VopF, a type III effector protein from a non-O1, non-O139 *Vibrio cholerae* strain, demonstrates toxicity in a *Saccharomyces cerevisiae* model

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Received 25 April 2009
Accepted 22 September 2009

VopF, a type III effector protein, has been identified as a contributory factor to the intestinal colonization of type III secretion system-positive, non-O1, non-O139 *Vibrio cholerae* strains. To gain more insight into the function of VopF, a yeast model was developed. Using this model, it was found that ectopic expression of VopF conferred toxicity in yeast.

INTRODUCTION

Vibrio cholerae, the aetiological agent of the diarrhoeal disease cholera, encompasses more than 200 serogroups, two of which (O1 and O139) are associated with cholera epidemics and pandemics. Studies on the pathogenesis of *V. cholerae* serogroup O1 and O139 have led to the identification of several critical virulence factors such as cholera toxin and the toxin co-regulated pilus. In addition, *V. cholerae* produces a major zinc-dependent metalloprotease known as haemagglutinin/protease. Strains belonging to the serogroups other than O1 and O139 are known collectively as the non-O1, non-O139 serogroup. Unlike their pathogenic counterparts O1 and O139, most non-O1, non-O139 strains are non-pathogenic and are found ubiquitously in the aquatic environment. Nevertheless, some members of this serogroup are capable of causing sporadic cases of moderate to severe gastroenteritis and extraintestinal infections in humans, despite the fact that the genes encoding toxin co-regulated pilus and cholera toxin are absent, thus raising increasing concern in endemic areas. In contrast to O1 and O139 strains, non-O1, non-O139 strains employ a distinct virulence strategy that remains largely undefined. Recently, much work has been carried out in an effort to understand the biology in the context of pathogenesis and quorum sensing-mediated physiological events in these strains of *V. cholerae* (Chen *et al.*, 2007; Dziejman *et al.*, 2005; Joelsson *et al.*, 2006; Raychaudhuri *et al.*, 2006). A comparative genomic microarray analysis has suggested that these strains are quite divergent from O1 and O139 strains. Moreover, sequence data mining has also revealed that the genes encoding the type III secretion system (T3SS) in these strains are related to the T3SS 2 gene cluster of *Vibrio parahaemolyticus* (Dziejman *et al.*, 2005). As non-O1, non-O139 strains are, by and large,

devoid of cholera toxin and toxin co-regulated pilus, it is therefore conceivable that the T3SS could contribute towards the pathogenesis of these strains. In a continuing effort, Mekalanos and co-workers have characterized a T3SS effector molecule, VopF (NT01VC2350, WH2 motif domain protein), which has been posited to play a key role in enhancing the intestinal colonization of non-O1, non-O139 strains (Tam *et al.*, 2007). Functionally, VopF belongs to the group of bacterial virulence factors that interfere with actin homeostasis. It is a protein of 530 aa, containing one formin homology 1 (FH1)-like domain and three WASP homology 2 (WH2) domains. A proline-rich motif bridges the second and third WH2 domains. The possible way in which VopF may promote intestinal colonization could either be by introducing a specific localized cytotoxic effect on intestinal epithelial cells or by altering the actin cytoskeleton of neutrophils to evade the innate immune response (Tam *et al.*, 2007).

Recent work has witnessed the introduction and development of *Saccharomyces cerevisiae* as a model genetic system to identify and characterize the function of virulence factors, especially those translocated by T3SSs (Valdivia, 2004). Indeed, the expression of various virulence determinants from pathogenic bacteria such as *Yersinia* (Nejedlik *et al.*, 2004; Von Pawel-Rammingen *et al.*, 2000; Yoon *et al.*, 2003), *Salmonella* (Lesser & Miller, 2001; Rodríguez-Pachón *et al.*, 2002), *Pseudomonas aeruginosa* (Rabin & Hauser, 2003; Sato *et al.*, 2003), *V. parahaemolyticus* (Trosky *et al.*, 2004), *Legionella* (Shohdy *et al.*, 2005), enteropathogenic *Escherichia coli* (Rodríguez-Escudero *et al.*, 2005), *Campylobacter jejuni* (Hassane *et al.*, 2001) and *Chlamydia pneumoniae* (Huang *et al.*, 2008) has been observed to inhibit the growth of budding yeast *S. cerevisiae* by targeting cellular processes that are conserved between yeast and mammals. Interestingly, such yeast models have also been exploited to screen small-molecule

Abbreviations: FACS, fluorescence-activated cell sorting; PI, propidium iodide; T3SS, type III secretion system.

inhibitors against bacterial virulence factors (Arnoldo *et al.*, 2008; Huang *et al.*, 2008).

In this study, we wanted to evaluate the suitability of budding yeast as a model system to study the function of VopF. Here, we report the effect of systemic heterologous expression of VopF from non-O1, non-O139 *V. cholerae* serogroup O34 in *S. cerevisiae*. Upon expression, VopF conferred growth inhibition coupled with cell-cycle arrest at the G₂/M transition in the budding yeast.

METHODS

Organisms. A clinical isolate of non-O1, non-O139 *V. cholerae* strain SC110 serogroup O34 served as a source for the type III effector molecule

VopF. *E. coli* NovaBlue strain was used for general cloning purposes. Both *E. coli* and *V. cholerae* were propagated at 37 °C in liquid with agitation in Luria broth or on solid 1.5 % L-agar unless stated otherwise. *S. cerevisiae* strain W303-1A (*MATa leu2-3,112 his3-11,15 ade2-1 ura3-1 trp1-1 can1-100 GAL SUC2*) was used in this study (Table 1).

Construction of strains. The *vopF* gene was amplified from the genomic DNA of *V. cholerae* strain SC110 using the primers *KasI* VopF and *XbaI* VopF (Table 2). The fidelity of the amplified product was verified by sequencing (GenBank accession no. EU862280). The product was further cloned into the *KasI/XbaI* sites of pYES260 under the control of the *GAL1* promoter. Positive clones were further confirmed by sequencing and maintained in *E. coli* strain NovaBlue cells. The recombinant construct, designated pSRT, was subsequently transformed into *S. cerevisiae* strain W303-1A (*his3-11 leu2-1 ade2-1 ura3-52 trp1-1*). The resultant strain was designated W303-SRT (*GAL1*–VopF).

Table 1. Strains and plasmids used in this study.

Strain/plasmid	Description	Source
Strains		
<i>S. cerevisiae</i>		
W303-1A	<i>MATa leu2-3,112 his3-11,15 ade2-1 ura3-1 trp1-1 can1-100 GAL SUC2</i>	This study
W303-SR	W303 + pYES260 vector, URA3 selection	This study
W303-MM	W303 + pEG(KG), URA3 selection	This study
W303-SRT	W303 + pSRT, URA3 selection	This study
W303-SRN1	W303 + pSRN1, URA3 selection	This study
W303-SRN2	W303 + pSRN2, URA3 selection	This study
W303-SRN3	W303 + pSRN3, URA3 selection	This study
W303-RC	W303 + pRC, URA3 selection	This study
W303-SRC	W303 + pSRC, URA3 selection	This study
W303-SWF	W303 + pSWF, URA3 selection	This study
W303-SRTmCherry	W303 + pSRTmCherry, URA3 selection	This study
DBY8728	W303α <i>cdc15-2^{ts}</i>	David Botstein, Princeton University, USA
<i>E. coli</i>		
NovaBlue	<i>E. coli</i> K-12 <i>recA endA lacI^q lacY</i>	Novagen
<i>V. cholerae</i>		
SC110	Non-O1, non-O139 strain of <i>V. cholerae</i> serogroup O34	This study (gift from Ranjan Nandy, National Institute of Cholera and Enteric Diseases)
Plasmids		
pYES260	URA3 selection	Maria Molina, Universidad Complutense de Madrid, Spain
pEG/KG	URA3 selection	
pSRT	1.593 kb <i>vopF</i> gene fragment amplified from genomic DNA by PCR with <i>KasI</i> VopF/ <i>XbaI</i> VopF primers and cloned into pYES260, which was digested with the same restriction enzymes	This study
pSRN1	pSRT + <i>vopFΔWH2</i> dm2, dm3	This study
pSRN2	pSRT + <i>vopFΔWH2</i> dm1, dm3	This study
pSRN3	pSRT + <i>vopFΔWH2</i> dm1, dm2	This study
pRC	pSRT + <i>vopFΔFH1</i>	This study
pSRC	pSRT + <i>vopFΔ3WH2</i> dm1, dm2, dm3	This study
pSWF	pSRT + <i>vopFΔ3WH2ΔFH1</i>	This study
pSRT-mCherry	<i>vopF</i> tagged with <i>mCherry</i> . <i>mCherry</i> and <i>vopF</i> were amplified from the pmCherry-C1 plasmid (Clontech) and pSRT with primer pairs O V mCherry Fwd/ <i>XbaI</i> mCherry V Rev and <i>Bam</i> HI Fwd VopF/O V mCherry Rev, respectively. The products were purified and overlapping PCR was performed with primer pair <i>Bam</i> HI Fwd VopF/ <i>XbaI</i> mCherry V Rev. The final product was cloned into the <i>Bam</i> HI/ <i>XbaI</i> sites of the pEG(KG) vector.	This study

Table 2. Primers used in this study

Primer	Sequence (5'→3')
KasI VopF	CCCCGGCGCCATGTTTAAAATTAGCGTATC
XbaI VopF	CTAGTCTAGACTATTTTGTAGTAAGTGCTG
FH1 Del Fwd	GCTCAATTAAACAAAAAGCCAC
FH1 Del Rev	AATTGAGCCATCTTGTTTTAC
WH2 Domain 1 Del Fwd	CAGCGCAGACTAGCAGAGCAACCCCGAAATC
WH2 Domain 1 Del Rev	GCTAGTCTGCGCTGCAGAC
WH2 Domain 2 Del Fwd	AAAAGCGCATCAAGCACATCAGATATTC
WH2 Domain 2 Del Rev	TGATGCGCTTTTTTCGGTC
WH2 Domain 3 Del Fwd	TGCTAGCAAACCTACTGCGGGTTCGTCAGAAACGGTG
WH2 Domain 3 Del Rev	AGTAGGTTTGCTAGCAAATG
XbaI mCherry V Rev	CTAGTCTAGATTACTTGTACAGCTCGTCCATG
O V mCherry Fwd	GCAGCACTTACTACAAAAATGGTGAGCAAGGGCGAG
O V mCherry Rev	CTCGCCCTTGCTCACCATTTTTGTAGTAAGTGCTGC
BamHI Fwd VopF	CAAGGATCCATGTTTAAAATTAGCGTATC

VopF contains one FH1 and three WH2 domains, which are required for actin nucleation and polymerization activity (Tam *et al.*, 2007). These domains were deleted individually, as well as in combination, using the GeneTailor site-directed mutagenesis system (Invitrogen). The primers were designed according to the instructions provided and are listed in Table 2. The mutant constructs were sequenced in their entirety to confirm the clone and the desired mutations at the corresponding positions. The recombinant constructs, designated pSRN1, pSRN2, pSRN3, pRC, pSRC and pSWF, were subsequently transformed into *S. cerevisiae* strain W303-1A to create *S. cerevisiae* W303-SRN1 (GAL1-VopFAWH2 dm2, dm3), *S. cerevisiae* W303-SRN2 (GAL1-VopFAWH2 dm1, dm3), *S. cerevisiae* W303-SRN3 (GAL1-VopFAWH2 dm1, dm2), *S. cerevisiae* W303-RC (GAL1-VopFAFH1), *S. cerevisiae* W303-SRC (GAL1-VopFA3WH2 dm1, dm2, dm3) and *S. cerevisiae* W303-SWF (GAL1-VopFA3WH2ΔFH1).

Growth assay. The yeast strain W303-1A was grown in YPD [1 % (w/v) yeast extract, 2 % (w/v) peptone, 2 % (w/v) glucose] broth or agar. To evaluate the functionality of VopF, a galactose induction experiment was carried out essentially as described previously (Rodríguez-Escudero *et al.*, 2005). Briefly, the transformed yeast strains W303-SR (harbouring pYES260 alone), W303-SRT, W303-SRN1, W303-SRN2, W303-SRN3, W303-RC, W303-SRC and W303-SWF were grown in synthetic selective medium (SC) containing 0.17 % (w/v) yeast nitrogen base without amino acids, 0.5 % (w/v) ammonium sulphate and 2 % (w/v) glucose, and supplemented with appropriate amino acids and nucleic acid bases. SC^{Gal} and SC^{Raf} were SC media containing 2 % (w/v) galactose or raffinose, respectively, instead of glucose. For induction experiments, W303-SR, W303-SRT, W303-SRN1, W303-SRN2, W303-SRN3, W303-RC, W303-SRC and W303-SWF were grown in SC^{Raf} medium at 30 °C to exponential phase, followed by addition of galactose to a final concentration of 2 % (w/v) and further growth for 6 h. The effect of the expression of VopF and its various derivatives on the growth of *S. cerevisiae* was examined by spotting equal numbers of cells onto SC and SC^{Gal} plates lacking the corresponding auxotrophic markers to maintain the plasmids. Growth was monitored after 2–3 days at 30 °C and photographed.

Measurement of yeast DNA content. It has been documented that ectopic expression of several bacterial virulence factors causes growth inhibition in yeast by cell-cycle arrest (Hassane *et al.*, 2001; Huang *et al.*, 2008; Rodríguez-Escudero *et al.*, 2005). In order to gain further insight into how VopF causes growth inhibition, the yeast DNA content was measured by propidium iodide (PI; Sigma) staining,

essentially as described previously (Hassane *et al.*, 2001). To achieve this, W303-SR, W303-SRT and W303-SWF were sampled at 10 h after induction, followed by fixing and treatment with PI. At least 10 000 events were assessed by fluorescence-activated cell sorting (FACS) for PI fluorescence intensity using a FACScan flow cytometer (Becton Dickinson).

Microscopy. As VopF perturbs the actin dynamics and cell morphology of human epithelial HEp-2 cells (Tam *et al.*, 2007), we wanted to examine the integrity of the actin network of yeast expressing VopF. To this end, recombinant yeast cells were harvested after galactose induction at stipulated time points and fixed in 3.7 % formaldehyde for 1 h at 4 °C as described elsewhere (Nejedlik *et al.*, 2004). After washing, cells were resuspended in PBS supplemented with FITC-labelled phalloidin (Sigma) to visualize the actin and examined under a confocal microscope (Zeiss). For the localization study, VopF was C-terminally tagged with mCherry by an overlapping PCR method. Briefly, *mCherry* and *vopF* were amplified from the pmCherry-C1 plasmid (Clontech) and pSRT (Table 1) with primer pairs O V mCherry Fwd/XbaI mCherry V Rev and BamHI V Fwd/O V mCherry Rev, respectively (Table 2). The products were purified and overlapping PCR was performed with primer pair BamHI Fwd VopF/XbaI mCherry V Rev. The final product was cloned into the BamHI/XbaI sites of the pEG(KG) vector. The resultant recombinant plasmid, pSRT-mCherry, was transformed into W303-1A to create W303-SRTmCherry. In a similar manner, W303-SRTmCherry was harvested after galactose induction, followed by fixing and staining with FITC-labelled phalloidin, and visualized under a confocal microscope.

RESULTS AND DISCUSSION

Expression of VopF in *S. cerevisiae* affects cell growth

The budding yeast *S. cerevisiae* has been exploited as a model genetic system to elaborate the function of virulence factors, especially those translocated by T3SSs (Valdivia, 2004). In the present work, we wanted to evaluate the suitability of *S. cerevisiae* as a genetic system to study the function of VopF. To investigate whether VopF possesses toxic activity, we expressed this protein ectopically under the control of the GAL1 promoter in an *S. cerevisiae* strain

W303-1A genetic background. Yeast cells transformed with pSRT (harbouring GAL1–VopF) grew normally in glucose, where the promoter is repressed. Upon switching to medium containing galactose, a strong inhibition of growth was observed compared with the strain transformed with vector alone (Fig. 1a).

VopF-induced toxicity depends on the FH1 and WH2 domains

It has been shown that VopF contains one FH1 domain and three WH2 domains, which are required for actin nucleation and polymerization activity (Tam *et al.*, 2007). To ascertain whether actin polymerization activity of VopF is essential to exert growth inhibition in yeast, the respective domains were deleted individually, as well as in combination, and the resulting constructs were transformed into *S. cerevisiae* strain W303-1A to create W303-SRN1 (GAL1–VopFΔWH2 dm2, dm3), W303-SRN2 (GAL1–VopFΔWH2 dm1, dm3), W303-SRN3 (GAL1–VopFΔWH2 dm1, dm2), W303-RC (GAL1–VopFΔFH1), W303-SRC (GAL1–VopFΔ3WH2 dm1, dm2, dm3) and W303-SWF (GAL1–VopFΔ3WH2 dm1, dm2, dm3 ΔFH1).

These recombinant strains were further subjected to growth assays in galactose-containing medium. Our data clearly revealed that the extent of growth inhibition exerted by VopF was dependent on the actin-binding domains, whereby W303-SRT (GAL1–VopF) exhibited maximum inhibition, followed by W303-RC (GAL1–VopFΔFH1) and W303-SRC (GAL1–VopFΔ3WH2) (Fig. 1a). There was no inhibition of growth of W303-SWF (GAL1–VopFΔ3WH2ΔFH1) compared with the vector control, which clearly suggested that actin polymerization activity of VopF is required for growth inhibition in *S. cerevisiae* (Fig. 1a). These data also suggested that the cumulative action of the three WH2 domains on the growth of budding yeast is stronger than that of the FH1 domain alone. To determine the contribution of individual WH2 domains, a series of VopF variant molecules were constructed by deleting double WH2 domains in wild-type VopF (Table 1). Subsequently, these variant VopF constructs harbouring FH1 and any one of the three WH2 domains were transformed into the budding yeast to test their growth inhibitory capacity. Notably, the growth assay with recombinant strains transformed with VopF variants containing single WH2 domains, namely W303-SRN1,

Fig. 1. (a) Serial dilution assay to study the effect of VopF on the growth of the yeast cells. The wild-type strain was transformed independently with regulatable expression constructs for full-length VopF (GAL1–VopF), as well as with different domains deleted independently and in combination under the control of GAL1 promoters so that expression could be induced by the addition of galactose to the medium. Cells transformed with vector alone were used as a negative control. The indicated strains were grown overnight in non-inducing selective synthetic medium (SC) containing 2 % raffinose as the carbon source. Serial dilutions of 10^4 , 10^3 , 10^2 and 10 cells per drop (left to right) were spotted onto SC (–Ura) (left panel, repressed by glucose) and SC^{Gal} (–Ura) (right panel, induced by galactose). The data shown are representative of three experiments. (b) Alignment of the WH2 domains of *V. cholerae* SC110 VopF (GenBank accession no. EU862280) and AM-19226 VopF (GenBank accession no. DQ124262). The consensus sequence is shown below the alignment. Asterisks indicate the natural mutations in SC110 VopF compared with AM-19226 VopF.

W303-SRN2 and W303-SRN3, revealed an intriguing result whereby W303-SRN3 exhibited less growth than W303-SRN1 and W303-SRN2, thus indicating that domain 3 exerts a stronger inhibitory effect than domains 1 and 2 (Fig. 1a).

Expression of VopF results in an accumulation of G₂-arrested cells

To understand whether VopF-induced growth inhibition was accompanied by a concomitant arrest of the cell cycle, a flow cytometric analysis was conducted to determine the DNA content of cells expressing GAL1-VopF and GAL1-VopFΔ3WH2ΔFH1 relative to the vector control. As shown in Fig. 2, cells containing vector alone (W303-SR) as well as cells expressing mutant VopF (W303-SWF) showed a balanced proportion of G₁ and G₂/M cells. In contrast, the strain expressing wild-type VopF (W303-SRT) exhibited an accumulation of G₂/M cells, thus suggesting a specific cell-cycle arrest.

Expression of VopF alters actin distribution

As VopF has been demonstrated to induce actin-rich protrusion formation by interfering with actin dynamics in tissue culture cells (Tam *et al.*, 2007), we wanted to determine whether expression of VopF altered the distribution of actin in the budding yeast *S. cerevisiae*. In yeast cells in which wild-type VopF was expressed from the GAL1 promoter (W303-SRT), actin showed a diffuse pattern with no cortical patches or cables visible, which was in sharp contrast to the organized actin pattern with distinct cortical patches and cables seen in yeast cells transformed with vector alone (W303-SR) (Fig. 3). These data indicated that the normal distribution of actin was affected by wild-type VopF.

Localization of VopF in yeast cells

To determine its localization, VopF was C-terminally tagged with mCherry. The resulting construct, pSRT-mCherry, was transformed to generate the recombinant yeast strain W303-SRTmCherry (GAL1-VopFmCherry). Previously, it has been documented that fusion of a fluorescent tag at the N- or C-terminus sometimes results in a non-functional derivative (Sturgill *et al.*, 2008). To evaluate the functionality of the VopF-mCherry fusion construct, the recombinant strain W303-SRTmCherry was subjected to a growth assay in a similar manner to that adopted for untagged strains such as W303-SRT. Upon induction, VopF-mCherry conferred growth inhibition in W303-SRTmCherry (Fig. 4a), thus indicating that the fusion of mCherry at the C terminus of VopF did not alter its activity. Next, we examined the cellular localization of VopF-mCherry. To achieve this, W303-SRTmCherry cells were harvested at 1 h time points following the addition of galactose and stained with FITC-labelled phalloidin to visualize actin. Our results revealed that mCherry-tagged VopF was dispersed throughout the cytoplasm and was also localized at the cell periphery (Fig. 4b). Further staining with FITC-labelled phalloidin suggested co-localization of VopF with cellular actin.

In conclusion, expression of VopF caused growth inhibition by arresting the cell cycle at the G₂/M phase in the budding yeast *S. cerevisiae*. Furthermore, we demonstrated that the VopF-mediated inhibitory growth phenomenon in yeast is likely to be dependent on the domains that are required for actin nucleation and polymerization activity. Interestingly, our domain-deletion analysis suggested that the VopF variant comprising the FH1 domain in combination with only domain 3 of WH2, and not domains 1 and 2, was more efficient at restricting the growth of budding yeast. One possible reason could be the

Fig. 2. FACS analysis of PI-stained yeast cells transformed independently with vector alone (W303-SR) or with the GAL1-VopF (W303-SRT) or GAL1-VopFΔ3WH2ΔFH1 (W303-SWF) regulatable expression construct. Expression of wild-type VopF led to G₂/M arrest in yeast cells. (a) Yeast cells transformed with vector pYES260 alone showed a mixed population of cells having both N and 2N DNA content. (b) Cells with expressed VopF showed a population predominantly with 2N DNA content, a hallmark of G₂/M arrest. (c) Cells transformed with the GAL1-VopFΔ3WH2ΔFH1 construct effectively rescued the G₂/M arrested phenotype, as apparent from the two peaks corresponding to N and 2N DNA content.

Fig. 3. Localization of actin in yeast expressing VopF. *S. cerevisiae* strains W303-SR (with empty vector pYES260) and W303-SRT (with *vopF* construct pSRT) were grown in selective medium containing 2% galactose to induce *vopF* gene expression. Aliquots were removed after 0 and 10 h of galactose induction and fixed with 3.7% formaldehyde for 2 h. FITC-labelled phalloidin was added to the cultures to visualize actin.

sequence diversity within each WH2 domain of VopF. The critical amino acids that are important for actin binding to WH2 domains have been determined previously (Quinlan *et al.*, 2005). Based on this knowledge, a consensus sequence has been generated (Liverman *et al.*, 2007). CLUSTAL W alignment of all three WH2 domains of VopF and the consensus sequence identified maximum homology with domain 3, rather than domains 1 and 2 (Liverman *et al.*, 2007; Fig. 1b). In addition to conserved residues, there is a varying number of non-conserved residues in each domain. As there remains a diversity of amino acid sequence in each WH2 domain, it would be interesting to ascertain the contribution of these amino acids in the functioning of VopF. This warrants further investigation.

Recent studies have produced a rich harvest of data that reveal how various pathogens have evolved with a myriad of virulence determinants containing eukaryotic actin-binding domains that in turn help them to usurp the host actin cytoskeleton machinery. Harnessing actin cytoskeleton dynamics promotes adherence, invasion and intracellular locomotion of pathogens. Virulence factors such as VopL from *V. parahaemolyticus* (Liverman *et al.*, 2007), TARP from *Chlamydia* (Jewett *et al.*, 2006), SipC from *Salmonella* (Hayward & Koronakis, 2002), Map from pathogenic *E. coli* (Dean *et al.*, 2005) and IpgB1, IpgB2 and IpaC from *Shigella* (Tran Van Nhieu *et al.*, 1999) have been shown to affect cytoskeletal organization either by

mimicking the signalling function of small G proteins or by modulating actin nucleation and the filamentation process. Functionally, VopF belongs to the category containing VopL, TARP, SipC and IpaC, which directly nucleate or modulate actin filaments. Our deletion analysis suggests that both WH2 and FH1 domains contributing to actin nucleation and polymerization are required to exert growth arrest in yeast. Further cell-cycle analysis revealed a G₂/M arrest that resulted in loss of viability. It has been documented that growth inhibition by certain virulence factors such as CopN, CDT and SigD^{R468A} is associated with G₂/M arrest in budding yeast. Interestingly, cell-cycle arrest in CopN- and CDT-expressing cells is mediated by a spindle assembly checkpoint and DNA damage checkpoint, respectively, whereas SigD^{R468A} causes cell-cycle arrest through a morphogenesis checkpoint.

It is now known that actin perturbation can trigger a morphogenesis checkpoint response in budding yeast (McMillan *et al.*, 1998). It seems likely that the growth inhibition in VopF-expressing yeast cells may be mediated through this checkpoint. In light of the present work, we surmise that this yeast model will be useful for analysis of VopF. This model will be a valuable tool to expedite the identification of the intracellular target of VopF and also useful to screen small-molecule inhibitors against this effector molecule. Additional studies are necessary to address these issues.

Fig. 4. (a) Serial dilution assay to study the functionality of the VopF-mCherry construct (pSRT-mCherry) on the growth of yeast cells. The wild-type strain was transformed with regulatable expression constructs for full-length *vopF-mCherry* (GAL1-VopFmCherry). Cells transformed with the vector pEG(KG) alone were used as a negative control. The indicated strains were grown overnight in non-inducing selective synthetic medium containing 2% raffinose as the carbon source. (a) Serial dilutions of 10^4 , 10^3 , 10^2 and 10 cells per drop (left to right) were spotted onto SC (-Ura) (left panel, repressed by glucose) and SC^{Gal} (-Ura) (right panel, induced by galactose). The data shown are representative of three experiments. (b) Co-localization of actin and VopF in yeast cells. C-terminally *mCherry*-tagged *vopF* was inducibly expressed in selective medium containing 2% galactose from the GAL1 promoter in *S. cerevisiae* cells. Cells were harvested after 1 h of induction and fixed with 3.7% formaldehyde for 2 h. FITC-labelled phalloidin was added to the cultures to visualize actin. The results showed that VopF co-localized with the actin patches (arrows), as well as being present in a diffuse fashion. RFP, Red fluorescent protein.

ACKNOWLEDGEMENTS

We are very much indebted to Professor David Botstein, Princeton University, for *S. cerevisiae* strain DBY8728, which was used as a control strain in the FACS assay. We are also grateful to Professor Maria Molina, Universidad Complutense de Madrid, Spain, for plasmid pEG(KG). We thank Vibhu Jain and Mitesh Dongre for helping us to compile the sequencing data and Ranjan Nandy for *V. cholerae* strain SC110. A token of appreciation goes to Deepak Bhatt and Shweta Jain for confocal imaging and FACS data analysis, respectively. This work was supported by grants from the Institute of Microbial Technology, Council of Scientific and Industrial Research (OLP-39 and SIP10). R.T., N.S.S. and S.H. acknowledge, respectively, the Indian Council of Medical Research (ICMR), Department of Biotechnology (DBT) and Council of Scientific and Industrial Research (CSIR), India, for fellowships.

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