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**Electrostatic interactions play a minor role in the binding of ExoS to 14-3-3.****Lubna Yasmin<sup>1\*‡</sup>, Jeffrey L. Veesenmeyer<sup>2\*</sup>, Maureen H Diaz<sup>2†</sup> Matthew S. Francis<sup>1,3</sup>  
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**Short title: A non-phosphorylated interaction between 14-3-3 and exoS**

**Abstract:**

14-3-3 proteins belong to a family of conserved molecules expressed in all eukaryotic cells that play an important role in a multitude of signaling pathways. 14-3-3 proteins bind to either phosphoserine/phosphothreonine residues or to sequence-specific non-phosphorylated motifs in more than 200 interaction partners (Pozuelo Rubio, Geraghty, Wong, Wood, Campbell, Morrice, Mackintosh. 2004, Biochemical Journal, 379:395-408). These interactions result in cell cycle regulation, apoptosis, stress responses, cell metabolism and malignant transformation. One example of a phosphorylation-independent interaction is the binding of 14-3-3 to exoenzyme S (ExoS), a bacterial ADP-ribosyltransferase toxin of *Pseudomonas (P) aeruginosa*. Here we have utilized additional biochemical and infection analyses to further define the structural basis of the interaction between ExoS and 14-3-3. An ExoS leucine substitution mutant dramatically reduced the interaction potential with 14-3-3 suggesting that ExoS Leucine residues-422, 423, 426, 428 are important for its interaction with 14-3-3, its enzymatic activity and cytotoxicity. However, ExoS substitution mutants of residues that interact with 14-3-3 through an electrostatic interaction such as Ser-416, His-418, and Asp-424 and Asp-427 showed no reduction in their interaction potential with 14-3-3. These ExoS substitution mutants were also as aggressive as wild type ExoS at inducing cell death and to modify endogenous ExoS target within the cell. In conclusion, electrostatic interaction between ExoS and 14-3-3 via polar residues (S416, H418, D424 and D427) appears to be of secondary importance. Thus, the interaction between the “roof” of the groove of 14-3-3 and ExoS relies more on hydrophobic interaction forces, which probably contributes to induce cell death after ExoS infection and activation.

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## Introduction

The highly conserved eukaryotic protein family 14-3-3 is capable of forming homo- or heterodimers and interacts with a diverse number of target proteins, although it has no enzymatic activity [1-5]. In humans, these partners play a role in physiological processes such as cell proliferation, cell cycle progression, protein trafficking, rearrangement of the cytoskeleton, apoptosis, and survival [6, 7]. Much attention has been focused on the conventional interaction between 14-3-3 and specific phosphoserine/phosphothreonine residues located on binding partners. Crystal structure analysis of 14-3-3 in this type of complex with peptides or native partners has revealed a basic amino acid cluster in the amphipathic groove of each monomer that mediates the interaction of 14-3-3 with the phospho-serine/threonine-residues of its binding partners [8, 9]. Each 14-3-3 dimer contains two binding pockets and can interact with canonical binding motifs, defined as mode I (RSXpSXP) and mode II (RXΦXpSXP) where Φ is an aromatic or aliphatic residue and X is any type of residue [6, 9, 10]. From the prominent structural similarity between the C-terminal 14-3-3 binding motif of the serotonin-N-acetyltransferase (AANAT, arylalkyl amine-N-acetyltransferase) and the plant plasma membrane H<sup>+</sup>-ATPase, a mode III consensus for 14-3-3 binding (pS/TX1-2-COOH) has been proposed [11, 12]. Furthermore, additional interactions between 14-3-3 protein and its target partner(s) probably occur outside the amphipathic groove and function to stabilize the three-dimensional arrangement and potentially change the conformation of the target [8, 10, 13].

However, 14-3-3 also interacts with proteins in a phosphorylation-independent manner, such as human telomerase and p190RhoGEF [14, 15]. Another example is the interaction between 14-3-3 and the specific peptide motif of the bacterial toxin exoenzyme S (ExoS) produced by the opportunistic pathogen *P. aeruginosa* [16-19]. The ExoS toxin is specifically translocated into target eukaryotic cells by the type III secretion system of *P. aeruginosa* and contributes to infections in immuno-compromised individuals including those suffering from cystic fibrosis, cancer, burns, wounds and respiratory compromise requiring mechanical ventilation ([20] and refs. therein). ExoS is regarded as a bifunctional toxin containing N-terminal Rho GTPase activating protein (GAP) activity [21, 22] and C-terminal encoded ADP-ribosyltransferase activity [23]. The latter activity seems to predominantly and promiscuously modify members of the RAS family [20, 24-26]. Furthermore, the intracellular function of ExoS (specifically the ADP-ribosylation activity) requires an interaction with 14-3-3 and leads to apoptosis by activation of death pathways [17, 19, 27-29]. Further, in a more biological relevant assay the ADP-ribosyltransferase activity of ExoS resulted in increased

bacterial persistence in the lung and decreased host survival in an animal model of pneumonia [30].

A recent structural analysis of the interaction between 14-3-3 and ExoS showed that the interaction occurs in a reversed orientation relative to previously described 14-3-3/ligand interactions. The binding was described as a non-phospho-mimicking interaction compared with mode I-III and was denoted mode IV. These results suggested that the interaction between ExoS and 14-3-3 was of a hydrophobic nature and that electrostatic forces may not contribute to the overall binding energy between ExoS and 14-3-3 [18]. The hydrophobic residues in the “roof” of the 14-3-3 amphipathic groove (P165, I166, Leu216, I217, L220, and L172) interacted with L422, L423, L426 and L428 of ExoS to provide the main attractive force between the proteins (Figure 1) [18]. This mechanism of binding is different from modes I-III, which predominantly rely on the electrostatic interaction between the phosphoserine/phosphothreonine residues of the client and a basic cluster of amino acids within the amphipathic groove of 14-3-3. In this cluster, two arginines (R56 and R60), one lysine (K49) and one tyrosine (Y128) residue coordinate the phosphate moiety [8, 9]. In ExoS, two negatively charged aspartate residues, D424 and D427, are within the 14-3-3 binding region, but structural analysis indicated that they do not mimic a phosphoryl group, as they are too far away from the positions occupied by the phosphorylated serine or phosphorylated threonine residues during consensus binding in mode I to III motifs [18]. However 14-3-3/ExoS binding was coordinated by several H-bonds, involving S416, H418, D424 and D427 of ExoS and K212, D213, K49 and Y128 of 14-3-3 (Figure 1)[18]. In this study, we have addressed the importance of these ExoS residues for the interaction with 14-3-3. Employing mutational analysis of ExoS, we performed GST-ExoS pull-down, biochemical, cytotoxicity, and virulence analyses. Our study shows that within the interface of ExoS and 14-3-3, hydrophobic interactions are more important than electrostatic interactions for binding, enzymatic activity and virulence.

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## Experimental procedures

### Cell cultures and lysis

HeLa cells were grown in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum and 100 units/mL penicillin. Following bacterial infection, cells were washed in ice-cold NaCl/Pi and lysed on ice in lysis buffer [1%(v/v) Triton x-100, 100 mM NaCl, 50 mM Tris/HCl (pH 7.5), 1 mM EDTA supplemented with protease inhibitors (Complete, #1697498, Roche Diagnostics, Basel, Switzerland)]. Lysates were subsequently cleared by centrifugation at 15 000 g for 10 min at 4 °C. Lysates were pre-cleared with glutathione S-transferase (GST) for 5 min before incubation with various GST-fusion proteins for 1 h prior to the addition of Glutathione Sepharose (GE Healthcare, Uppsala, Sweden) for 30 min. After three washes in lysis buffer, samples were boiled for 5 min in SDS/PAGE sample buffer.

### Western analysis, peptides and antibodies

Anti-14-3-3 (K-19):SC-629 was purchased from Santa Cruz (New York, NY, USA) and monoclonal Ras (cat 610002) was obtained from BD Biosciences (Stockholm, Sweden). RAP, RAS, p-ERK, p-PKB antibodies were obtained from Transduction laboratories (cat: R22020 and R02120) and Cell signaling technologies (cat: 9271 and 9101), respectively. Anti-ExoS was from Agrisera AB, Sweden. Immunoblotting was performed according to the manufacturer's instructions using secondary antibodies conjugated to horseradish peroxidase sheep anti-mouse or rabbit antibodies (Pierce, Rockford, IL, USA, and ECL Plus, Amersham-Biosciences).

### Plasmids

pGEX-ExoS( $\Delta$ ) was constructed as a derivative of pGEX-ExoS(88–453), both of which have been described previously [16]. The substitution mutants (Table 1) pGEX-ExoS(S416A), pGEX-ExoS(H418A), pGEX-ExoS(S416A;H418A), pGEX-ExoS(H418A;D424A;D427A) and pGEX-ExoS(L422A;L423A;L426A; L428A) were constructed by digesting pGEX-ExoS ( $\Delta$ ) with NdeI/NheI and PstI/NheI and inserting oligomers (Supplementary Table 2) corresponding to the appropriate amino acid substitutions as outlined in Table 1. All constructs were confirmed by sequencing with DYEnamic ET terminal cycle sequencing kit (Amersham-Biosciences).

### Construction of arabinose inducible ExoS derivatives and infection of cells

Mutant alleles were co-expressed with *orf1* to ensure protein stability of full-length ExoS derivatives, encoding the cognate non-secreted chaperone of ExoS. In all cases, DNA was amplified by PCR as described previously [31]. Construction of pMF384 containing arabinose inducible *exoS*(WT) [16] and pMF516 containing arabinose inducible *exoS*(LDL426-428) [19] have both been described previously. Arabinose inducible *exoS* variants on the plasmids pMF743, pMF744, pMF745, pMF746, and pMF747 were obtained by replacing the C-terminal *Cl*I/*K*pnl *exoS* fragment from pMF384 with DNA amplified and restriction enzyme cut with *Cl*I/*K*pnl from pGEX-ExoS(S416A), pGEX-ExoS(H418A), pGEX-ExoS(S416A;H418A), pGEX-ExoS(L422A;L423A;L426A;L428A), and pGEX-ExoS(H418A;D424A;D427A) respectively (Supplementary Table 2) using the *exoS*-specific primers, *pexoS*seq3 (position 973991; forward): 5'-AAGTGATGGCGCTTGGTCT-3' and *pexoS*d (reverse): 5'-ATGCATGGTACCTCAGGCCAGATCAAGGCCGCG-3'. All constructs were maintained in *Escherichia coli* DH5 and were confirmed by sequence analysis using the DYEnamic ET terminator cycle sequencing kit (Amersham Biosciences). Stable induction of protein expression in strains grown in the presence of 0.02% L(+)-arabinose was confirmed by western analysis, using polyclonal rabbit anti-ExoS. Bacterial infection of cells was performed in the presence of 0.1% L(+)-arabinose, as described previously [26].

#### **Construction and maintenance of *P. aeruginosa* mutants.**

The *P. aeruginosa* strains PA99null, which secretes no known effectors, PA99null+S, which secretes only wild type ExoS, PA99null+S(L426A/D427A/L428A), which secretes ExoS with substitutions in L426, D427, and L428, and PA99null +S(E379A;E381A), which secretes ExoS with substitutions E379 and E381 were generated in previous studies from the clinical strain PA99, which naturally encodes the *exoS*, *exoT* and *exoU* genes but not the *exoY* gene [18, 30, 32]. Strains secreting the ExoS variants ExoS(L422A), ExoS(L423A), ExoS(L422A/L423A), ExoS(L422A/L423A/L426A/L428A), ExoS(H418A), and ExoS(D424A/D427A) were generated by integrating mutated copies of the *exoS* allele, under the control of its endogenous promoter, into the chromosome of PA99null. First, specific mutations were generated within the *exoS* allele of the mini-CTX1-ExoS plasmid [30] using the QuickChange II XL site directed mutagenesis method (Stratagene). Plasmids encoding mutated alleles were then transformed into the *Escherichia coli* strain S17.1 and subsequently conjugated into the PA99null where the promoter and *exoS* sequences were integrated into the *Pseudomonas* chromosome at the *attB* locus using the method of Hoang et al. [33]. The

presence of appropriately mutated sequences was verified by nucleotide sequencing, and protein secretion was verified by growth in low-calcium medium followed by immunoblot screening of culture supernatants using ExoS-specific antibodies. Strains were maintained on Luria-Bertani agar or Vogel-Bonner minimal (VBM) agar. When necessary medium was supplemented with antibiotics at the following concentrations: carbenicillin 500 µg/ml, gentamicin 100 µg/ml and tetracycline 100 µg/ml.

#### **Mouse model of acute pneumonia**

Studies of acute pneumonia were conducted using the aspiration mouse model described by Comolli et al. [34]. Briefly, bacteria were collected by centrifugation and resuspended to the appropriate concentration in phosphate-buffered saline (PBS). Six- to eight-week-old female BALB/c mice were anesthetized by intraperitoneal injection of a mixture of ketamine (100 mg/ml) and xylazine (20 mg/ml). Mice were intranasally inoculated with  $6 \times 10^6$  CFU of bacteria in 50 µL PBS, as determined by optical density. Inocula were confirmed by plating of serial dilutions. Survival was monitored for seven days after infection. Mice were killed when severe illness developed and were scored as dead. Animals were purchased from Harlan and housed in the containment ward of the Center for Comparative Medicine at Northwestern University. All experiments were performed in accordance with the guidelines of the Northwestern University Animal Care and Use Committee.



## Result and discussion

### Leucine residues of ExoS are important for interaction with 14-3-3

The interaction between 14-3-3 and ExoS is essential for the ADP-ribosyltransferase activity of ExoS [25, 27]. ExoS amino acid residues 419 to 428 are known to be important for this interaction [16]. Although previous pull-down and cytotoxicity analyses indicate that D424 and D427 are not important for the binding of ExoS to 14-3-3 or activation of ExoS-mediated cytotoxicity [19], recent structural analysis indicated that the ExoS peptide is coordinated by H-bonds involving D424 and D427 as well as S416 and H418 of ExoS and D213, K49 and Y128 of 14-3-3 $\zeta$  [18]. Thus, it was of interest to analyze whether the aspartic acids either alone or in combination with S416 and H418 were important for the interaction and ExoS enzymatic activity.

A set of single, double, triple, and quadruple substitution ExoS mutants were constructed to investigate which ExoS residues were important for the electrostatic interaction with 14-3-3. Together with previously constructed variants, the new ExoS variants were fused to glutamine S-transferase (GST) giving rise to the following collection of fusion proteins: GST-ExoS(WT) [17], GST-ExoS(S $\Delta$ ) [16], GST-ExoS(DALDL424-428AAAAA) [19], GST-ExoS(L422A;L423A;L426A;L428A), GST-ExoS(D424A; D427A) [19], GST-ExoS(H418A;D424A;D427A), GST-ExoS(S416A;H418A), GST-ExoS(S416A), GST-ExoS(H418A), GST-ExoS(L422A) [19], GST-ExoS(L423A) [19], GST-ExoS(L426A) [19], and GST-ExoS(L428A) [19] (Table 1). All GST-ExoS derivatives were expressed, purified, and employed in protein pull-down experiments (Figure 2). HeLa cells were harvested and the lysates were pre-cleared with GST beads prior to 1-h incubation with each of the indicated GST-ExoS-fusion proteins. Samples were subsequently washed and run on SDS/PAGE followed by immunoblotting with 14-3-3 antibodies. Since we used a pan-14-3-3 antibody, we did not investigate binding or specificity of different 14-3-3 isoforms in this study. It was observed that GST-ExoS(WT) interacts with 14-3-3, but not GST-beads alone (Figure 2, lane 2 and 3). As previously described, GST-ExoS(DALDL424-428AAAAA) was unable to interact with 14-3-3 proteins from HeLa whole cell lysates (Figure 2, lane 4). Similarly, interaction of GST-ExoS(L422A;L423A;L426A;L428A) with 14-3-3 proteins from HeLa whole cell lysates was clearly reduced (Figure 2, lane 13). The new substitution mutants ExoS(S416A) and ExoS(H418A) agreed with previous data [19] showing that single amino acid substitutions of GST-ExoS between amino acid 419–428 did not obviously reduce their ability to interact with endogenous 14-3-3 proteins (Figure 2, lanes 5 and 6). The double substitution mutants GST-ExoS(D424A; D427A) (Figure 2, lane 12) and GST-

ExoS(S416A;H418A) (Figure 2, lane 11) and the triple mutant GST-ExoS(H418A;D424A;D427A) (Figure 2, lane 14) also showed no reduced ability to interact with 14-3-3 proteins. Thus, our pull down analysis strongly suggests that the four leucine residues are important for ExoS mediated interaction with 14-3-3 but that electrostatic interactions play at most a minor role.

### **Leucine 422, 423, 426, and 428 are important for the induction of cell death by the ADP-ribosyltransferase domain of ExoS.**

A live/dead cell assay was utilized to further investigate the functional significance of the ExoS residues in the 14-3-3 binding domain. HeLa cells were infected for 2 h with the surrogate bacterium *Yersinia pseudotuberculosis* lacking endogenous toxins and engineered to express and translocate ExoS (wild type as well as several single, double, triple, and quadruple amino acid substitution variants) into target cells under the control of arabinose [26, 35]. To ensure that ExoS variants were indeed delivered to infected cells, this assay capitalized on the fact that the GAP activity of ExoS causes transient cell rounding due to disruption of actin microfilaments prior to ADP-ribosylation mediated cell death [36]. Infection with bacteria secreting each of the ExoS variants resulted in a cytotoxic phenotype, (e.g., rounded and semi-detached cells) due to the N-terminal GAP activity of ExoS [21, 36], indicating that all ExoS variants were being expressed and injected (data not shown). Both detached and semi-detached cytotoxic cells were washed free from bacteria and transferred to a new Petri dish and incubated overnight with medium containing gentamicin. Cell death was quantified by a trypan blue exclusion assay performed 24 h after infection. Infection with wild-type ExoS [ExoS(WT)] mediated an irreversible change in cell morphology that ultimately leads to cell death (Figure 3), consistent with the results of previous studies [19, 26]. In fact, when normalized to the survival of uninfected cells, only 9% of ExoS(WT) infected cells survived (Figure 3). It was also observed that the single substitutions of serine at 416 [ExoS(S416A)] and histidine at 418 [ExoS(H418A)], as well as the double mutant ExoS(S416A;H418A) and the triple mutant ExoS(H418A;D424A;D427A) were as potent as ExoS(WT) in their ability to induce cell death (Figure 3). These results indicate that negatively charged amino acids at positions 424 and 427 were not crucial for cytotoxicity and, as previously suggested, probably do not mimic phosphorylated serine motifs [18]. The basic charged amino acids at positions 416 and 418, which also were not required for interaction with 14-3-3 proteins, resulted in a phenotype very similar to that of wild type ExoS. However, the quadruple mutant ExoS(L422A;L423A;L426A; L428A), which was unable to

interact with 14-3-3 proteins in pull-down experiments, was significantly impaired in its ability to induce cell death (93% survival) (Figure 3) after 2 h infection. Similar results were observed for bacteria expressing the ExoS(LDL426–428AAA) mutant (Figure 3), as previously reported [18]. These results indicate that ExoS(L422A;L423A;L426A;L428A) has reduced ADP-ribosylation activity similar to ExoS(LDL426-428AAA). This phenotype was reminiscent of cells transiently infected with the ADP-ribosylation mutant ExoS(E381A), which recover their original cell structure and morphology overnight due to the transient nature of ExoS GAP-mediated effects [26, 36]. In our initial study of the triple mutant ExoS(LDL426-428AA) [19], the possible involvement of the aspartic acid at position 427 could not be excluded. However, the new quadruple mutant excludes the involvement of aspartic acid 427. Thus, results from the cell death and GST-pull-down assays indicated that the four leucine residues are essential for ExoS activity.

#### **Leu-422, 423, 426, 428 of ExoS are critical for ADP-ribosylation of RAS and RAP1.**

Ras is modified by the ADP-ribosyltransferase activity of ExoS when expressed and delivered into eukaryotic cells by *Y. pseudotuberculosis* devoid of endogenous toxins [26]. This assay was used to further assess the *in vivo* biological activity of ExoS variants. HeLa cells were infected for 2 h with *Y. pseudotuberculosis* induced by arabinose to express and translocate ExoS(WT), ExoS(S416A), ExoS(H418A), ExoS(S416A;H418A), ExoS(H418A;D424A;D427A), ExoS(LDL426-428AAA), and ExoS(L422A;L423A;L426A;L428A) into target cells. The cells were then harvested and the resultant lysates were separated by SDS/PAGE followed by immunoblotting with anti-Ras, anti-Rap1 and anti-pan-Erk antibodies as a loading control (Figure 4). As evidenced by an increase in apparent molecular mass, Ras and Rap1 were modified in cells infected with bacteria expressing either wild-type ExoS, ExoS(S416A), ExoS(H418A), ExoS(S416A;H418A) or ExoS(H418A;D424A;D427A) (Figure 4, lanes 3, 5, 6, 7, and 9). Significantly, no modification of Ras and Rap1 were observed in bacteria translocating either ExoS(L422A;L423A;L426A;L428A) or ExoS(LDL426–428AAA) into infected cells (Figure 4, lanes 4 and 8). Thus, the results indicated that loss of the hydrophobic surface of the 14-3-3 binding motif of ExoS dramatically reduces the ability of 14-3-3 to interact with ExoS and act as a cofactor for the ExoS enzymatic activity.

#### **Leu-422, 423, 426, 428 of ExoS are essential for inhibition of Ras activation and Ras downstream signaling.**

Activation of Ras normally results in phosphorylation of the downstream signaling molecules such as Erk and PKB/Akt [26]. Therefore, it was examined if ADP-ribosylation of Ras by ExoS resulted in functional inactivation upon stimulation with EGF of RAS and loss of Erk and PKB/Akt phosphorylation (Figure 4). Infection of cells with bacteria expressing wild-type ExoS markedly prevented phosphorylation of Erk and PKB/Akt (Figure 4, lane 3) whereas ExoS(LDL426-428AAA) and ExoS(L422A;L423A;L426A; L428A) caused normal levels of phosphorylation (Figure 4, lanes 4 and 8). Further, all other tested ExoS mutants able to modify Ras and Rap1 [i.e., ExoS(S416A), ExoS(H418A), ExoS(S416A;H418A) and ExoS(H418A;D424A;D427A)] were also able to inhibit phosphorylation of PKB/AKT in a similar manner as ExoS(WT) (Figure 4, lanes 3, 5, 6, 7, and 9). Thus, S416, H418, D424 and D427 were not critical for interaction between 14-3-3 and ExoS and subsequent ExoS ADP-ribosyltransferase activity.

#### **Leu-422, 423, 426, 428 are required for ExoS-mediated virulence in a mouse model of acute pneumonia.**

To investigate the biological significance of Leu-422, 423, 426, and 428 of ExoS in a clinically relevant model, we generated *P. aeruginosa* strains that secreted ExoS variants harboring substitutions in residues within the 14-3-3 binding region of ExoS. Variants of the *exoS* gene were introduced into a neutral site in the chromosome of *P. aeruginosa* strain PA99null, a strain derived from clinical isolate PA99 that does not secrete any type III effectors. ExoS containing a single amino acid substitution (either L422A, L423A, or H418A), two substitutions (L422A/L423A or D424A/D427A), three substitutions (L426A/D427A/L428A) or four substitutions (L422A/L423A/L426A/L428A) were generated and were compared to PA99null (no ExoS), PA99null secreting wild-type ExoS, and PA99null secreting an ExoS variant with two substitutions in the ADP-ribosyltransferase catalytic site (E379A/E381A). To examine the contribution of residues affecting interaction of ExoS with 14-3-3 in pathogenesis of *P. aeruginosa*, mice were intranasally inoculated with *P. aeruginosa* variants and survival was monitored over time. *P. aeruginosa* secreting ExoS variants L422A, L423A, L422A/L423A, D424A/D427A, or H418A rapidly induced severe illness similar to wild-type ExoS (Figure 5). In contrast, all mice infected with *P. aeruginosa* secreting ExoS(L426A/D427A/L428A) or ExoS(L422A/L423A/L426A/L428A) survived the course of the experiment. The severe attenuation of these strains in mice indicates that the ExoS leucine residues required for interaction with 14-3-3 are relevant in the pathogenesis *P. aeruginosa* infection. These results further support a critical role for ExoS C-terminal leucine

residues in interaction with 14-3-3 and ExoS activity, contrasting with an at most minimal role for charged residues.

The combination of the above results verifies our hypothesis that hydrophobic interactions are important for both the interaction between 14-3-3 and ExoS and for the ExoS ADP-ribosyltransferase activity. However, the mechanism by which binding of 14-3-3 activates ExoS remains unclear. It was previously suggested that Leu428 is involved in an ExoS conformational change from an inactive to an active enzyme, but this remains to be investigated [19]. The data clearly suggest that the phosphorylation-independent ExoS/14-3-3 interaction is complex and is likely to involve other interactive surfaces beyond the hydrophobic interaction in the “roof of the groove” of 14-3-3. Further structural and biochemical analyses would enlighten this matter. In conclusion, this study verifies that 14-3-3 and ExoS interact in a novel fashion that does not involve phospho-mimicking electrostatic interactions but is dependent on the hydrophobic interaction surface between 14-3-3 and ExoS (mode IV).

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### Figure legends

**Figure 1.** Crystal structure of ExoS binding to 14-3-3 $\zeta$ . The 14-3-3 interacting motif of ExoS (residues 416-430) is shown as yellow sticks (carbon atoms) with nitrogen and oxygen coloured in blue and red, respectively. 14-3-3 $\zeta$  is shown as grey ribbon with semitransparent surface. Residues of 14-3-3 $\zeta$  that are directly contacting ExoS are shown as green sticks. Electrostatic interactions are displayed as dotted lines and the hydrophobic 'roof' of the groove of 14-3-3 $\zeta$  is shown as green semitransparent surface

**Figure 2.** Interaction of GST-ExoS variants with endogenous 14-3-3 proteins. HeLa cells were harvested and 1.25  $\mu$ g of lysates were subjected to 'pull-down' analysis with 1.25  $\mu$ g of individual GST-fusion proteins. Samples were separated on a SDS/PAGE followed by immunoblotting with 14-3-3 antibodies. Upper panel: Lane 1, control HeLa cell lysate, 2  $\mu$ g; lane 2, GST alone; lane 3, GST-ExoS(WT); lane 4, GST-ExoS(DALDL424–428AAAAA); lane 5, GST-ExoS(S416A); lane 6, GST-ExoS(H418A); lane 7, GST-ExoS(L422A); lane 8, GST-ExoS(L423A); lane 9, GST-ExoS(L426A); lane 10, GST-ExoS(L428A); lane 11, GST-ExoS(S416A;H418A); lane 12, GST-ExoS(D424A;D427A); lane 13, GST-ExoS(L422A;L423A;L426A;L428A); and lane 14, GST-ExoS(H418A;D424A;D427A). Lower panel: Coomassie blue stained SDS/PAGE showing the purified GST-fusion proteins used in this study. The order corresponds to lanes 2–14 above.

**Figure 3.** Cytotoxicity analysis of mammalian cells infected with ExoS variants. Viability of HeLa cells was expressed as survival percentage after 24 h intoxication with ExoS variants. HeLa cells were infected for 2 h in the presence of 0.1% L(+)-arabinose with *Yersinia* (YPIII/pIB251) carrying plasmids expressing different ExoS variants under the control of arabinose-inducible promoters as indicated in the figure. Both detached and semi-detached cytotoxic cells were washed free from bacteria and transferred to a new Petri dish and incubated overnight with fresh medium containing gentamicin. A trypan blue exclusion assay was performed 24 h after infection to quantify the percentage of dead cells. Error bars represent s.e.m. Each bar represents the mean values of three independent experiments.

**Figure 4.** Protein modification in host mammalian cells following infection with variants of ExoS. HeLa cells were infected for 2 h in the presence of 0.1% L(+)-arabinose with *Yersinia* (YPIII/pIB251) carrying plasmids expressing different ExoS variants under the control of

arabinose-inducible promoters. Lane 1, uninfected cells; lane 2, uninfected cells, lane 3, YPIII(pMF384) expressing ExoS(WT); lane 4, YPIII(pMF516) expressing ExoS(LDL426-428AAA); lane 5, YPIII(pMF743) expressing ExoS(S416A); lane 6, YPIII(pMF744) expressing ExoS(H418A); lane 7, YPIII(pMF745) expressing ExoS(S416A;H418A); lane 8, YPIII(pMF746) expressing ExoS(L422A;L423A;L426A;L428A) and lane 9, YPIII(pMF747) expressing ExoS(H418A;D424A;D427A). ExoS-mediated ADP-ribosylation of HeLa cell proteins following infection and stimulation with EGF (50 ng/ml) for 3 min before harvesting, as indicated in figure. HeLa cells were lysed and western blot analysis was performed on immunoblotted filters with anti-RAS, anti-RAP1, anti-phospho-ERK1/2, anti-phospho-PKB/Akt, and anti-pan-ERK antibodies as indicated in figure.

**Figure 5:** Survival of mice infected with *P. aeruginosa* secreting ExoS variants. Mice were infected with strain PA99null secreting the indicated ExoS variant. Each symbol represents the number of mice surviving in each experimental group over time. Results are from a representative experiment (n=5); similar results were obtained in at least two independent experiments. Survival of mice infected with PA99null+ExoS(L426A/D427A/L428A) or PA99null+ExoS(L422A/L423A/L426A/L428A) differed significantly from that of mice infected with PA99null+ExoS ( $P < 0.01$ , log rank test for equality of survivor functions).



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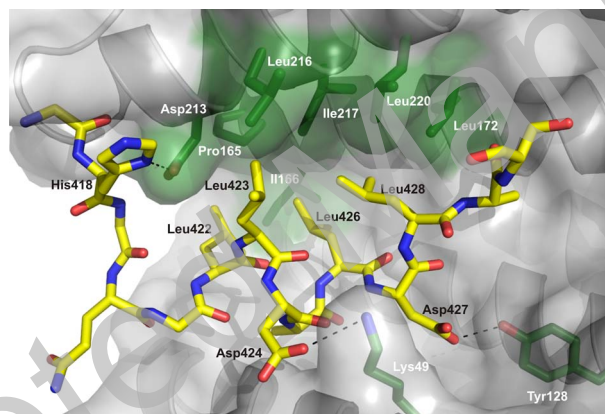
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**Table 1:** Summary of recombinant ExoS expression plasmids and bacterial strains used in this study. Substituted amino acid(s) are underlined. With respect to the GST-ExoS fusions, GST-ExoS(88–453) was the parental allele (WT) such that all other alleles listed differ only by the amino acid substitution indicated in parentheses. The number in front of plasmid indicates the serial number.

Plasmid or Bacterial strain (mutation)	Substituted amino acid(s)	Reference
1. GST alone	Amersham	
2. GST-ExoS(88–453), wild type	S <sup>416</sup> GHSQGLLDALDL <sup>428</sup>	[37]
3. GST-ExoS(88–453; SΔ)	M <sup>419</sup> AAAA <sup>428</sup>	[16]
4. GST-ExoS(H418A;D424A;D427A)	S <sup>416</sup> GASQGLLAALAL <sup>428</sup>	This study
5. GST-ExoS(L422A;L423A;L426A;L428A)	S <sup>416</sup> GHSQGAADAADA <sup>428</sup>	This study
6. GST-ExoS(DALDL424–428AAAAA)	S <sup>416</sup> GHSQGLLA <sup>428</sup>	[19]
7. GST-ExoS(D424A;D427A)	S <sup>416</sup> GHSQGLLAALAL <sup>428</sup>	[19]
8. GST-ExoS(S416A)	A <sup>416</sup> GHSQGLLDALDL <sup>428</sup>	This study
9. GST-ExoS(H418A)	S <sup>416</sup> GASQGLLDALDL <sup>428</sup>	This study
10. GST-ExoS(L422A)	S <sup>416</sup> GHSQGLDALDL <sup>428</sup>	[19]
11. GST-ExoS(L423A)	S <sup>416</sup> GHSQGLADALDL <sup>428</sup>	[19]
12. GST-ExoS(L426A)	S <sup>416</sup> GHSQGLLDAADL <sup>428</sup>	[19]
13. GST-ExoS(L428A)	S <sup>416</sup> GHSQGLLDALDA <sup>428</sup>	[19]
14. Mini-CTX1-ExoS, wild type	S <sup>416</sup> GHSQGLLDALDL <sup>428</sup>	[28]
15. Mini-CTX1-ExoS (L422A)	S <sup>416</sup> GHSQGLDALDL <sup>428</sup>	This study
16. Mini-CTX1-ExoS (L423A)	S <sup>416</sup> GHSQGLADALDL <sup>428</sup>	This study
17. Mini-CTX1-ExoS (L422A;L423A)	S <sup>416</sup> GHSQGAADALDL <sup>428</sup>	This study
18. Mini-CTX1-ExoS (L422A;L423A;L426A;L428A)	S <sup>416</sup> GHSQGAADAADA <sup>428</sup>	This study
19. Mini-CTX1-ExoS (H418A)	S <sup>416</sup> GASQGLLDALDL <sup>428</sup>	This study
20. Mini-CTX1-ExoS (D424A;D427A)	S <sup>416</sup> GHSQGLLAALAL <sup>428</sup>	This study
pMF384 (arabinose inducible <i>exoS</i> (WT))	S <sup>416</sup> GHSQGLLDALDL <sup>428</sup>	[16]
pMF516 (arabinose inducible <i>exoS</i> (LDL426-428AAA))	S <sup>416</sup> GHSQGLLDAAA <sup>428</sup>	[19]
pMF743 (arabinose inducible <i>exoS</i> (S416A))	A <sup>416</sup> GHSQGLLDALDL <sup>428</sup>	This study
pMF744 (arabinose inducible <i>exoS</i> (H418A))	S <sup>416</sup> GASQGLLDALDL <sup>428</sup>	This study
pMF745 (arabinose inducible <i>exoS</i> (S416A;H418A))	A <sup>416</sup> GASQGLLDALDL <sup>428</sup>	This study
pMF746 (arabinose inducible <i>exoS</i> (L422A;L423A;L426A;L428A))	S <sup>416</sup> GHSQGAADAADA <sup>428</sup>	This study
pMF747 (arabinose inducible <i>exoS</i> (H418A;D424A;D427A))	S <sup>416</sup> GASQGLLAALAL <sup>428</sup>	This study
<i>P. aeruginosa</i> strains		
1. PA99ΔUTS/ExoS, wild type	S <sup>416</sup> GHSQGLLDALDL <sup>428</sup>	[28]
2. PA99ΔUTS/ExoS (E379A;E381A)	S <sup>416</sup> GHSQGLLDALDL <sup>428</sup>	[16]
3. PA99ΔUTS/ExoS (L426A;D427A;L428A)	S <sup>416</sup> GHSQGLLDAAA <sup>428</sup>	[16]
4. PA99ΔUTS/ExoS (L422A)	S <sup>416</sup> GHSQGLDALDL <sup>428</sup>	This study
5. PA99ΔUTS/ExoS (L423A)	S <sup>416</sup> GHSQGLADALDL <sup>428</sup>	This study

6. PA99ΔUTS/ExoS (L422A;L423A)	S <sup>416</sup> GHSQGAADALDL <sup>428</sup>	This study
7. PA99ΔUTS/ExoS (L422A;L423A;L426A;L428A)	S <sup>416</sup> GHSQGAADAADA <sup>428</sup>	This study
8. PA99ΔUTS/ExoS (H418A)	S <sup>416</sup> GASQGLLDALDL <sup>428</sup>	This study
9. PA99ΔUTS/ExoS (D424A;D427A)	S <sup>416</sup> GHSQGLLAALAL <sup>428</sup>	This study
<i>Y. pseudotuberculosis</i> strain		
YPIII/pIB251 ( $\Delta yopE$ , $\Delta yopH$ )		[38]



**Figure 1**

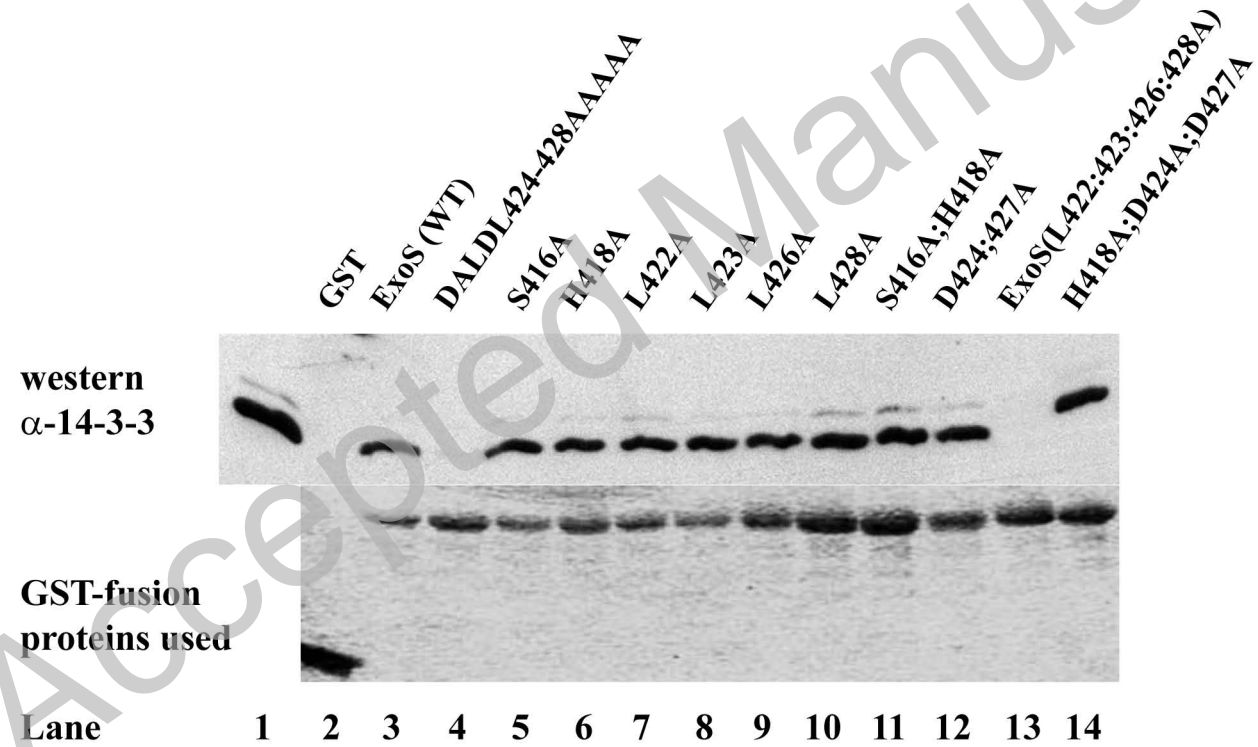
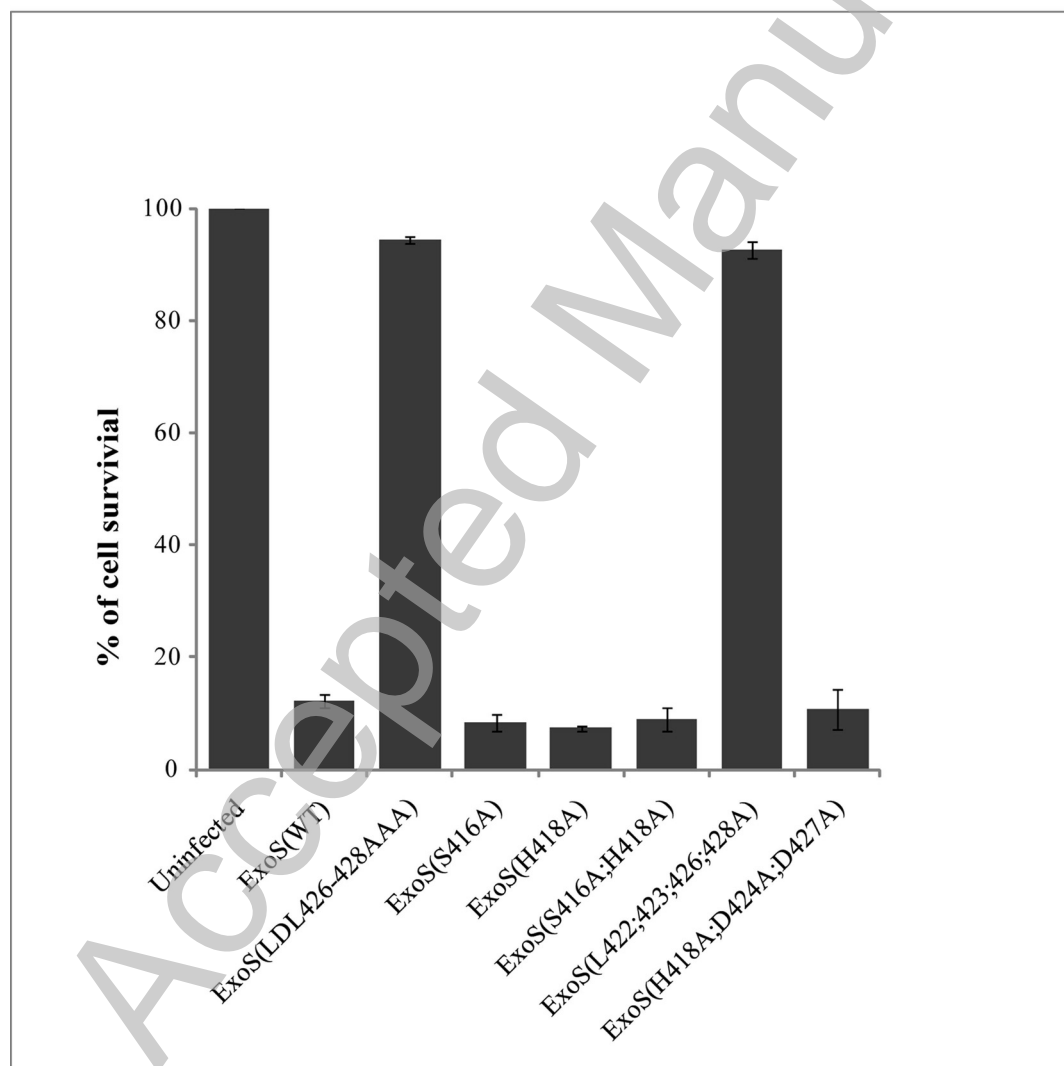




Figure 3



**Figure 4**