Effects of the type III secreted pseudomonal toxin ExoS in the yeast *Saccharomyces cerevisiae*

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Pseudomonas aeruginosa secretes a number of toxins by a type III system, and these are important in virulence. One of them, ExoS, is a bifunctional toxin, with a GTPase-activating protein domain, as well as ADP ribosyltransferase (ADPRT) activity. These two domains have numerous potential cellular targets, but the overall mechanism of ExoS action remains unclear. The effects of ExoS in a simple eukaryotic system, the yeast *Saccharomyces cerevisiae*, using a tetracycline-regulated expression system were studied. This system allowed controlled expression of ExoS in yeast, which was not possible using a galactose-induced system. ExoS was found to be an extremely potent inhibitor of yeast growth, and to be largely dependent on the activity of its ADPRT domain. ExoS produced a dramatic alteration in actin distribution, with the appearance of large aggregates of cortical actin, and thickened disorganized cables, entirely dependent on the ADPRT domain. This phenotype is suggestive of actin stabilization, which was verified by showing that the cortical aggregates of actin induced by ExoS were resistant to treatment with latrunculin A, an agent that prevents actin polymerization. ExoS increased the numbers of mating projections produced following growth arrest with mating pheromone, and prevented subsequent DNA replication, an effect that is again dependent on the ADPRT domain. Following pheromone removal, ExoS produced altered development of the mating projections, which became elongated with a swollen bud-like tip. These results suggest alternative pathways for ExoS action in eukaryotic cells that may result from activation of small GTPases, and this yeast expression system is well suited to explore these pathways.

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INTRODUCTION

Many Gram-negative bacteria have evolved a sophisticated system to introduce toxins directly into eukaryotic cells by a process called type III secretion (Cornelis, 2000; Cornelis & Van Gijsegem, 2000). The bacteria elaborate a multi-subunit apparatus, the injectisome, which acts as a conduit for secreted toxins. The toxins secreted by these type III secretion systems (TTSSs) usually act to subvert or modify normal eukaryotic cellular processes, to favour survival of the invading pathogen (Cornelis, 2002; Mota & Cornelis, 2005).

In many cases, a particular biochemical activity has been identified for individual type III secreted toxins. However, how this activity then translates into the observed phenotypic effects of the toxin on cells is often much less clear. Part of the problem in analysing toxin action in mammalian cells is the difficulty in identifying the crucial target of toxin action. Alternative models of infection have thus been

developed to study toxin action in a tractable genetic system. One such model uses the yeast *Saccharomyces cerevisiae* (Lesser & Miller, 2001). Although a simple eukaryote with 6591 ORFs, there is significant conservation of fundamental cellular processes between yeast and human cells. Indeed, up to 30 % of positionally cloned genes implicated in human disease have yeast homologues (Foury, 1997). *S. cerevisiae* is easily transformed with DNA, undergoes homologous replication with high efficiency, and its complete gene sequence is known and well annotated. Importantly, its cell cycle is extremely well understood, and cells can be readily synchronized at specific stages.

The human pathogen *Pseudomonas aeruginosa* is an important cause of nosocomial infection in those with impaired immune function, in patients with cystic fibrosis, and in those with burns (Chastre & Fagon, 2002; Lyczak *et al.*, 2002; Richards *et al.*, 1999). It secretes four toxins by a TTSS: ExoS, ExoT, ExoU and ExoY (Barbieri & Sun, 2004; Finck-Barbancon *et al.*, 1997; Frank, 1997; Hauser *et al.*, 1998; Yahr *et al.*, 1996, 1998). ExoS can induce a cytotoxic effect (Olson *et al.*, 1999), and is associated with the ability to produce lung damage and poor outcome from *P. aeruginosa* infection (Roy-Burman *et al.*, 2001). ExoS is a bifunctional toxin,

Abbreviations: ADPRT, ADP ribosyltransferase; GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor; PI, propidium iodide TTSS type III secretion system.

with N-terminal GTPase-activating protein (GAP) activity, and a C-terminal ADP ribosyl transferase (ADPRT) domain (Barbieri, 2000). The latter domain has been more clearly linked with the cytotoxic effects of ExoS, and has the ability to inhibit DNA synthesis in cultured cells (Fraylick *et al.*, 2001). The GAP domain of ExoS had no effect on DNA synthesis. Many targets of the ExoS ADPRT domain action have been identified, including members of the small GTPase family proteins, such as Ras, Rab, Ral and Rap, as well as vimentin, and the GAP domain within its own N-terminal region (Aktories *et al.*, 2000; Barbieri & Sun, 2004). These studies have demonstrated the effects of ExoS on the activation and interaction of small GTPases with downstream effectors. Targeting of Ras pathways, in particular, would provide a link between the actions of ExoS and inhibiting cell proliferation (Henriksson *et al.*, 2000, 2002; McGuffie *et al.*, 1998). However, it is not clear which of the small GTPase pathways is crucial in the cytotoxic effects of ExoS. Moreover, ADP ribosylation of Ras can be prevented with no effect on the cytotoxicity of ExoS (Pederson *et al.*, 2002). In addition, in epithelial cells, ADP-ribosylation by ExoS can lead to activation of the small GTPase Rac (Rocha *et al.*, 2005), which could act to increase cell proliferation through p21-activated kinase activity.

Given that the mechanisms of ExoS action in mammalian cells remain unclear, we sought to develop an alternative simpler system to analyse ExoS action using the yeast *S. cerevisiae*. We describe here a model system for the expression of ExoS in *S. cerevisiae* under tetracycline regulation. Using this system, we demonstrate that ExoS inhibits yeast cell growth, an effect largely dependent on the activity of the ADPRT domain. ExoS expression produces an unusual effect on actin within yeast cells, resulting in large cortical patches and thickened cables, again dependent on ExoS ADPRT activity. ExoS increased the numbers of mating projections formed following treatment of haploid yeast cells with mating pheromone. Following release of yeast cells from growth arrest with mating factor, ExoS prevented DNA replication, and resulted in unusual elongated projections with expanded tips. These studies demonstrate a unique phenotype of *S. cerevisiae* following ExoS expression, and suggest previously unsuspected targets for ExoS action within the cell.

METHODS

All chemicals were supplied by Sigma-Aldrich. Griener Bio-One supplied the plasticware, unless otherwise stated. The primers were synthesized by Operon Biotechnologies, and DNA sequencing was performed by the Dundee University Sequencing Service (Dundee, UK).

***S. cerevisiae* strains.** The strains of *S. cerevisiae* used in this study were the diploid strain INVSc1 (Invitrogen), and the haploid strain BMA64-1A (Euroscarf). The genotypes of these strains are: INVSc1, *his3Δ1/his3Δ1 leu2/leu2 trp1-289/trp1-289 ura3-52/ura3-52*; and BMA64-1A, *MATa ura3-52 trp1Δ2 leu2-3_112 his3-11 ade2-1 can1-100*.

Maintenance and growth of *S. cerevisiae*. Yeast strains were grown in yeast peptone glucose (YPD) broth containing 1% yeast extract (Oxoid), 2% peptone (Oxoid), and 2% glucose. Transformed yeast cells were grown in synthetic dropout (SD) medium to maintain the selective pressure on the plasmid. For plates, 4% agar (Oxoid) was added to the YPD and SD media. All yeast incubations were carried out at 30 °C, with shaking at 250 r.p.m. for liquid cultures. Working stock plates of yeast were kept for up to 2 months at 4 °C, and, for long-term storage, yeast strains were stored in 25% (v/v) glycerol at –80 °C.

Construction of plasmids. This study utilized three yeast expression vectors to examine the effect of ExoS, and the GAP and ADPRT domains of ExoS, on *S. cerevisiae*. The three vectors were pYES2/NT (Invitrogen), pYC2/NT (Invitrogen) and pCM252 (Euroscarf). pYES2/NT and pYC2/NT contain the *URA3* gene, and pCM252 contains the *TRP1* gene; therefore, *S. cerevisiae* cells transformed with these plasmids were selected for on SD medium lacking uracil and tryptophan, respectively. Wild-type ExoS, ExoS with the GAP domain mutated, ExoS with the ADPRT domain mutated, and ExoS with GAP and ADPRT domains mutated, were expressed from all three vectors. Table 1 summarizes how each construct was made and Table 2 lists the primers utilized.

Amplification of *exoS*. *PfuTurbo* Hotstart DNA polymerase (2.5 U; Stratagene) was used to PCR amplify *exoS* from PA01 genomic DNA in a reaction mixture containing 1× cloned *Pfu* DNA polymerase reaction buffer (Stratagene), 25 mM of each dNTP, 1 pmol μl^{–1} ExoS-Fw primer, 1 pmol μl^{–1} ExoS-Rev primer (Table 2), and 5% DMSO, in a total volume of 100 μl. The mixture was subjected to one denaturing cycle of 5 min at 94 °C, and then 30 cycles using the following conditions: a 45 s denaturing step at 94 °C, followed by a 45 s annealing step at 52 °C, and a 1 min extension step at 72 °C, and completed by a final extension cycle of 10 min at 72 °C. After cloning into the *KpnI* and *XhoI* sites of pYES2/NTA, the sequence of *exoS* was confirmed by sequencing with the T7prom, CYC1R and ExoS-Int primers (Table 2).

Site-directed mutagenesis. The QuikChange site-directed mutagenesis kit (Stratagene) was used to incorporate the R146A and E379A-E381A mutations into ExoS in pYES2/NT-ExoS, according to the manufacturer's instructions. For each site-directed mutagenesis reaction, two complementary primers were designed (Table 2) with nucleotide mutations that would result in the desired amino acid substitutions, and would also introduce a new restriction site into pYES2/NT-ExoS. Mutations were subsequently confirmed by sequencing the constructs with the T7 prom, CYC1R and ExoS-Int primers (Table 2).

Yeast transformation. *S. cerevisiae* cells were transformed with plasmids using the Frozen-EZ Yeast Transformation II kit (Zymo Research), according to the manufacturer's instructions. Briefly, competent cells were prepared by centrifuging 10 ml of mid-exponential phase yeast cells at 500 g for 4 min, then washing the pellet in 10 ml EZ1 solution, and resuspending the cells in 1 ml EZ2 solution. For transformation, 0.2–1 μg of plasmid DNA was mixed with 50 μl of competent cells, and 500 μl EZ3 solution. The transformation reaction was incubated at 30 °C for 45 min, and subjected to vigorous mixing three times during incubation. A 100 μl volume of the transformation mixture was plated onto appropriate SD agar, and allowed to grow at 30 °C for 2–4 days.

Integration of pCM242 into the *LEU2* locus of the *S. cerevisiae* genome. The pCM242 plasmid was integrated into the genome of INVSc1 and BMA64-1A by homologous recombination between the functional *LEU2* gene in pCM242, and the mutated *LEU2* locus in the yeast chromosomes. The pCM242 plasmid

Table 1. Plasmid construction

Plasmid	Construction
pYES2/NT-ExoS	The <i>exoS</i> gene was amplified from PA01 genomic DNA using the ExoS-Fw and ExoS-Rev primers, and cloned into the <i>KpnI</i> and <i>XhoI</i> sites of pYES2/NTA
pYES2/NT-ExoS_GAPM	The R146A mutation was introduced into the GAP domain of ExoS by site-directed mutagenesis using the R146A-Fw and R146A-Rev primers
pYES2/NT-ExoS_ADPRM	The E379A and E381A mutations were introduced into the ADPRM domain of ExoS by site-directed mutagenesis using the E379A-E381A-Fw and E379A-E381A-Rev primers
pYES2/NT-ExoS_GAPM_ADPRM	The <i>SacII</i> – <i>XhoI</i> fragment from pYES2/NT-ExoS_ADPRM was subcloned into the <i>SacII</i> / <i>XhoI</i> sites of pYES2/NT-ExoS_GAPM
pYC2/NT-ExoS	<i>exoS</i> was subcloned from pYES2/NT-ExoS into the <i>KpnI</i> and <i>XhoI</i> sites of pYC2/NTA
pYC2/NT-ExoS_GAPM	<i>exoS</i> _GAPM was subcloned from pYES2/NT-ExoS_GAPM into the <i>KpnI</i> and <i>XhoI</i> sites of pYC2/NTA
pYC2/NT-ExoS_ADPRM	<i>exoS</i> _ADPRM was subcloned from pYES2/NT-ExoS_ADPRM into the <i>KpnI</i> and <i>XhoI</i> sites of pYC2/NTA
pYC2/NT-ExoS_GAPM_ADPRM	<i>exoS</i> _GAPM_ADPRM was subcloned from pYES2/NT-ExoS_GAPM_ADPRM into the <i>KpnI</i> and <i>XhoI</i> sites of pYC2/NTA
pUC19-ExoS	<i>exoS</i> was subcloned from pYES2/NT-ExoS into the <i>KpnI</i> and <i>XbaI</i> sites of pUC19
pUC19-ExoS_GAPM	<i>exoS</i> _GAPM was subcloned from pYES2/NT-ExoS_GAPM into the <i>KpnI</i> and <i>XbaI</i> sites of pUC19
pUC19-ExoS_ADPRM	<i>exoS</i> _ADPRM was subcloned from pYES2/NT-ExoS_ADPRM into the <i>KpnI</i> and <i>XbaI</i> sites of pUC19
pUC19-ExoS_GAPM_ADPRM	<i>exoS</i> _GAPM_ADPRM was subcloned from pYES2/NT-ExoS_GAPM_ADPRM into the <i>KpnI</i> and <i>XbaI</i> sites of pUC19.
pCM252-ExoS	<i>exoS</i> was subcloned from pUCP19-ExoS (blunt <i>KpnI</i> and <i>PstI</i>) into the blunt <i>StuI</i> and <i>PstI</i> sites of pCM252
pCM252-ExoS_GAPM	<i>exoS</i> _GAPM was subcloned from pUCP19-ExoS_GAPM (blunt <i>KpnI</i> and <i>PstI</i>) into the blunt <i>StuI</i> and <i>PstI</i> sites of pCM252
pCM252-ExoS_ADPRM	<i>exoS</i> _ADPRM was subcloned from pUCP19-ExoS_ADPRM (blunt <i>KpnI</i> and <i>PstI</i>) into the blunt <i>StuI</i> and <i>PstI</i> sites of pCM252
pCM252-ExoS_GAPM_ADPRM	<i>exoS</i> _GAPM_ADPRM was subcloned from pUCP19-ExoS_GAPM_ADPRM (blunt <i>KpnI</i> and <i>PstI</i>) into the blunt <i>StuI</i> and <i>PstI</i> sites of pCM252
pCM252-ExoS-Xpress	<i>exoS</i> was subcloned from pYES2/NT-ExoS (<i>HindIII</i> and <i>XbaI</i>) into the <i>HindIII</i> and <i>XbaI</i> sites of pCM252
pCM252-ExoS-Xpress_GAPM	<i>exoS</i> _GAPM was subcloned from pYES2/NT-ExoS _GAPM (<i>HindIII</i> and <i>XbaI</i>) into the <i>HindIII</i> and <i>XbaI</i> sites of pCM252
pCM252-ExoS-Xpress _ADPRM	<i>exoS</i> _ADPRM was subcloned from pYES2/NT-ExoS_ADPRM (<i>HindIII</i> and <i>XbaI</i>) into the <i>HindIII</i> and <i>XbaI</i> sites of pCM252
pCM252-ExoS-Xpress _GAPM_ADPRM	<i>exoS</i> _GAPM_ADPRM was subcloned from pYES2/NT-ExoS _GAPM_ADPRM (<i>HindIII</i> and <i>XbaI</i>) into the <i>HindIII</i> and <i>XbaI</i> sites of pCM252

was digested with *EcoRV*, and purified using the QIAquick PCR purification kit (Qiagen), according to the manufacturer's instructions. The *EcoRV*-linearized pCM242 was transformed into INVSc1 or BMA64-1A, and the yeast cells that had successfully integrated the plasmid were selected on SD agar lacking Leu, with glucose added (SD–Leu + glucose).

Growth assays. For the plate growth assays, overnight cultures of yeast grown in SD medium lacking Leu and Trp, with glucose added (SD–Leu–Trp + glucose), were diluted in PBS (10 mM Na₂HPO₄, 137 mM NaCl, 2.7 mM KCl, 1.47 mM KH₂PO₄) to an OD₆₀₀ of 1. These cultures were serially 10-fold diluted four times in PBS, and 5 µl of each dilution was spotted onto SD–Leu–Trp + glucose agar supplemented with doxycycline as indicated. The plates were incubated at 30 °C for 4 days before being photographed using a Kodak DX4530 digital camera.

For the liquid growth assay, cultures of INVSc1(pCM242), transformed with either pCM252 or pCM252-ExoS, were grown overnight in SD–Leu–Trp + glucose. These cultures were diluted in fresh media to an OD₆₀₀ of 0.3. After 4 h growth, doxycycline was added at a final concentration of 2 µg ml^{−1}. OD₆₀₀ readings were taken every hour to assess the growth of the cultures.

Analysis of ExoS protein expression. ExoS and individual domain mutants with the Xpress expression tag were constructed in pCM252, as outlined in Table 1. Plasmids were transformed into INVSc1(pCM242), and protein expression was induced by the addition of 2 µg doxycycline ml^{−1}, as described above. Four hours after doxycycline addition, 1.5 ml of yeast culture was centrifuged at 3000 g for 5 min at 4 °C, and the pellet was resuspended in Y-PER (Pierce) at 5 µl (mg pellet)^{−1}, vortexed, and agitated by rotation at room temperature for 20 min. An equal volume of SDS loading

Table 2. Primers

Primer	Sequence (5'–3')
ExoS-Fw	GCGGTACCTCAAGCATATGCATATTCAATCGCTTCAGCAG
ExoS-Rev	CTCTCGAGGGATCCGCTGCCGAGCCAAGAATC
R146A-Fw	CGGAGATGGGGCGCTAGCTTCGCTGAGCACCG
R146A-Rev	CGGTGCTCAGCGAAGCTAGCGCCCCATCTCCG
E379A-E381A-Fw	CGAACTACAAGAATGCAAAAGCGATTCTCTATAACAAAG
E379A-E381A-Rev	CTTTGTTATAGAGAATCGCTTTTGCATTCTTGAGTTTCG
T7prom	GTAATACGACTCACTATAGGGC
CYC1R	GCGTGAATGTAAGCGTGAC
ExoS-Int	CGTGTTCAGCAGATGGTG

buffer was added, and the samples were denatured by heating at 100 °C for 3 min. After centrifugation, equal volumes were separated on SDS-polyacrylamide gels, and immunoblotted with anti-Xpress antibody (Invitrogen) and ECL detection reagents (Amersham).

Fluorescence analysis of yeast actin. Cultures of INVSc1-(pCM242) containing a pCM252-based plasmid were grown and induced as described for the liquid growth assay. Four hours after the addition of doxycycline, the *S. cerevisiae* cells were fixed by adding 5% formaldehyde to 5 ml of culture, and incubating for 30 min at room temperature, with occasional inversion. The cells were centrifuged at 1500 g for 5 min, and the pellets were washed three times in PBS. The pellets were resuspended in 200–800 µl PBS (to roughly normalize cell density), and 150 µl aliquots were washed in 1 ml solution B (100 mM K₂HPO₄, 100mM KH₂PO₄ and 1.2 M sorbitol). Each pellet was incubated in 1 ml solution B, containing 0.2% 2-mercaptoethanol and 2 µg lyticase ml^{−1}, at 37 °C for 30 min, to permeabilize the yeast. After permeabilization, the cells were centrifuged at 500 g, and washed once in 1 ml solution B. To stain for actin, the fixed and permeabilized *S. cerevisiae* were incubated in 50 µl solution B, containing 0.4 U Alexa Fluor 488 Phalloidin (Invitrogen), at 37 °C overnight, in the dark. The cells were washed three times in 1 × PBS before being spread onto a Poly Prep slide (Sigma-Aldrich), and allowed to dry for 10 min. A drop of Vectashield (Vector Laboratories) was used to mount the yeast under a coverslip. The slides were viewed using a Nikon Eclipse E600 microscope with a Nikon pan Fluor ×100 lens, and captured using an Optronics digital camera and MagnaFire software (Meyor Instruments).

Effects of latrunculin A on yeast actin cytoskeleton. INVSc1-(pCM242) with pCM252 alone, or expressing ExoS, was induced to express ExoS with 2.0 µg doxycycline ml^{−1} for 4 h, as described above. Samples were removed for actin staining (time 0), and latrunculin A (Biomol) was added at 200 µM. Samples of 1 ml were removed at various times after addition of latrunculin A, fixed by addition of formaldehyde to 5% final concentration, washed three times in PBS, and then stained for actin with 0.4 U Alexa Fluor 488 phalloidin, in a final volume of 100 µl PBS, at 37 °C overnight in the dark, with shaking. Cells were washed three times with PBS, spread on poly L-lysine coverslips, and analysed by a Zeiss Confocal microscope and associated software. Since, in this protocol, not every cell stained for actin, quantification of the effects of latrunculin A on yeast actin was normalized to the numbers of cells showing actin staining of their cytoskeleton prior to the addition of latrunculin A (time 0).

Synchronization of *S. cerevisiae*. The haploid MATa strain BMA64-1A, containing the integrated pCM242 plasmid and a pCM252-based plasmid, was grown to mid-exponential phase in

SD–Leu–Trp+glucose. The cultures were diluted to an OD₆₀₀ of 0.2, and arrested in G1 with 20 µg α-factor ml^{−1} (Zymo Research). Two hours after addition of the α-factor, ExoS or mutant ExoS was induced by adding 2 µg doxycycline ml^{−1}. The yeast were released from α-factor-arrest an hour after the doxycycline was added, by centrifuging the cells at 1500 g for 5 min, then washing twice in pre-warmed SD–Leu–Trp+glucose containing 2 µg doxycycline ml^{−1}. The cells were resuspended in pre-warmed SD–Leu–Trp+glucose containing 2 µg doxycycline ml^{−1}, and cells were fixed for immunofluorescence or flow cytometry analysis every 30 min.

Flow cytometry analysis. Synchronized BMA64-1A(pCM242), containing a pCM252-based plasmid, was prepared for flow cytometry analysis. The cells from 1 ml of culture were harvested by centrifugation at 500 g for 5 min, and the pellet was resuspended in 1.5 ml double-distilled water (DDW). The yeast cells were fixed by adding 3.5 ml 95% ethanol, and incubating overnight at 4 °C. The cells were centrifuged at 500 g for 5 min, and washed in 1 ml DDW. The RNA was degraded by incubating the yeast with 0.5 ml 2 mg RNase A ml^{−1} in 50 mM Tris/HCl, pH 8.0, for 1–2 h at 37 °C. The cells were centrifuged at 500 g for 5 min, and the pellet was resuspended in 200 µl 5 mg pepsin ml^{−1} and 0.45% conc. HCl, and incubated for 30–60 min at 37 °C to degrade the proteins. The yeast cells were harvested by centrifugation at 500 g for 5 min, and the pellet was resuspended in 0.5 ml 1 × propidium iodide (PI) solution (180 mM NaCl, 70 mM MgCl₂, 75 µM PI, 100 mM Tris/HCl, pH 7.5). The yeast cells were incubated in the 1 × PI solution overnight at 4 °C to stain the DNA. A 50 µl aliquot of cells was diluted in 0.1 × PI solution diluted in 50 mM Tris/HCl, pH 7.5, for flow cytometry analysis. Samples were analysed on a FACscan flow cytometer using CellQuest software to obtain and analyse the data (BD Biosciences).

RESULTS

Tetracycline-inducible expression of ExoS in *S. cerevisiae*

We first attempted to express ExoS using the expression vector pYES2/NT, a high-copy-number vector that allows gene expression to be controlled by altering the carbon source from glucose to galactose. Using this system, we were unable to isolate any yeast transformants containing the ExoS plasmid (data not shown). We also attempted to express ExoS in the low-copy-number vector pYC2/NT, but similarly were unable to isolate transformants containing this plasmid. This suggested that there was some leakiness of

expression of ExoS from these vectors, even under repressed conditions in the presence of glucose, and this was sufficient to prevent recovery of transformed *S. cerevisiae*. These glucose-repressed systems have been used successfully to express ExoU and ExoT under galactose control in yeast (Garrity-Ryan *et al.*, 2004; Rabin & Hauser, 2003), so we concluded that ExoS must be more toxic to yeast than these other TTSS toxins.

To overcome this problem, we expressed ExoS under the control of a tetracycline-regulated activator–repressor dual system (Belli *et al.*, 1998). This required chromosomal integration of an expression plasmid (pCM242) for the wild-type tetracycline repressor *tetR* fused to *Ssn6*, a component of the general repressor complex in yeast. We generated this chromosomal integration at the *LEU2* locus of the *S. cerevisiae* strain INVSc1, yielding the strain INVSc1(pCM242). We then transformed this strain with ExoS constructs in the expression plasmid pCM252. This vector expresses inserted genes under the control of the tetracycline-responsive *tetO* promoter, and is maintained episomally. It also expresses a fusion protein of *tetR*–VP16 transactivator that will only bind and activate the *tetO* promoter in the presence of tetracycline. This system provides very tight control of transcription of exogenous genes under the control of tetracycline (Belli *et al.*, 1998). In the absence of tetracycline, the TetR–Ssn6 fusion protein binds to the *tetO* promoter constitutively, and potently inhibits transcription. In the presence of tetracycline, this repression is lifted, and the TetR'–VP16 transactivator can bind to the *tetO* promoter, resulting in high-level expression of the introduced gene.

Using this system, we were able successfully to introduce the *exoS* gene and a variety of mutants into *S. cerevisiae*, and express them under the control of tetracycline. We transformed INVSc1(pCM242) with pCM252 alone, or pCM252 containing wild-type ExoS (ExoS), ExoS with the active site of the GAP domain mutated (GAPM), ExoS with the ADPRT active sites mutated (ADPRTM), or ExoS with both these alterations (GAPM+ADPRTM). In the absence of tetracycline, none of these constructs produced any effect on yeast growth, as assayed by spotting serial dilutions of each strain on agar (Fig. 1). Virtually identical numbers of each transformed strain were detected in this assay. When these dilutions of each strain were spotted on plates containing 0.1 µg tetracycline ml^{−1}, yeast transformed with pCM252 containing wild-type ExoS were profoundly growth-inhibited compared with yeast transformed with plasmid alone. Further increases in tetracycline concentration did not produce appreciable increases in this growth inhibitory effect (Fig. 1). Yeast transformed with the GAPM mutant were similarly profoundly growth inhibited by 0.1 µg tetracycline ml^{−1}, with no growth observable beyond the first yeast dilution spotted. Transformation with the ADPRTM mutant was much less toxic towards the yeast, with detectable growth inhibition only seen when the concentration of tetracycline was greater than 1 µg ml^{−1}

Fig. 1. Toxicity of ExoS, and the GAP and ADPRT domains of ExoS, in *S. cerevisiae*. INVSc1 with pCM242 integrated into its *LEU2* locus was transformed with the empty vector pCM252, or pCM252 encoding ExoS, the ExoS GAP mutant (GAPM), the ExoS ADPRT mutant (ADPRTM), or the ExoS GAP and ADPRT mutant (GAPM+ADPRTM). The toxicity of each construct to *S. cerevisiae* was assessed by spotting 10-fold serial dilutions of culture onto agar containing varying concentrations of doxycycline (Conc. dox), and assessing growth after 3 days. Identical results were obtained in three separate experiments.

(Fig. 1). The double mutant GAPM+ADPRTM showed no toxicity compared with yeast transformed with vector pCM252 alone. Thus, ExoS shows clear toxicity towards *S. cerevisiae* in this system, with virtually all of this effect being dependent on an intact ADPRT domain. The GAP domain did have some toxicity, however, as the ADPRTM mutant did inhibit yeast growth when expression levels were elevated by increased tetracycline concentrations. This inhibitory effect of ExoS was also observed in liquid culture following induction of ExoS expression by the addition of tetracycline (Fig. 2).

Fig. 2. Toxicity of ExoS in liquid culture. Growth curves of INVSc1 with pCM242 integrated into the *LEU2* containing either the empty pCM252 vector or pCM252-ExoS. Overnight cultures were diluted to an OD₆₀₀ of approximately 0.3, and, after 4 h growth, ExoS expression was induced by the addition of 2 µg doxycycline ml⁻¹. The experiment was performed in triplicate. Mean OD values are plotted; error bars represent standard deviation of the mean.

Effect of ExoS expression on the actin cytoskeleton in *S. cerevisiae*

Next, we examined the effect of ExoS expression in this system on the actin cytoskeleton of yeast. In the presence of the inducer tetracycline, yeast transformed with the pCM252 plasmid alone showed a normal distribution of actin, with cortical patches that showed a marked polarized distribution in nascent buds, and delicate actin cables extending between mother and daughter cell (Fig. 3A). This did not differ from untransformed yeast (data not shown). Following induction of ExoS, there was a marked change in the distribution of filamentous actin within the yeast cells. Patches of actin were much larger and brighter than those seen in the cells transformed with pCM252 alone (Fig. 3B). The images shown in Fig. 3 were each photographed with the same settings to allow direct comparison of actin intensity. Many of these large actin patches aggregated in areas of the cell. The number of cells showing buds did not appear to differ from yeast transformed with vector alone. However, the degree of polarization of actin was disrupted. Although actin patches did tend to accumulate within the nascent daughter cell, patches were still visible within the mother in cells expressing ExoS. In addition, actin cables were thickened and disorganized, and did not exhibit the normal

Fig. 3. Effect of ExoS on the actin cytoskeleton of *S. cerevisiae*. Fluorescent staining of the actin cytoskeleton with phalloidin in INVSc1 containing pCM242 and the empty pCM252 vector (A), or pCM252 encoding ExoS (B, C), the GAP domain mutant (D), the ADPRT domain mutant (E), or the GAP and ADPRT mutant of ExoS (F), 4 h after ExoS induction with doxycycline (2 µg ml⁻¹). The area boxed in (B) is enlarged in (C). (C) The arrowhead shows a thickened disorganized actin cable. Images are representative of three separate experiments; bar, 1 µm.

polarized distribution along the mother–daughter axis. This is seen in the cell boxed in Fig. 3(B), and it is enlarged in Fig. 3(C). A disorganized actin cable is shown by an arrowhead.

To define which domain of ExoS was responsible for this alteration in actin cytoskeleton, we expressed mutants of ExoS with the active site of the GAP domain mutated (GAPM), the active sites of the ADPRT domain mutated (ADPRTM), or both (GAPM + ADPRTM), and analysed the distribution of actin following toxin expression (Fig. 3D–E). Yeast expressing ExoS containing the functioning ADPRT domain alone had the same alterations in actin as seen with the wild-type ExoS (Fig. 3D, GAPM). Expression of ExoS with a mutation of the ADPRT domain produced no discernible difference in actin staining from control cells (ADPRTM, Fig. 3E, compared with pCM252, Fig. 3A). Expression of ExoS with mutation of both the ADPRT and GAP domains (Fig. 3F) also gave an identical pattern of actin staining to the vector control (Fig. 3A). Thus, we concluded that the changes in yeast actin produced following expression of ExoS resulted from the activity of the ADPRT domain.

Expression of ExoS and ExoS mutant proteins

To be certain that the lack of effect of the ExoS ADPRT domain mutant was not due to a lack of expression of this protein, we analysed the levels of expression of ExoS and the different domain mutants 4 h after induction in yeast, the time at which the effects upon the actin cytoskeleton were determined in Fig. 3. As shown in Fig. 4, we did not detect any expression of the intact ExoS or GAPM ExoS at this time point, as we would expect, given the high toxicity of both these forms of ExoS to the yeast (Fig. 1). However, we were able to detect both the ADPRTM and ADPRTM + GAPM ExoS proteins (Fig. 4) in amounts that reflected their relative toxicity within the cell; levels of both proteins were

Fig. 4. Protein expression of ExoS and mutants. INVSc1-(pCM242) transformed with pCM252 alone, or with the indicated Xpress-tagged ExoS constructs, was induced for protein expression with doxycycline ($2 \mu\text{g ml}^{-1}$) for 4 h. Protein expression was quantified by immunoblot using the Xpress epitope, as described in the Methods. Equal weights of yeast were loaded in each lane.

sustained for up to 24 h (data not shown). Thus, ExoS with only the GAP domain active (ADPRTM) was expressed within yeast at a time when no effect on actin organization was detected.

Effects of ExoS on actin turnover

The large cortical actin aggregates and thickened disorganized actin cables seen in yeast expressing ExoS, suggested that the effect of the toxin might be to stabilize actin, in a fashion similar to that seen in cells expressing a mutant actin that inhibits depolymerization (Belmont & Drubin, 1998). To explore this possibility, we utilized the effects of the drug latrunculin A on the yeast actin cytoskeleton. This drug sequesters actin monomers, preventing new filamentous actin formation (Ayscough *et al.*, 1997), although not affecting filament disassembly that occurs within minutes in *S. cerevisiae* (Ayscough *et al.*, 1997). Thus, factors that lead to stabilization of actin filaments result in actin structures that are relatively resistant to latrunculin-A-induced actin depolymerization. Fig. 5 shows the effects of ExoS expression on the stability of cortical actin patches in the presence of latrunculin A. ExoS expression dramatically stabilized the actin cortical patches that disappeared in wild-type cells following just 1 min treatment with latrunculin A (Fig. 5). Thus, the changes in actin organization following ExoS expression appear to result from stabilization of actin filaments by ExoS.

Effect of ExoS on actin distribution and DNA synthesis following release from growth arrest with mating pheromone

ExoS expression in yeast results in growth inhibition, a marked alteration in actin distribution and polarity, but no apparent reduction in the number of cells with buds. Given these findings, we hypothesized that ExoS could be affecting small GTPase proteins controlling actin distribution and cell growth. To examine this more closely, we analysed what effect ExoS expression had on actin distribution and cell division when expressed in cells synchronized in G1 of the cell cycle. To synchronize cells at this point, we utilized the mating pheromone α -factor, which arrests haploid yeast cells of mating type *MATa* just prior to START in the G1 phase of the cell cycle. We therefore constructed the tetracycline-inducible activator–repressor system in the haploid strain BMA64-1A by stable integration of the Tet-repressor plasmid pCM242, and expression of ExoS in the pCM252 vector. We confirmed that, in this haploid strain, ExoS expression inhibited yeast growth at $0.1 \mu\text{g doxycycline ml}^{-1}$, as seen with ExoS in the diploid INVSc1 strain (data not shown). We then studied this haploid strain BMA64-1A(pCM242) arrested using α -factor, and transformed with either the pCM252 vector containing ExoS, or the pCM252 vector alone as a control, as described in Methods.

First, we examined the effect of ExoS expression on the ability of yeast to replicate their DNA in the first S phase

Fig. 5. Effects of latrunculin A on yeast actin following ExoS expression. (A–D) Filamentous actin, as assayed by phalloidin staining following doxycycline treatment ($2 \mu\text{g ml}^{-1}$), of cells transformed with vector alone (pCM252; A, C) or ExoS (ExoS; B, D). Cells were analysed prior to latrunculin A treatment (0 min; A, B), or 3 min afterwards (C, D). (E) The percentage of cells showing actin cytoskeletal elements at the indicated times following latrunculin A treatment, normalized to the numbers of cells stained at time 0. Results are the means of triplicate determinations counting at least 150 cells; error bars are 1 SD. ExoS-expressing cells had significantly more polymerized actin compared to vector controls at all time points (t test, * $P < 0.01$, ** $P < 0.001$). Black bars, pCM252; grey bars, pCM252-ExoS.

following mating-factor growth arrest. The BMA64-1A(pCM242) strain was transformed with either pCM252 or pCM252-ExoS, growth arrested for 2 h with α -factor, and induced with doxycycline in the continuing presence of

α -factor, for another 1 h. The α -factor was then removed, and the DNA content of the yeast was then followed for the next 3 h. At time 0, yeast transformed with vector alone had a haploid content of DNA (Fig. 6, pCM242). Sixty minutes after mating-factor release, a very small number of the pCM242-transformed yeast had replicated their DNA, but, at 90 min, the majority of the cells had undergone DNA replication. At 120 and 150 min following α -factor release, a significant number of yeast still had a diploid DNA content, but, at 180 min, virtually all of the yeast cells had divided and had returned to a haploid DNA content (Fig. 6, pCM242). The pattern of DNA replication in cells expressing ExoS was dramatically different. Following α -factor release, only about 15 % of the yeast cells replicated their DNA at the peak times of 120 and 150 min (Fig. 6, ExoS). At 180 min, the diploid peak had subsided, suggested that those cells that did replicate their DNA eventually went on to divide (Fig. 6, ExoS). However, the majority of cells expressing ExoS did not replicate their DNA.

To define which domain of ExoS was responsible for this inhibition of DNA synthesis, we repeated this experiment using yeast transformed with pCM242 expressing ExoS with mutations in the GAP domain (GAPM), the ADPRT domain (ADPRTM) or both (G + AM). The resulting patterns of DNA synthesis following removal of α -factor clearly showed that the ADPRT domain was entirely responsible for this inhibition of DNA replication (Fig. 6).

Next, we examined the distribution of filamentous actin within the yeast using fluorescent phalloidin. Following α -factor arrest, yeast form mating projections or 'schmoos', and these did not differ in form or actin distribution between cells expressing ExoS or vector controls (Fig. 7, time 0). However, in cells expressing ExoS, we found that there was an increase in the number of yeast cells expressing more than one mating projection, as can be seen in Fig. 7 (pCM252-ExoS) at time 0. We quantified this difference, as shown in Fig. 8, and showed that this was significantly different between yeast transformed with pCM252 alone, and those expressing ExoS ($P < 0.001$, χ^2 test). Following removal of α -factor, yeast transformed with vector alone showed a normal polarization of actin at a nascent bud at 30–60 min after α -factor release. At 90 min, polarized buds were larger, and continued to increase in size up to 120 min after α -factor removal (Fig. 7, pCM252). In contrast, cells expressing ExoS had a grossly abnormal morphology following α -factor release. Initial polarization of the actin cytoskeleton was not inhibited at 30 min following α -factor removal; indeed, it often appeared rather more marked than in the control cells transformed with vector alone (Fig. 7). However, rather than the small nascent buds apparent in the control cells, the expression of ExoS produced polarized actin within a markedly elongated projection. This became more pronounced at later times following α -factor removal. Thus, at the 60 and 120 min time points, the projections were longer, and had a terminal bud-like swelling (Fig. 7). However, there was virtually no sign of actin accumulating

Fig. 6. Effect of ExoS on DNA synthesis in *S. cerevisiae*. BMA64-1A containing pCM242 and the pCM252 empty vector (pCM252), or pCM252 encoding ExoS (ExoS), the GAP mutant of ExoS (GAPM), the ADPRT mutant of ExoS (ADPRTM), or the GAP and ADPRT mutant of ExoS (G+AM), was arrested at G1 with α -factor, induced with doxycycline ($2 \mu\text{g ml}^{-1}$), and released from α -factor arrest. At various time points, the cells were fixed and stained with PI, and subjected to flow cytometry analysis. 1N and 2N indicate the DNA complement of the cells. Traces are representative of three separate experiments.

at the neck of the process, and many of the cells showed the large bright staining actin patches already seen in non-synchronized cells expressing ExoS (Fig. 2).

DISCUSSION

We have developed a *S. cerevisiae* model in which to study the pseudomonas TTSS toxin ExoS, and used this model to show that ExoS produces profound inhibition of yeast growth. Although both the GAP and ADPRT domains were toxic, the ADPRT domain accounted for the extreme toxicity of ExoS. We demonstrated that ExoS disrupted the actin cytoskeleton of yeast, and that the ADPRT domain was responsible for this phenotype. Additionally, we showed that the ADPRT domain of ExoS inhibits DNA synthesis in this model system. Finally, we found that ExoS increased the number of mating projections formed when *S. cerevisiae* was arrested with α -factor, and that ExoS interfered with the normal bud development after release from α -factor.

The use of this tetracycline-controlled yeast expression system offers several advantages over the glucose/galactose-based system. Firstly, the degree of repression of uninduced gene expression is greater than glucose repression of the galactose-controlled *GAL1* promoter. It has been demonstrated that the *tetR*-Ssn6 repressor decreases expression of a *lacZ* control gene under the control of the *tetO₇* promoter to virtually undetectable levels (≤ 0.10 Miller units of β -galactosidase activity) (Belli *et al.*, 1998). This is compared with the *GAL1* promoter system, which only represses β -galactosidase activity to 2 Miller units when repressed by growth on glucose (Belli *et al.*, 1998). In the work reported here, tetracycline-controlled yeast expression allowed the use of *S. cerevisiae* to express ExoS, which could not be expressed using the glucose/galactose system because of the

inhibitory effects of even tiny amounts of ExoS expression. In addition to its tight repression, the tetracycline-regulated activator-repressor dual system is also capable of inducing expression of regulated genes to high levels. For example, maximal *lacZ* expression levels from this system are 10-fold higher than with the *MET3*-based promoter system (a system that is induced by the removal of methionine), and 70 % of that achieved by the *GAL1*-based promoter system (Belli *et al.*, 1998; Gari *et al.*, 1997). Controlling gene expression by the addition (or removal) of tetracycline has another advantage over the *GAL*- and *MET3*-based systems. The *GAL*- and *MET3*-based systems require a nutrient change to confer regulation, either a switch from glucose to galactose, or the removal of Met, and this has pleiotropic effects on yeast metabolism. Thus, it is hard to confirm which phenotypes are due to the regulated gene, and which are the consequence of the nutrient change. In contrast, doxycycline has no effect on the growth rate, cell morphology or global gene expression of *S. cerevisiae*, so any phenotypic change will be the result of the regulated gene (Wishart *et al.*, 2005). Finally, the tetracycline-regulated activator-repressor dual system enables rapid gene induction without the requirement for a change of medium. It has been demonstrated that this system can induce detectable levels of protein expression by 30 min after antibiotic addition (Belli *et al.*, 1998).

The ability of the ADPRT domain of ExoS to disrupt the actin cytoskeleton in *S. cerevisiae* parallels what is observed in mammalian cell culture systems, where the ADPRT domain has been shown to cause cell morphology changes in both epithelial and macrophage cell lines (Fraylick *et al.*, 2001; Rocha *et al.*, 2005). Although it is not clear how the ADPRT domain of ExoS leads to the observed disruption of the actin structures in *S. cerevisiae*, a clue to its possible

Fig. 7. Effect of ExoS on the actin cytoskeleton of *S. cerevisiae* after α -factor arrest. BMA64-1A containing pCM242 and the pCM252 empty vector, or pCM252 encoding ExoS, was arrested at G1 with α -factor, induced with doxycycline ($2 \mu\text{g ml}^{-1}$), and released from α -factor arrest. At the various time points indicated, the cells were fixed, and fluorescent staining of the actin cytoskeleton with phalloidin was carried out. Images are representative of three separate experiments; bar, $1 \mu\text{m}$.

mechanism of action comes from the yeast V159N actin mutant (Belmont & Drubin, 1998). When actin with a Val 159 to Asn mutation is expressed in *S. cerevisiae* as the sole source of actin, the resulting phenotype is very similar to that which we observed after expression of the ADPRT domain of ExoS. The V159N actin mutation results in these phenotypes because it forms actin filaments that are exceptionally stable due to their slow depolymerization (Belmont & Drubin, 1998). ATP-bound actin monomers polymerize

Fig. 8. Effect of ExoS on the number of mating projections. The percentage of BMA64-1A(pCM242) containing pCM252 (light grey bars), or pCM252-ExoS (dark grey bars) cells with 0, 1, 2 or 3 mating projections, after 3 h α -factor arrest, and 1 h ExoS induction. Values are the mean of three fields of view ($\pm$ SD). For each field, at least 50 cells were examined. The differences in the numbers of mating projections between ExoS-expressing cells and control were highly significant ($P < 0.001$, χ^2 test).

to form filamentous actin, the ATP is then hydrolysed, and the release of P_i leads to a conformational change that destabilizes the actin filament, and promotes disassembly (Carrier, 1990). The V159N mutation results in actin that depolymerizes slowly because the filamentous actin fails to undergo a conformation change after P_i release (Belmont *et al.*, 1999). The similarity of the V159N actin and ExoS ADPRT-domain-induced actin disruption suggests that the ADPRT domain of ExoS may also stabilize filamentous actin in some way. This is supported by experiments using latrunculin A as an inhibitor of actin polymerization (Fig. 4). Under these conditions, ExoS dramatically stabilized the actin cortical patches within the yeast, consistent with an ExoS-induced stabilization of actin filaments, as was also found for the *Salmonella* SspA protein when expressed in yeast (Lesser & Miller, 2001).

Our results also showed that ExoS expression led to the formation of more mating projections after α -factor treatment, and the disruption of normal bud formation after α -factor release. Such polarized growth in *S. cerevisiae* is dependent on small GTPases, such as Cdc42 (Ziman *et al.*, 1991). When *MATa* yeast are treated with a high concentration of the α -factor pheromone, they initiate and terminate growth of mating projections with regular periodicity (Bucking-Throm *et al.*, 1973). It has been

demonstrated that the regulators of Cdc42 activity control the initiation of mating projection formation (Bidlingmaier & Snyder, 2004). Mutation of Cdc24, the guanine nucleotide exchange factor (GEF) for Cdc42, results in a longer mating projection initiation period, and mutation of Bem3, which is a GAP for Cdc42, leads to a shorter mating projection period (Bidlingmaier & Snyder, 2004). Therefore, it is apparent that Cdc42 activation by its GEF, Cdc24, initiates mating projection formation, and this initiation is inhibited by the inactivation of Cdc42 by its GAP, Bem3. As detailed above, ExoS has been shown to ADP-ribosylate Rac1 and Cdc42 *in vivo*, and ADP-ribosylation of Rac1 by ExoS activates this Rho GTPase. One could therefore speculate that ExoS might ADP-ribosylate and activate Cdc42 in *S. cerevisiae*, leading to an increase in the initiation of α -factor-induced mating projection formation.

Effects of ExoS on Cdc42 may also provide an explanation for the strange bud growth observed after α -factor release in yeast expressing ExoS. ExoS expression resulted in many *S. cerevisiae* cells with large buds and elongated necks. Although we have been unable to discover in the literature any yeast mutants that have a similar phenotype, it is slightly reminiscent of the filamentous growth that results during cell stress (Pruyne & Bretscher, 2000). During filamentous growth, Cdc42 does not redistribute over the bud surface during G2-M, but remains localized at the tip of the bud, and directs further apical growth. Also, constitutive activation of Cdc42, or loss of its GAPs, Bem3 and Rga1, locks Cdc42 into a polarized distribution, and hyperpolarizes growth (Stevenson *et al.*, 1995; Ziman *et al.*, 1991). Therefore, if ExoS activated Cdc42 by ADP-ribosylation, this may lead to the strange bud-growth phenotypes we observed.

When we released *S. cerevisiae* from α -factor arrest, yeast not expressing an active ADPRT domain of ExoS were able to exit G1, and proceed with DNA synthesis in S phase. However, when ExoS with an active ADPRT domain was expressed in yeast, no DNA synthesis was observed. This finding is consistent with what has been observed in mammalian cells, where ExoS inhibits DNA synthesis, and the ADPRT domain was shown to be responsible for this phenotype (Fraylick *et al.*, 2001; Olson *et al.*, 1999). It is not clear which target(s) of the ExoS ADPRT domain is responsible for this inhibition of DNA synthesis. Ras is integral to signal transduction pathways that affect DNA synthesis, so it may be the target (Takai *et al.*, 2001). GTP-bound Ras binds and activates its effector, Raf. Raf is a protein kinase that induces gene expression through the mitogen-activated protein kinase cascade, and this drives the cell cycle. Therefore, inhibition of Ras by ADP-ribosylation may result in the observed inhibition of DNA synthesis. It has been demonstrated that ADP-ribosylation of Ras by ExoS is not required for the cytotoxicity of this protein (Pederson *et al.*, 2002). Therefore, if inhibition of DNA synthesis is the trigger for ExoS-induced cell death, another protein that is involved in inducing DNA synthesis must be inactivated by ExoS ADP-ribosylation.

What of the role of the GAP domain of ExoS? The experiments reported here show that, unlike the *Yersinia* type III secreted toxin YopE with GAP activity, the ExoS GAP domain produces no discernible effect on yeast actin (Lesser & Miller, 2001). However, the ExoS GAP domain did inhibit yeast growth to a lesser degree than the ADPRT domain, and was expressed (Figs 1 and 4). Thus, although ExoS and YopE have a 54 % amino-acid identity over the N-terminal GAP domain (Frithz-Lindsten *et al.*, 1997), they appear functionally distinct. This is also borne out by studies in mammalian cells. YopE produces disruption of the actin cytoskeleton in a number of cell types (Andor *et al.*, 2001; Von Pawel-Rammingen *et al.*, 2000). The GAP domain of ExoS, however, although producing an expected rounding of cells when introduced by transfection, only gave minimal effects when directly introduced into cells by type III secretion (Fraylick *et al.*, 2001; Pederson & Barbieri, 1998). Thus, although, biochemically, the GAP domain of ExoS has a wide number of targets, when introduced into living cells, its effects seem much more limited. The effects of the GAP domain on yeast growth, although less than the ADPRT domain, clearly show an inhibitory effect (Fig. 1). However, the GAP domain did not prevent DNA synthesis following mating-factor-induced growth arrest (Fig. 6). The mechanism of its growth inhibitory effect remains unclear.

In conclusion, we have developed for the first time a model of ExoS function in the yeast *S. cerevisiae*. ExoS is a powerful inhibitor of yeast growth, largely due to its ADPRT activity, which also produces an unusual change in actin distribution, suggestive of actin stabilization, and alterations in the numbers and development of mating-factor projections. This suggests that activation of small GTPases might play a more important role in ExoS action than previously thought. The model system described here will be very useful in analysing these changes in more detail.

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REFERENCES

- Aktories, K., Schmidt, G. & Just, I. (2000). Rho GTPases as targets of bacterial protein toxins. *Biol Chem* **381**, 421–426.
- Andor, A., Trulzsch, K., Essler, M., Roggenkamp, A., Wiedemann, A., Heesemann, J. & Aepfelbacher, M. (2001). YopE of *Yersinia*, a GAP for Rho GTPases, selectively modulates Rac-dependent actin structures in endothelial cells. *Cell Microbiol* **3**, 301–310.
- Ayscough, K. R., Stryker, J., Pokala, N., Sanders, M., Crews, P. & Drubin, D. G. (1997). High rates of actin filament turnover in budding yeast and roles for actin in establishment and maintenance of cell polarity revealed using the actin inhibitor latrunculin A. *J Cell Biol* **137**, 399–416.

- Barbieri, J. T. (2000). *Pseudomonas aeruginosa* exoenzyme S, a bifunctional type-III secreted cytotoxin. *Int J Med Microbiol* **290**, 381–387.
- Barbieri, J. T. & Sun, J. (2004). *Pseudomonas aeruginosa* ExoS and ExoT. *Rev Physiol Biochem Pharmacol* **152**, 79–92.
- Belli, G., Gari, E., Piedrafito, L., Aldea, M. & Herrero, E. (1998). An activator/repressor dual system allows tight tetracycline-regulated gene expression in budding yeast. *Nucleic Acids Res* **26**, 942–947.
- Belmont, L. D. & Drubin, D. G. (1998). The yeast V159N actin mutant reveals roles for actin dynamics *in vivo*. *J Cell Biol* **142**, 1289–1299.
- Belmont, L. D., Orlova, A., Drubin, D. G. & Egelman, E. H. (1999). A change in actin conformation associated with filament instability after Pi release. *Proc Natl Acad Sci U S A* **96**, 29–34.
- Bidlingmaier, S. & Snyder, M. (2004). Regulation of polarized growth initiation and termination cycles by the polarisome and Cdc42 regulators. *J Cell Biol* **164**, 207–218.
- Bucking-Throm, E., Duntze, W., Hartwell, L. H. & Manney, T. R. (1973). Reversible arrest of haploid yeast cells in the initiation of DNA synthesis by a diffusible sex factor. *Exp Cell Res* **76**, 99–110.
- Carlier, M. F. (1990). Actin polymerization and ATP hydrolysis. *Adv Biophys* **26**, 51–73.
- Chastre, J. & Fagon, J. Y. (2002). Ventilator-associated pneumonia. *Am J Respir Crit Care Med* **165**, 867–903.
- Cornelis, G. R. (2000). Type III secretion: a bacterial device for close combat with cells of their eukaryotic host. *Philos Trans R Soc Lond B Biol Sci* **355**, 681–693.
- Cornelis, G. R. (2002). *Yersinia* type III secretion: send in the effectors. *J Cell Biol* **158**, 401–408.
- Cornelis, G. R. & Van Gijsegem, F. (2000). Assembly and function of type III secretory systems. *Annu Rev Microbiol* **54**, 735–774.
- Finck-Barbancon, V., Goranson, J., Zhu, L., Sawa, T., Wiener-Kronish, J. P., Fleiszig, S. M., Wu, C., Mende-Mueller, L. & Frank, D. W. (1997). ExoU expression by *Pseudomonas aeruginosa* correlates with acute cytotoxicity and epithelial injury. *Mol Microbiol* **25**, 547–557.
- Foury, F. (1997). Human genetic diseases: a cross-talk between man and yeast. *Gene* **195**, 1–10.
- Frank, D. W. (1997). The exoenzyme S regulon of *Pseudomonas aeruginosa*. *Mol Microbiol* **26**, 621–629.
- Fraylick, J. E., La Rocque, J. R., Vincent, T. S. & Olson, J. C. (2001). Independent and coordinate effects of ADP-ribosyltransferase and GTPase-activating activities of exoenzyme S on HT-29 epithelial cell function. *Infect Immun* **69**, 5318–5328.
- Frithz-Lindsten, E., Du, Y., Rosqvist, R. & Forsberg, A. (1997). Intracellular targeting of exoenzyme S of *Pseudomonas aeruginosa* via type III-dependent translocation induces phagocytosis resistance, cytotoxicity and disruption of actin microfilaments. *Mol Microbiol* **25**, 1125–1139.
- Gari, E., Piedrafito, L., Aldea, M. & Herrero, E. (1997). A set of vectors with a tetracycline-regulatable promoter system for modulated gene expression in *Saccharomyces cerevisiae*. *Yeast* **13**, 837–848.
- Garrity-Ryan, L., Shafikhani, S., Balachandran, P. & 8 other authors (2004). The ADP ribosyltransferase domain of *Pseudomonas aeruginosa* ExoT contributes to its biological activities. *Infect Immun* **72**, 546–558.
- Hauser, A. R., Kang, P. J. & Engel, J. N. (1998). PepA, a secreted protein of *Pseudomonas aeruginosa*, is necessary for cytotoxicity and virulence. *Mol Microbiol* **27**, 807–818.
- Henriksson, M. L., Rosqvist, R., Telepnev, M., Wolf-Watz, H. & Hallberg, B. (2000). Ras effector pathway activation by epidermal growth factor is inhibited *in vivo* by exoenzyme S ADP-ribosylation of Ras. *Biochem J* **347**, 217–222.
- Henriksson, M. L., Sundin, C., Jansson, A. L., Forsberg, A., Palmer, R. H. & Hallberg, B. (2002). Exoenzyme S shows selective ADP-ribosylation and GTPase-activating protein (GAP) activities towards small GTPases *in vivo*. *Biochem J* **367**, 617–628.
- Lesser, C. F. & Miller, S. I. (2001). Expression of microbial virulence proteins in *Saccharomyces cerevisiae* models mammalian infection. *Embo J* **20**, 1840–1849.
- Lyczak, J. B., Cannon, C. L. & Pier, G. B. (2002). Lung infections associated with cystic fibrosis. *Clin Microbiol Rev* **15**, 194–222.
- McGuffie, E. M., Frank, D. W., Vincent, T. S. & Olson, J. C. (1998). Modification of Ras in eukaryotic cells by *Pseudomonas aeruginosa* exoenzyme S. *Infect Immun* **66**, 2607–2613.
- Mota, L. J. & Cornelis, G. R. (2005). The bacterial injection kit: type III secretion systems. *Ann Med* **37**, 234–249.
- Olson, J. C., Fraylick, J. E., McGuffie, E. M., Dolan, K. M., Yahr, T. L., Frank, D. W. & Vincent, T. S. (1999). Interruption of multiple cellular processes in HT-29 epithelial cells by *Pseudomonas aeruginosa* exoenzyme S. *Infect Immun* **67**, 2847–2854.
- Pederson, K. J. & Barbieri, J. T. (1998). Intracellular expression of the ADP-ribosyltransferase domain of *Pseudomonas* exoenzyme S is cytotoxic to eukaryotic cells. *Mol Microbiol* **30**, 751–759.
- Pederson, K. J., Krall, R., Riese, M. J. & Barbieri, J. T. (2002). Intracellular localization modulates targeting of ExoS, a type III cytotoxin, to eukaryotic signalling proteins. *Mol Microbiol* **46**, 1381–1390.
- Pruyne, D. & Bretscher, A. (2000). Polarization of cell growth in yeast. I. Establishment and maintenance of polarity states. *J Cell Sci* **113**, 365–375.
- Rabin, S. D. & Hauser, A. R. (2003). *Pseudomonas aeruginosa* ExoU, a toxin transported by the type III secretion system, kills *Saccharomyces cerevisiae*. *Infect Immun* **71**, 4144–4150.
- Richards, M. J., Edwards, J. R., Culver, D. H. & Gaynes, R. P. (1999). Nosocomial infections in medical intensive care units in the United States. National Nosocomial Infections Surveillance System. *Crit Care Med* **27**, 887–892.
- Rocha, C. L., Rucks, E. A., Vincent, D. M. & Olson, J. C. (2005). Examination of the coordinate effects of *Pseudomonas aeruginosa* ExoS on Rac1. *Infect Immun* **73**, 5458–5467.
- Roy-Burman, A., Savel, R. H., Racine, S., Swanson, B. L., Revadigar, N. S., Fujimoto, J., Sawa, T., Frank, D. W. & Wiener-Kronish, J. P. (2001). Type III protein secretion is associated with death in lower respiratory and systemic *Pseudomonas aeruginosa* infections. *J Infect Dis* **183**, 1767–1774.
- Stevenson, B. J., Ferguson, B., De Virgilio, C., Bi, E., Pringle, J. R., Ammerer, G. & Sprague, G. F., Jr (1995). Mutation of RGA1, which encodes a putative GTPase-activating protein for the polarity-establishment protein Cdc42p, activates the pheromone-response pathway in the yeast *Saccharomyces cerevisiae*. *Genes Dev* **9**, 2949–2963.
- Takai, Y., Sasaki, T. & Matozaki, T. (2001). Small GTP-binding proteins. *Physiol Rev* **81**, 153–208.
- Von Pawel-Rammingen, U., Telepnev, M. V., Schmidt, G., Aktories, K., Wolf-Watz, H. & Rosqvist, R. (2000). GAP activity of the *Yersinia* YopE cytotoxin specifically targets the Rho pathway: a mechanism for disruption of actin microfilament structure. *Mol Microbiol* **36**, 737–748.
- Wishart, J. A., Hayes, A., Wardleworth, L., Zhang, N. & Oliver, S. G. (2005). Doxycycline, the drug used to control the tet-regulatable promoter system, has no effect on global gene expression in *Saccharomyces cerevisiae*. *Yeast* **22**, 565–569.
- Yahr, T. L., Goranson, J. & Frank, D. W. (1996). Exoenzyme S of *Pseudomonas aeruginosa* is secreted by a type III pathway. *Mol Microbiol* **22**, 991–1003.

Yahr, T. L., Vallis, A. J., Hancock, M. K., Barbieri, J. T. & Frank, D. W. (1998). ExoY, an adenylate cyclase secreted by the *Pseudomonas aeruginosa* type III system. *Proc Natl Acad Sci U S A* **95**, 13899–13904.

Ziman, M., O'Brien, J. M., Ouellette, L. A., Church, W. R. & Johnson, D. I. (1991). Mutational analysis of *CDC42Sc*, a *Saccharomyces cerevisiae* gene that encodes a putative GTP-binding protein involved in the control of cell polarity. *Mol Cell Biol* **11**, 3537–3544.