. Cells infected with the strain expressing ExoT(E385A), which is functional for GAP-activity but lacks ADP-ribosyltransferase activity, regained normal cell morphology as well as actin microfilament structure (Figs. 2B and 3B). This indicates that the effect caused by the GAP-activity is reversible which correlates well with the non-covalent modification of RhoA associated with the GAP-activity of both ExoS and ExoT. In

### Table 1

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevant genotype</th>
<th>Reference</th>
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<tbody>
<tr>
<td>pCS100</td>
<td>orf1 and exoT cloned into pUC19</td>
<td>[16]</td>
</tr>
<tr>
<td>pCS101</td>
<td>orf1 and exoT(R149K) cloned into pUC19</td>
<td>[16]</td>
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<tr>
<td>pCS102</td>
<td>orf1 and exoT(E385A) cloned into pUC19</td>
<td>This study</td>
</tr>
<tr>
<td>pCS103</td>
<td>orf1 and exoT(R149K, E385A) cloned into pUC19</td>
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contrast, cells infected with strains expressing ExoT and ExoT(R149K) which both have ADP-riboosyltransferase activity did not regain normal cell morphology or intact actin microfilaments (Figs. 2B and 3B). This strongly argues that ExoT irreversibly ADP-ribosylates and modifies a cellular target required for integrity of actin microfilaments and cell morphology.

During the course of this study, evidence was presented that ExoT in vitro specifically ADP-ribosylated the adaptor proteins Crk-I and Crk-II [21]. Furthermore, it was shown that the specific activity of ExoT in ADP-ribosylation of Crk-I is high and comparable to that of ExoS on proteins of the Ras family. Even if it remains to be shown that the biological activities of ExoT are mediated through inactivation of Crk-I, these findings are interesting and important. ExoT appears to be encoded by most clinical isolates studied [8,9, Sundin et al. unpublished results] and expression of ExoT has also been correlated to the ability of P. aeruginosa to prevent phagocytosis by macrophages [19]. This is in agreement with the fact that Crk-I has been suggested to be involved in phagocytosis and that P. aeruginosa multiplication during infection mainly occurs extracellularly [22]. The TTSSs of P. aeruginosa and pathogenic Yersinia species are closely related and the systems are tightly regulated to deliver effector proteins rapidly into host cells in order to prevent uptake. Interestingly, YopE and YopH, two of the effector proteins of Yersinia also target host cell signalling to prevent phagocytosis. Similar to ExoT, YopE has RhoGAP activity [23,24], and YopH prevents uptake by inactivating focal adhesion proteins critical for uptake by a mechanism that involves dephosphorylation [25,26]. If ExoT targets and inactivates Crk-I the biological effect should be similar to that of YopH as signalling pathways required to promote phagocytosis would be disrupted. The challenge of the future work will be to show that the ADP-ribosylation of Crk-I by ExoT is responsible for the anti-internalisation activity of P. aeruginosa.

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References


