Enteropathogenic *Escherichia coli* type III effectors alter cytoskeletal function and signalling in *Saccharomyces cerevisiae*

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Enteropathogenic *Escherichia coli* (EPEC) strains cause attaching/effacing lesions in enterocytes through the development of actin-supported pedestals at the site of bacterial adhesion. Pathogenesis requires a type III secretion system (TTSS), which injects into the host cell the intimin receptor, Tir, as well as other effectors called Esp (Escherichia secreted proteins). The genes encoding TTSS structural components and EspS are found within a pathogenicity island called the locus of enterocyte effacement (LEE). This paper describes the application of *Saccharomyces cerevisiae* as a model to probe the functions of LEE-encoded genes. In a systematic approach, the LEE-encoded translocator and effector proteins were endogenously expressed in yeast and their effects on cell growth, cytoskeletal function and signalling pathways were studied. EspD, EspG and Map inhibited growth by depolarizing the actin cortical cytoskeleton, whereas EspF expression altered the septin cytoskeleton. Specific yeast MAP kinase pathways were activated by EspF, EspG, EspH and Map. The yeast system was used to define functional domains in Map by expressing truncated versions; it was concluded that the C-terminal region of the protein is necessary for actin disruption and toxicity, but not for mitochondrial localization. The utility of the yeast model for functional analyses of EPEC pathogenesis is discussed.

**INTRODUCTION**

Enteropathogenic *Escherichia coli* (EPEC) causes severe diarrhoea in children in developing countries (Vallance *et al.*, 2002; Clarke *et al.*, 2003). These strains colonize the intestinal mucosa through adhesion to enterocytes, causing lesions known as attaching-and-effacing (A/E). A/E lesions are characterized by destruction of microvilli and the formation of local dynamic actin-based structures, known as 'pedestals', below the area of intimate bacterial adhesion [reviewed by Kaper, (1998); Goosney *et al.*, (2000); Nougayrede *et al.* (2003)]. The proteins responsible for disease are encoded in part by a 35 kb pathogenicity island called the locus of enterocyte effacement (LEE) (McDaniel *et al.*, 1995). LEE function and regulation is conserved among related pathogens such as enterohaemorrhagic *E. coli* (EHEC) and *Citrobacter rodentium* (Deng *et al.*, 2004). The LEE encodes a type III secretion system (TTSS) required for the direct delivery of virulence factors responsible for modifying the host cell, as well as some of these type III effectors and their cognate chaperones. Among the genes included in this pathogenicity island are those encoding the hollow appendage that connects the bacterium to the host cell (EspA) (Kenny *et al.*, 1996; Sekiya *et al.*, 2001), the two translocon components that insert into the host plasma membrane, constituting the 'tip' of the injecting needle [EspB and EspD (Knutton *et al.*, 1998; Wachter *et al.*, 1999)], and at least five translocated effectors [EspF, EspG, EspH, Tir and Map (Goosney *et al.*, 2000; Clarke *et al.*, 2003)]. Tir-mediated intimate adhesion allows translocation of the other bacterial effectors via the TTSS (Crane *et al.*, 2001; Elliott *et al.*, 2001; Kenny *et al.*, 2002; Tu *et al.*, 2003). Through still unclear mechanisms such effectors mediate cytotoxicity, reduction in tight junction permeability, and, ultimately, diarrhoea.

The budding yeast *Saccharomyces cerevisiae* has already been established as a model system to study virulence-related proteins from pathogenic bacteria (reviewed by Valdivia, 2004). Basic signalling modules and cytoskeletal components are conserved, making the yeast model a feasible approach to analyse the effects induced by bacterial virulence.
factors in mammalian cells. Secreted effectors from Yersinia (Pawel-Rammingen et al., 2000; Lesser & Miller, 2001; Skrzypek et al., 2003; Yoon et al., 2003; Nejedlik et al., 2004), Salmonella (Lesser & Miller, 2001; Rodriguez-Pachon et al., 2002), Pseudomonas (Rabin & Hauser, 2003; Sato et al., 2003), Vibrio (Trosky et al., 2004) and Legionella (Shohdy et al., 2005) have been expressed in yeast and found to interfere with cellular functions related to their proposed targets within the host cell, such as Rho and Cdc42 small GTPases, mitogen-activated protein kinase (MAPK) cascades, membrane trafficking and the actin cytoskeleton. Here we report the systematic heterologous expression of all known LEE-encoded EPEC translocator and effector proteins in the yeast system. We show that these bacterial proteins cause differential phenotypic effects on cell growth, cytoskeletal function and signalling pathways. Furthermore, by expressing mutant versions of Map, we provide evidence that expression of bacterial TTSS effectors in yeast can be a useful tool to identify functional domains in these proteins.

**METHODS**

**Bacterial and yeast strains, media and growth conditions.** EPEC genes were amplified from *E. coli* E2348/69 (Levine et al., 1978). The *S. cerevisiae* strain used in all experiments was YPH499 (MATa ade 2-101 trp1-63 leu2-1 ura3-52 his3-200 lys2-801 (P. Hieter). Deletions in *SIC1* and *KSL1* in this background were provided by J. M. Rodriguez-Pachon and H. Martin (Departamento de Microbiología II, Universidad Complutense de Madrid). Strain VCY1 cdc10-11 (Cid et al., 1998) and isogenic wild-type 1784 (MATa leu2-3,112 trp1-1 ura3-52 his3) were used as the appropriate amino acids and nucleic acid bases. SCGal and SCRaf were formed by growing cells in SCRaf medium at 30°C and adjusted to an OD600 of 0.5. Samples were taken every 2 h and OD600

**Table 1. Oligonucleotides**

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*Restriction sites used for cloning are marked in bold.*
fractionation by SDS-PAGE and transfer to nitrocellulose membranes. Rabbit anti-phospho-p42/p44 MAPK (T202/Y204) antibody (New England Biolabs) was used to detect dual phosphorylated Slt2, Kss1 and Fus3 MAPKs. Actin was detected using a mouse anti-actin (clone C4) antibody (MP Biomedicals). GST fusion proteins were detected using a rabbit anti-GST antibody (Santa Cruz Biotechnology). The primary antibodies were detected using a horseradish-peroxidase-conjugated anti-rabbit or anti-mouse antibody with the ECL detection system (Amersham Biosciences).

**RESULTS**

**Microscopy and immunofluorescence.** For fluorescence microscopy on live cells for the localization of GFP, cells from exponentially growing cultures were centrifuged gently, washed once with sterile water and observed. Localization of actin in yeast cells with FITC-conjugated phalloidin (Sigma) was performed as previously described (Jimenez et al., 1998). For chitin staining, cells were treated with Calcofluor white (Fluorescent Brightener 28; Sigma) as described by Pringle (1991). Visualization of mitochondria by *in vivo* DAPI staining (Williamson & Fennell, 1979) was performed by adding DAPI (Sigma) to cells resuspended in PBS at a final concentration of 10 μg ml⁻¹ and incubating for 5 min. For statistics on cell populations, 100–200 cells were counted for each experiment.

Indirect immunofluorescence on yeast cells was performed as previously described (Cid et al., 2001b). Primary antibodies in immunofluorescence experiments were used as follows: rabbit anti-GST antibodies (Santa Cruz Biotechnology) at a 1 : 500 dilution; rat anti-alpha-tubulin (YOL1/34) antibodies (Serotec) at a 1 : 500 dilution; mouse anti-V-ATPase (Molecular Probes) at 20 μg ml⁻¹; mouse anti-myc (Covance Research Products) at a 1 : 250 dilution. As secondary antibodies, Cy3-conjugated goat anti-rabbit IgG (Chemicon International) and Cy5-conjugated goat anti-rabbit IgG (Jackson Immuno-Research) were used at a 1 : 500 dilution; FITC-conjugated goat anti-rat IgG (Sigma) and FITC-conjugated goat anti-mouse (Sigma) were used at a 1 : 200 dilution. For phase-contrast, fluorescence microscopy and indirect immunofluorescence, cells were examined with an Eclipse TE2000U microscope (Nikon). Digital images were acquired with an Orca C4742-95-12ER charge-coupled device camera (Hamamatsu) and Aquacosmos Imaging Systems software.

**Expression of EPEC TTSS EspD, EspF, EspG and Map proteins in *S. cerevisiae* affects cell growth**

We amplified the *espA*, *espB*, *espD*, *espF*, *espG*, *espH*, *map* and *tir* ORFs from the EPEC strain E2348/69 and generated N-terminal fusions to glutathione S-transferase (GST) under the control of the *S. cerevisiae GAL1* galactose-inducible promoter in a 2μ-based vector. Galactose-dependent expression of the heterologous proteins was detected via immunoblotting with anti-GST antibodies (Fig. 1a). All fusion proteins were produced in yeast at detectable levels, although GST-EspH was less abundant in the lysates. Yeast cells transformed with these constructions grew normally in glucose, where the promoter is repressed (Fig. 1b, left panel). When cells were grown in galactose (Fig. 1b, right panel), a strong inhibition of growth was observed for GST-EspG- and GST-Map-expressing cells. A moderate inhibition was also observed for GST-EspD- and GST-EspF-expressing transformants, whereas the expression of GST-EspA, GST-EspB, GST-EspH and GST-Tir did not have any apparent effect on cell growth. Accordingly, in liquid media, both GST-Map and GST-EspG expression strongly inhibited growth, while GST-EspD and GST-EspF increased generation times slightly over the control (data not shown).

**EspB, EspD, EspH and Tir accumulate at discrete subcellular compartments**

We analysed the subcellular localization of the expressed EPEC proteins using immunofluorescence with anti-GST antibodies on fixed yeast cells (Fig. 2). GST-EspA, GST-EspG and GST-Map were randomly distributed in the cytoplasm (data not shown), as observed for GST alone (Fig. 2a). GST-EspF was also generally found throughout the cytoplasm, but it was also found to form amorphous...
patches associated with the bud neck and polarity areas (Fig. 2b). In contrast, GST-Tir, GST-EspB, GST-EspD and GST-EspH showed a definite specific localization. GST-EspH appeared as punctate or rod-shaped structures in the cytoplasm (Fig. 2c). However, in agreement with the low amount of GST-EspH detected in cell extracts by immunoblotting, only 10% of the population showed a detectable fluorescence signal. GST-EspB and GST-Tir accumulated at spherical cytoplasmic compartments with a diameter approximately half that of the nucleus, usually one or two per cell, but occasionally more. This could correspond to a vacuolar localization. To test this possibility, we detected vacuoles in cells expressing GST-Tir and GST-EspB by simultaneous immunofluorescence with anti-GST and anti-vacuolar ATPase antibodies. As shown in Figs 2(d) and (e), neither GST-EspB- nor GST-Tir-containing compartments.

Fig. 2. Subcellular localization of heterologously expressed GST-fused EPEC proteins in S. cerevisiae YPH499 as indicated. Cells were fixed 6 h after induction in galactose medium. In all panels, immunofluorescence with anti-GST antibodies is shown in red and nuclear staining with DAPI is shown in blue. In (d) and (e), immunostaining with anti-vacuolar ATPase antibodies is shown in green. In (f), immunostaining with anti-myc antibodies to detect Sec63-myc as an endoplasmic reticulum marker is shown in green. Representative cells from each experiment were selected. Bars, 5 μm.
co-stained with the areas recognized by the vacuolar marker. GST-EspD was clearly associated with the nuclear membrane, consistent with a possible localization to the endoplasmic reticulum (Fig. 2f). We co-transformed into GST-EspD-expressing cells a plasmid encoding Sec63-myc, a typical endoplasmic reticulum marker (Lyman & Schekman, 1997), and stained with anti-GST and anti-myc antibodies in a co-immunofluorescence experiment. As shown in Fig. 2(f), Sec63-myc and GST-EspD co-localized in the same compartment. Retention at certain stages of the secretory pathway or formation of aggregates could be expected for EspB, EspD and Tir, which are known to contain transmembrane domains. Nevertheless, despite the evolutionary distance between yeast and mammals and the fact that the bacterial proteins are expressed as GST fusions, the distinct localization patterns observed might reflect recognition of potential different targets for these bacterial proteins within the eukaryotic host cell.

EspF, EspG and Map expression alter morphogenesis at different stages

The microscopic studies shown above revealed alterations in yeast morphology induced by GST-EspF, GST-EspG and GST-Map. Calculation of the budding index of the corresponding cultures revealed that expression of both GST-EspF and GST-Map increased the proportion of unbudded cells, whereas yeast expressing GST-EspG accumulated small-budded cells (Fig. 3a). Flow cytometry analyses indicated that these effects were not accompanied by an accumulation of cells in either G1/S or G2/M stages in cells expressing EspF and Map, although an increase in G2/M population was observed in EspG-expressing cells (Fig. 3b). We stained cells with DAPI and anti-tubulin antibodies to visualize nuclei and mitotic spindles respectively. In contrast to control cells expressing GST alone, in GST-EspG-expressing populations we observed that 60% of small-budded cells had mitotic spindles or two unsegregated nuclei (Fig. 3b). In GST-Map-expressing cells we found that 12% of unbudded cells had also entered into

**Fig. 3.** EspF, EspG and Map alter budding. (a) Budding index of cells expressing indicated GST-effector fusions. Cells in the population were included in each of the following categories, as depicted in the pictures on top: unbudded cells (black bars), cells with a bud smaller than half the size of the mother cell (dashed bars) or cells with a bud of a size equivalent to half the mother cell or larger (white bars). Cells were analysed 6 h after induction in galactose medium. Data are the means of three experiments and about 200 cells were counted for each experiment. (b) EspG and Map desynchronize budding and nuclear division. Cells expressing GST, GST-EspG and GST-Map were collected after 6 h of induction, fixed, stained with DAPI to visualize nuclei and treated with anti-tubulin antibodies (anti-Tub) to observe the microtubular apparatus, as indicated. (c–e) Phase-contrast (left) and CW staining (right) of cells expressing GST (c), GST-Map (d) or GST-EspF (e). CW staining shows chitin-rich areas at the bud neck (c, top panels) or at scars from previous budding events (c, bottom panels), chitin enrichment of cell walls and loss of bud scars in GST-Map-expressing cells (d), and a range of morphogenetic alterations in cells expressing GST-EspF (e, arrows). Bars, 5 μm.
mitosis (Fig. 3b). This revealed that mitosis occurred even in the absence of proper bud morphogenesis. Therefore, Map and, especially, EspG expression causes loss of coordination of morphogenetic and nuclear events in yeast cells.

Although expression of either GST-EspF and GST-Map led to accumulation of unbudded cells, differences in morphology were apparent. Cells expressing GST-EspF displayed angular shapes due to the presence of one or several bumps on the cell surface in about 45% of unbudded cells (Fig. 3e, first row), whereas those expressing GST-Map were spherical rather than ellipsoidal (Fig. 3d). We stained cells with calcofluor white (CW), a fluorochrome that binds specifically to chitin-rich areas in the cell wall, namely septa and bud scars, as shown in Fig. 3(c) for control cells expressing GST. More than 95% of cells expressing GST-Map were very intensely stained with CW, suggesting increased chitin biosynthesis (Fig. 3d). Neither buds nor scars from previous budding events were observed in these cells. When cells expressing GST-EspF were stained with CW, as shown in Fig. 3(e), the above-mentioned characteristic surface bumps did not show the crater-like aspect of bud scars of wild-type cells (Fig. 3c, second row). Therefore, we interpreted them as abortive bud sites, suggesting that EspF interferes with some particular aspect of bud emergence. In GST-EspF cells able to bud, we observed elongated or non-constricted necks, as well as erratic chitin accumulation at the shoulders of the mother cell (Fig. 3e, second and third rows, arrows) or at the tip of the bud (Fig. 3e, fourth row, arrows). Thus, EspF also seems to affect morphogenesis at stages beyond bud emergence.

Expression of EspD, EspG and Map in yeast alters cortical actin function

Budding is a morphogenetic programme specific to yeast, but cytoskeletal structures involved in this process are conserved in mammalian cells. To determine whether the observed morphological alterations were related to interference of the EPEC effectors with the yeast actin cytoskeleton, we examined actin distribution on fixed cells stained with fluorochrome-conjugated phalloidin. During bud development, actin patches accumulate at the growing bud in control cells expressing GST. However, cells with mislocalized cortical actin were observed when expressing GST-EspD, GST-EspG or GST-Map (Fig. 4). We have previously reported that EspG disrupted cytoskeletal function in yeast, causing actin depolarization (Hardwidge et al., 2005). A significant proportion of small-budded cells expressing GST-EspD showed loss of actin polarization to the bud (Fig. 4a). This effect is similar to that reported for EspG, although quantitatively less severe (Fig. 4b). The proportion of cells with random cortical actin distribution was higher for GST-Map-expressing transformants, indicating that Map strongly interferes with basic cell polarity mechanisms (Fig. 4a, b).

Map and EspF cause different patterns of septin dysfunction

Proper bud formation in yeast depends on the coordinated function of actin and septin structures (Gladfelter et al., 2001). Septins are conserved structural proteins that in yeast assemble at the presumptive bud site as a ring of filaments and persist as a collar at the bud neck that eventually duplicates during septation and disappears after cytokinesis. Mammalian counterparts have defined roles in diverse cellular processes such as cytokinesis and exocytosis (Kinoshita, 2003). We studied in vivo septin distribution in yeast cells expressing EPEC proteins by using a Cdc10 septin-GFP fusion. Septin rings appeared normal in yeasts expressing GST alone (Fig. 5a) or EPEC proteins, except for GST-Map and GST-EspF. Expression of GST-Map led to the appearance of abnormal septin rings of short diameter, as well as to randomly distributed septin patches that did not conform to ring-like structures (Fig. 5b). In cells expressing...
GST-EspF, a different pattern of septin mislocalization was observed. Single rings assembled in unbudded cells, but they were irregular and discontinuous (Fig. 5c, first row). In cells showing the characteristic bumps, dense septin patches appeared at these sites but never formed proper rings around these structures (Fig. 5c, second row, arrows). Finally, in budding cells, Cdc10-GFP abnormally localized to the tip of growing buds or as thick lateral patches in cells with elongated bud necks (Fig. 5c, arrows in third and fourth rows, respectively). If EspF interferes with proper septin function, as suggested from these experiments, it could be expected that its toxicity would be enhanced in a background in which septin function is impaired. To test this, we expressed GST-EspF in a cdc10-11 background, which has a partial defect in septin function (Cid et al., 1998), as well as in an isogenic wild-type strain as a control.

As observed in Fig. 5(d), growth inhibition by expression of EspF was enhanced in the septin mutant background. In summary, Map seems to interfere with cell polarity establishment mechanisms that involve both actin and septin function, blocking bud site assembly, whereas EspF specifically disturbs septin function, causing a variety of morphogenetic failures.

### EspF, EspG, EspH and Map activate yeast MAPK pathways

TTSS-mediated invasion of enterocytes by EPEC and other enteric pathogens is known to depend on reprogramming of host cell signalling (de Grado et al., 2001). In yeast, as in higher eukaryotes, small GTPases like Cdc42 and Rho1, and phosphorylation cascades that involve mitogen-activated

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**Fig. 5.** Map and EspF interfere with septin ring assembly and function. Cells expressing GST (a), GST-Map (b) and GST-EspF (c) were co-transformed with GFP-Cdc10 and observed by fluorescence microscopy. In (c), phase-contrast images of the same field are shown to correlate septin localization with aberrant cell shape. Cells were observed 6 h after induction in galactose medium. Bars, 5 μm. See text for details of arrowed features. (d) Expression of GST-EspF impairs growth of a septin mutant (cdc10-11) background. Wild-type (1784) and VCY1 strains were transformed with the GST-expressing construct and incubated at 28 °C under conditions optimum for promoter repression (glucose) or induction (galactose), as indicated.
protein kinases (MAPKs), are essential for signal transduction in response to environmental changes [see Gustin et al. (1998) for a review]. Slt2, also known as Mpk1, is the MAPK operating in the cell integrity pathway, which monitors cell wall morphogenesis and actin function, in a Rho1-dependent manner (Kamada et al., 1996; Martin et al., 2000; Harrison et al., 2001). The MAPK Kss1 acts downstream of Cdc42 and is also involved in signal transduction related to morphogenesis and cell integrity (Cook et al., 1997; Rodriguez-Pachon et al., 2002). Activation of the Slt2 and Kss1 pathways can be monitored by using specific antibodies against the dually phosphorylated activation domain of mammalian p42/44 MAPK (Martin et al., 2000).

Expression of GST-EspF, GST-EspG, GST-EspH and GST-Map led to higher levels of Slt2 phosphorylation, compared to cells expressing GST alone (Fig. 6). Interestingly, GST-Map expression also activated the Kss1 MAPK.

**Toxicity of Map requires the integrity of a region located at its C-terminus**

Among all effectors expressed in yeast, Map most dramatically interferes with growth, cytoskeletal function and signalling. Map has been shown to target to host cell mitochondria, and such localization is suspected to account for its toxicity (Kenny & Jepson, 2000). However, as reported above, we were unable to detect a specific subcellular localization for the GST-Map fusion. We hypothesized that N-terminal tagging with GST might mask localization signals within Map. We therefore constructed a version of Map tagged with GFP at its C-terminus. Map-GFP was cloned in a yeast expression vector, again under the control of the galactose-inducible promoter GALI. As shown in Fig. 7(a), Map-GFP localized specifically to mitochondria in yeast cells. In vivo DAPI staining was used to visualize mitochondria in these experiments. Surprisingly, Map-GFP lacked toxicity: its overexpression did not cause growth inhibition, actin disruption or any of the effects reported above for GST-Map (data not shown). Although we cannot rule out that loss of toxicity is due to sequestration in the mitochondria, this result suggests that mitochondrial localization and toxicity in yeast may be separable features.

The fact that fusing GFP at the C-terminus of Map led to an innocuous peptide might indicate that regions of Map essential for toxicity are located in this region. To characterize this further, we developed three truncated versions of GST-Map: one lacking 55 residues, a second one lacking 15 residues and a third one lacking three residues from the C-terminus. Such truncations were intended to disrupt regions that are highly conserved among EPEC Map and orthologous sequences from EHEC and *Citrobacter rodentium*. Deletion of the last three amino acids (Map1–200) would eliminate a putative C-terminal microbodies-targeting signal (Keller et al., 1991), as predicted by PROSITE. These truncated versions were expressed in levels comparable to those of GST-Map in yeast cells (Fig. 7c). As shown in Fig. 7(b), deletion of the last 55 amino acids from the Map sequence (GST-Map1–149) yielded a non-toxic peptide. The same result was obtained by expressing GST-Map1–188, whereas elimination of the last three residues (GST-Map1–200) had no effect on the toxicity of Map, implicating the C-terminal 15 amino acids in growth inhibition. Only Map versions which are toxic for yeast, namely full-length GST-Map1–203 and GST-Map1–200, caused activation of the Kss1 MAPK (Fig. 7c). These data provide evidence that a region important for the function of Map lies close to the C-terminus of the protein and that the putative microbodies targeting signal does not contribute to toxicity.

**DISCUSSION**

**Development of the yeast model to study EPEC pathogenesis**

We report here what we believe to be the first systematic heterologous expression of LEE-encoded EPEC secreted translocator and effector proteins in yeast and the resultant phenotypes, and we discuss their functional implications. With the exception of Tir, the contribution of these proteins to virulence is not well understood. Hence, we believe that functional hints provided by the use of yeast as a simplified host cell model can provide valuable information. The advantages and caveats of yeast as a model for the study of bacterial virulence factors have been recently reviewed (Valdivia, 2004). It is generally accepted that conservation of cytoskeletal components and signalling pathways between yeast and humans facilitates elucidation of protein function. Actin nucleation mechanisms, as well as GTPases and...
MAPK cascades, for instance, are essentially conserved from lower eukaryotes to mammals. For example, we have previously reported that *Salmonella* translocated effectors that modulate either positively or negatively the small GTPase Cdc42 maintain such function in yeast, leading respectively to activation or inactivation of MAPKs Slt2, Kss1 and Fus3 (Rodriguez-Pachon et al., 2002). The ease of genetic screens and mutational analyses is a major advantage of the yeast model. The work presented here opens the possibility of designing such strategies in the future.

Expression of certain translocator proteins (EspD) and, remarkably, TTSS effectors EspF, EspG, EspH and Map led to a variety of effects in yeast, each protein causing a distinct phenotype, ranging from severe to subtle effects on growth, signalling and cytoskeletal rearrangements. We will discuss these results below, but to facilitate interpretation, a comprehensive view of the phenotypic systematic analyses carried out in this work is presented in Table 2, and a graphic overview is provided in Fig. 8.

**EspD alters actin function in yeast**

When expressing in yeast structural components of the TTSS needle, we found that EspA, the constituent of the intercellular filament (Daniell et al., 2001), is innocuous, apparently behaving as a soluble cytoplasmic protein. In contrast, EspB and EspD, which are thought to make up the pore inserted into the host cell plasma membrane at the end of the TTSS needle (Ide et al., 2001), accumulate in different compartments in the yeast cytoplasm. Due to this and to the fact that these proteins do not seem to reach the plasma membrane, it is unlikely that EspB–EspD pores can be assembled in yeast. Accordingly, we failed to detect enhanced toxicity in cells co-expressing both peptides (data not shown). It has been speculated that EspB from EHEC strains can act both as a translocator and as an effector. EHEC EspB binds alpha-catenin (Kodama et al., 2002) and disrupts actin-rich structures when expressed endogenously in HeLa cells (Taylor et al., 1999). When expressed in yeast cells, EPEC EspB did not alter actin function, whereas the other component of the translocon, EspD, interfered with actin polarization. However, this effect did not seem to be strong enough to cause morphogenetic defects or activation of the Slt2 pathway. Although evidence for EspD acting as an effector in mammalian cells has not been provided so far, the results observed in the yeast model leave open the possibility that it could participate in actin cytoskeletal rearrangements. Nevertheless, we cannot discount the possibility that such an effect on actin is secondary to a secretory failure of modulators of actin dynamics due to the apparent accumulation of EspD in the endoplasmic reticulum.

**EspF interferes with yeast morphogenesis and septin ring integrity**

Expression of EspF caused a very peculiar effect in yeast: although actin polarity seemed normal, the accumulation of aborted tiny buds suggests that slow growth of an EspF-expressing cell population is due to an abandonment of pre-selected bud sites. Chitin distribution in the cell wall was abnormal and the MAPK pathway that monitors cell wall integrity was activated. Also, budding cells often had...
unusually long or unconstricted bud necks, consistent with the failure of EspF-expressing cells to assemble proper septin rings. Supporting this view, expression of EspF was more deleterious in cells with a compromised septin function, such as cdc10-11 mutants. Septin filaments regulate bud morphogenesis and cytokinesis. In the earliest stages of budding, once the bud site has been selected, a ring of septins encircles the spot in which actin starts to accumulate to direct polarized secretion for bud emergence (for reviews, see Gladfelter et al., 2001; Faty et al., 2002). It is likely that EspF is able to sequester an important element for bud site assembly, hampering formation of the ring. EspF is a small proline-rich peptide (McNamara & Donnenberg, 1998), so it could interact with proteins containing SH3 domains related to bud development, like Sla1 or Abp1 (Drubin et al., 1990; Holtzman et al., 1993), or to cytokinesis such as Hof1 (Naqvi et al., 2001). In addition, certain mutations in the small GTPase Cdc42, which is responsible for both actin and septin assembly at presumptive bud sites, perturb septins without altering actin function (Gladfelter et al., 2002). Therefore, it cannot be discounted that EspF interferes with Cdc42-related pathways in yeast.

During infection, EspF reportedly disrupts tight junctions in basolateral membranes of epithelial cells (McNamara et al., 2001). One of the properties of tight junctions is to block diffusion of proteins and lipids in the plane of the plasma membrane. The septin ring serves this function in yeast during cell budding, dividing the plasma membrane into two domains, the morphogenetically active daughter cell compartment and the quiescent mother cell (Barral et al., 2000). Therefore, the modulation of septin ring assembly in yeast by EspF is consistent with its proposed role in tight junction disruption in mammalian cells. Two different groups have recently reported that EspF localized to host cell mitochondria, causing initiation of the mitochondrial death pathway (Nougayrede & Donnenberg, 2004; Nagai et al., 2004). Both groups also map an N-terminal mitochondrial localization signal in EspF. We did not detect such localization in yeast, probably because we expressed EspF as an N-terminal GST fusion. The fact that EspF causes cytoskeletal dysfunction in yeast when deprived of its mitochondrial localization suggests that EspF might be multifunctional. In fact, EspF has also recently been found to interact with the intermediate filament component cytokeratin 18 and adaptor 14-3-3 proteins (Batchelor et al., 2004; Viswanathan et al., 2004).

EspG impairs actin function in yeast and uncouples nuclear division from budding

We also expressed in S. cerevisiae two recently reported proteins translocated by EPEC into host cells, EspG and EspH. The function of EspH remains to be clarified, but it might somehow interact with the host actin cytoskeleton (Tu et al., 2003). However, its expression in yeast did not apparently affect actin function or cell growth, although it caused activation of the cell integrity MAPK pathway. EspH was produced in yeast in lower levels than the other EPEC proteins and was retained in a small compartment in the yeast cytoplasm, perhaps precluding its interaction with actin-associated targets at the plasma membrane.

EspG is homologous to the Shigella protein VirA, which interacts with mammalian tubulin and helps trigger host cytoskeletal remodelling prior to invasion (Yoshida et al., 2002). When expressed in yeast, EspG strongly depolarized actin patches without affecting septin assembly, causing an

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**Table 2. Summary of the systematic phenotypic analyses of expression of EPEC LEE genes in yeast**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Growth inhibition</th>
<th>MAPK activation</th>
<th>Cortical Septins</th>
<th>Localization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Slt2</td>
<td>Kss1</td>
<td></td>
</tr>
<tr>
<td>EspA</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Normal</td>
</tr>
<tr>
<td>EspB</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Normal</td>
</tr>
<tr>
<td>EspD</td>
<td>Slight</td>
<td>No</td>
<td>No</td>
<td>Depolarized</td>
</tr>
<tr>
<td>EspF</td>
<td>Slight</td>
<td>Yes</td>
<td>No</td>
<td>Normal</td>
</tr>
<tr>
<td>EspG</td>
<td>Strong</td>
<td>Yes</td>
<td>No</td>
<td>Normal</td>
</tr>
<tr>
<td>EspH</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>Normal</td>
</tr>
<tr>
<td>Map</td>
<td>Strong</td>
<td>Yes</td>
<td>Yes</td>
<td>Depolarized</td>
</tr>
<tr>
<td>Tir</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Normal</td>
</tr>
</tbody>
</table>

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**Fig. 8.** Scheme of the main interactions of endogenously expressed LEE-encoded EPEC proteins with cytoskeletal function and signalling in yeast.
activation of the Slt2 MAPK pathway. We show here that cultures of cells expressing EspG accumulate small-budded cells, and microscopic analysis of their nuclei and microtubular apparatus revealed a loss of coordination between bud development and nuclear division, processes that are tightly synchronized in yeast (for reviews, see Cid et al., 2002; Lew & Burke, 2003). We have recently reported that EspG is able to interact with mammalian tubulin and affects yeast cytoplasmic microtubules (Hardwidge et al., 2005). Another recent report presents evidence that local microtubular destabilization triggers assembly of actin stress fibres via activation of RhoA in mammalian cells (Matsuzawa et al., 2004). The RhoA yeast homologue Rho1 is an upstream component of the Slt2 pathway. Therefore, it is tempting to hypothesize that a similar mechanism occurs in yeast cells expressing EspG, leading to the activation of Slt2.

Map blocks yeast cell polarity and morphogenesis

Map, a small peptide showing slight homology to the Shigella IpgB protein, induces multiple interesting phenotypes when expressed in yeast. Map interference with the yeast actin cytoskeleton is more drastic than that of EspD and EspG. Cells expressing Map lose their normal ellipsoidal shape, turning spherical, substantially alter their cell wall composition, and completely fail to bud. Actin cortical patches are randomly distributed along the surface of Map-expressing cells, whereas septins adopt a patchy pattern, failing to assemble proper rings. These phenotypes are reminiscent of cells that have lost Cdc42 function (Johnson & Pringle, 1990), suggesting that Map might inhibit the function of this GTPase when expressed in yeast. Nevertheless, not all the effects of Map on yeast can be explained by inhibition of Cdc42. First, inability to bud in yeast Cdc42-defective cells leads to isodiametric growth, with cells reaching a large size, whereas Map-expressing cells are round and unbudded, but not over sized. Thus, some other phenomena required for growth must be affected. Second, Map-expressing cells show a very strong activation of the Slt2 MAPK, as in the case of other EPEC effectors, but also of the Kss1 MAPK. Activation of both MAPKs might reflect the existence of an altered cell wall in the Map-expressing cells, as CW staining suggests. However, Kss1 acts downstream of Cdc42 and therefore its phosphorylation could derive from a Map-mediated activation rather than an inhibition of this GTPase. This would be consistent with the proposed role of Map in the formation of filopodia in mammalian cells, a process that requires Cdc42 (Kenny et al., 2002). However, Map is also involved in the inhibition of Tir-dependent actin recruitment for pedestal formation by an as yet unknown mechanism (Kenny et al., 2002), and its overproduction promotes the recently described ability of EPEC to invade cultured cells (Jepson et al., 2003). The fact that Map causes diverse effects in yeast supports the idea that Map is involved in multiple regulatory events.

Also supporting the view that Map is a multifunctional protein, we provide evidence that targeting of Map to host cell mitochondria and its toxicity can be dissected. A C-terminal GFP-tagged version of Map specifically localizes to yeast mitochondria but is not toxic, whereas N-terminal GST-tagged versions of Map do not stain mitochondria but are highly toxic. Moreover, toxicity can be eliminated by deleting the last 15 residues of Map. This deletion disrupts a C-terminal putative α-helix secondary structure that is highly conserved in Map orthologues from related enteropathogenic bacteria. Further studies will help elucidate the molecular interactions in which this region is involved. This mutational analysis outlines the power of yeast genetics as a useful approach for clarifying the role of bacterial effectors in human disease.

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Characterization of translocation pores


MAP kinase pathways in the yeast


