Molecular Microbiology (2006) **60**(1), 51–66

doi:10.1111/j.1365-2958.2006.05074.x
First published online 16 February 2006

Multifunctional analysis of *Chlamydia*-specific genes in a yeast expression system

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Summary

Our understanding of how obligate intracellular pathogens co-opt eukaryotic cellular functions has been limited by their intractability to genetic manipulation and by the abundance of pathogen-specific genes with no known functional homologues. In this report we describe a gene expression system to characterize proteins of unknown function from the obligate intracellular bacterial pathogen *Chlamydia trachomatis*. We have devised a homologous recombination-based cloning strategy to construct an ordered array of *Saccharomyces cerevisiae* strains expressing all *Chlamydia*-specific genes. These strains were screened to identify chlamydial proteins that impaired various yeast cellular functions or that displayed tropism towards eukaryotic organelles. In addition, to identify bacterial factors that are secreted into the host cell, recombinant chlamydial proteins were screened for reactivity towards antisera raised against vacuolar membranes purified from infected mammalian cells. We report the identification of 34 *C. trachomatis* proteins that impact yeast cellular functions or are tropic for a range of eukaryotic organelles including mitochondria, nucleus and cytoplasmic lipid droplets, and a new family of *Chlamydia*-specific proteins that are exported from the parasitopherous vacuole. The versatility of molecular manipulations and protein expression in yeast allows for the rapid construction of comprehensive protein expression arrays to explore the function of pathogen-specific gene products from microorganisms that are difficult to genetically manipulate, grow in culture or too dangerous for routine analysis in the laboratory.

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Introduction

A common theme in the pathogenesis of invasive bacteria is the evolution of sophisticated mechanisms to manipulate the cell biology of their hosts. Pathogens as diverse as *Listeria*, *Salmonella*, *Legionella* and *Mycobacteria*, synthesize proteins and lipids that mimic or modify mammalian factors to influence processes such as cytoskeletal dynamics, endoplasmic reticulum (ER) function and endosomal maturation (reviewed in Hackstadt, 1998; 2000; Salcedo and Holden, 2005).

Among the pathogens best equipped to usurp eukaryotic cellular functions are the obligate intracellular pathogens (Hackstadt, 1998). However, because these organisms are not currently amenable to genetic manipulation, little progress has been made in understanding the molecular basis for their pathogenesis. Nonetheless, the infectious cycle and cell biology of some obligate intracellular pathogens have been described in great detail. For example, *Chlamydia trachomatis*, the causative agent of infectious blindness (trachoma) and the most common sexually transmitted pathogen (Schachter, 1999), displays a biphasic life cycle with an infectious, environmentally stable form, the elementary body (EB) and a vegetative, metabolically active form, the reticular body (RB) (Hackstadt, 1999; Hatch, 1999). The RB form of the parasite multiplies within a membrane bound compartment (termed the 'inclusion') that is largely devoid of known endosomal, lysosomal or Golgi markers (Scidmore *et al.*, 1996; 2003; Fields and Hackstadt, 2002; Carabeo *et al.*, 2003). Although the chlamydial inclusion does not fuse with classical endomembrane compartments, *C. trachomatis* efficiently acquires eukaryotic-derived membrane lipids including sphingolipids and cholesterol (Scidmore *et al.*, 1996; van Ooij *et al.*, 2000; Carabeo *et al.*, 2003). In addition, *C. trachomatis* manipulates the actin cytoskeleton (Carabeo *et al.*, 2002), co-opts microtubule-based motors (Grieshaber *et al.*, 2003), inhibits lysosomal recognition of the inclusion (Fields and Hackstadt, 2002), activates the ERK/MEK signalling pathways (Su *et al.*, 2004), and prevents the onset of programmed cell death (Fan *et al.*, 1998). Remarkably, all of these functions are achieved with a genome that encodes < 900 proteins (Stephens *et al.*, 1998).

Indeed, because of their restricted obligate intracellular niche, the *Chlamydiae* have lost the genetic material other

bacteria require for extracellular growth and instead acquired genes that enhance their intracellular survival and dissemination. Furthermore, a comparison of the genomic sequences of various *Chlamydia*, *Chlamydophila* and an environmental isolate of *Parachlamydia acanthamoeba* indicate that the Chlamydiacea have undergone limited genetic exchange and arrived at a genetic solution to successful intracellular parasitism early during eukaryotic evolution (Stephens *et al*., 1998; Kalman *et al*., 1999; Read *et al*., 2000; 2003; Horn *et al*., 2004; Thomson *et al*., 2005). Because of their small genomes (1–2 Mb) and complex interactions with the host cellular machinery, we hypothesize that the chlamydial genomes encode a high density of virulence-related proteins. As a result, these organisms provide unique research opportunities to identify novel proteins whose structure has been shaped through evolution to interact with eukaryotic proteins and modulate their function.

It has been estimated that >15% of the *C. trachomatis* genome encodes proteins that can be exported across the inclusion membrane (IM) (Bannantine *et al*., 2000; Rockey *et al*., 2002; Subtil *et al*., 2005). Because several of these proteins are conserved among the *Chlamydiae* but have no orthologues in other bacteria, we predict that a significant proportion of these proteins participate in the manipulation of eukaryotic cellular functions. Many of these proteins are likely translocated by a chlamydial type III secretion system (TTSS) (Fields *et al*., 2003). Targets of this TTSS include >25 conserved soluble proteins and a large family of integral membrane proteins of unknown function (~40–50 proteins) that localize to the IM (Inc proteins) (Fields *et al*., 2003; Ho and Starnbach, 2005; Subtil *et al*., 2005). In addition, *Chlamydia* may also secrete a large number of proteins into the host's cytoplasm in a TTSS-independent manner. For example, the protease CPAF (CT858), which is responsible for the degradation of the transcription factor RFX5 and cytokeratins, contains a classical bacterial secretion signal (Zhong *et al*., 2001; Fan *et al*., 2002; Shaw *et al*., 2002; Dong *et al*., 2004a). How proteins like CPAF are translocated from the inclusion lumen to the host cell cytoplasm is unknown.

Even though it is likely that >80 chlamydial proteins reside at the IM or are translocated into the cytoplasm of infected cells, a function has been assigned to very few of these proteins. Because the Chlamydiacea have been parasitizing eukaryotic cells for >700 million years (Horn *et al*., 2004), we reasoned that the cellular targets of several *C. trachomatis* virulence factors would be conserved among all eukaryotes, including the budding yeast *Saccharomyces cerevisiae*. Indeed, *S. cerevisiae* has recently emerged as an attractive system in which to model the function of microbial virulence factors because of the wealth of genetic, biochemical and genomic tools available to characterize the function of bacterial toxins and their corresponding eukaryotic targets (Lesser and Miller, 2001; Valdivia, 2004; Campodonico *et al*., 2005; Rodriguez-Escudero *et al*., 2005; Shohdy *et al*., 2005).

In the following report, we describe a homologous recombination-based cloning system for the rapid construction of a comprehensive *C. trachomatis* protein expression array in *S. cerevisiae*. We have used this protein expression system to (i) identify *C. trachomatis* proteins that induce phenotypes in yeast consistent with the disruption of eukaryotic cellular processes, (ii) monitor the tropism of bacterial proteins for eukaryotic subcellular organelles and (iii) screen for proteins that are translocated into mammalian cells during infection.

Results

Construction of a yeast-based chlamydial protein expression array

The *C. trachomatis* genome encodes 318 *Chlamydia*-specific open reading frames (ORFs) and ORFs with homologues in other bacteria, but for which no function has been ascertained (Stephens *et al*., 1998). We targeted 236 *Chlamydia*-specific or conserved hypothetical ORFs of unknown function for expression in yeast. Integral membrane proteins with cytoplasmic domains shorter than 50 amino acids were excluded from analysis. Included among the *Chlamydia*-specific ORFs are 33 putative membrane proteins (Inc proteins) containing a 40–60 amino acid hydrophobic signature motif (Bannantine *et al*., 2000) that predicts localization to the IM. However, because the lack of sorting signals and appropriate protein folding chaperones can cause ectopically expressed integral membrane proteins to misfold or insert incorrectly into membranes, we expressed only the putative cytoplasmic domains of Inc proteins. In addition, we expressed 34 ORFs with homology to virulence factors in other bacteria or proteins with putative activities that may participate in the modulation of host cellular functions [e.g. eukaryotic-like Ser/Thr kinases, proteases, GTPases and phospholipase D (PLD)-like proteins].

Chlamydial ORFs were cloned into yeast expression vectors by homologous recombination. In brief, oligonucleotide primers (~60–70 ntd.) with a 5′-terminus homologous to the multiple cloning site of the galactose-inducible yeast expression vector pSDY-8 (2μ *URA3* P_{GAL} *-GFP*) and a 3′-terminus specific for the gene of interest were used to amplify the ORF from *C. trachomatis* serovar D by the polymerase chain reaction (PCR) (Fig. 1A). Each PCR product was transformed into yeast with a linearized pSDY-8. The two DNA molecules were efficiently spliced by homologous recombination to generate a replication competent plasmid and an in frame gene fusion between

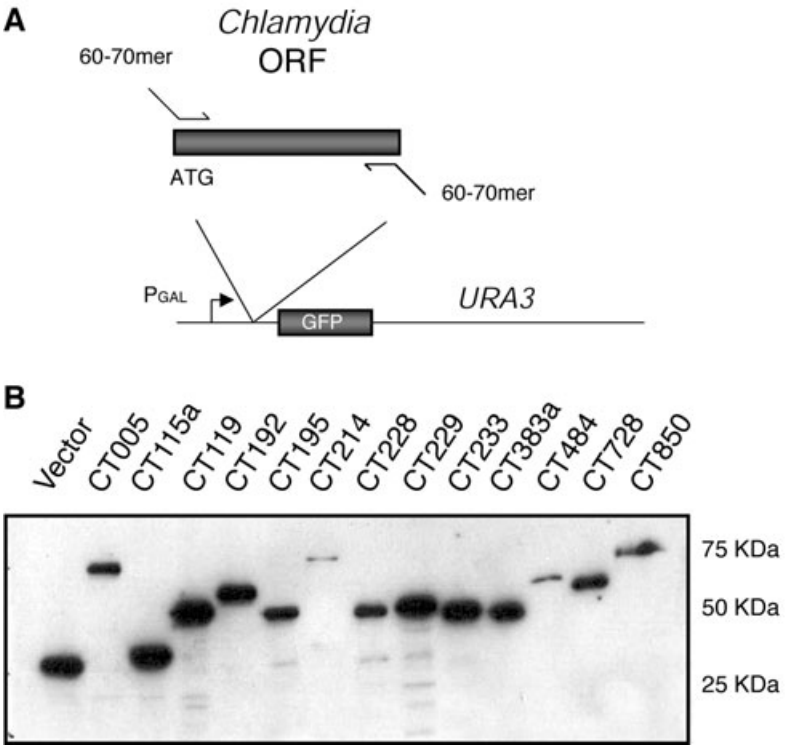

Fig. 1. Construction of *C. trachomatis* expression libraries.
A. Schematic of homologous recombination-based cloning of *C. trachomatis* genes. Bacterial ORFs were amplified by PCR with oligonucleotide primers where the 5′ end is homologous to a cloning site upstream of a GFP gene and a 3′ end specific for the gene of interest. Upon transformation into yeast, the PCR product and the expression vector (pSDY-8) are spliced together by homologous recombination to generate an in frame fusion between the bacterial ORF and GFP. Expression of the recombinant proteins is placed under the control of a galactose-inducible promoter (P_{GAL}).
B. Expression of the cytoplasmic domains of putative IM proteins (Inc) in yeast. The putative cytoplasmic domains of Inc proteins were amplified by PCR and fused to GFP as described above. Expression of recombinant proteins was induced by addition of 2% galactose for 5 h. Total proteins were extracted, resolved by SDS-PAGE and immunoblotted with anti-GFP antibodies. A representative sample is shown.

the *Chlamydia* ORF and green fluorescent protein (GFP). Plasmids were recovered from Ura^+ transformants, sequenced to confirm the correct splicing and re-transformed into yeast. Overall, 235 PCR products representing 216 ORFs and ORF fragments were successfully cloned in yeast (see Table S1 for details).

To induce the expression of *C. trachomatis* proteins, strains were shifted to media supplemented with 2% galactose. Immunoblot analysis of the resulting strains indicated that 168/235 protein fusions were expressed robustly (see Fig. 1B for a representative blot). Fluorescence microscopy analysis indicated that an additional 44 ORFs were expressed at lower levels.

Phenotypic characterization of yeast strains expressing chlamydial proteins

Because *C. trachomatis* can modulate essential cellular processes such as cytoskeletal dynamics and vesicular traffic, we expected that a subset of bacterial proteins would interfere with events as diverse as G-protein function, actin and tubulin dynamics, recruitment of Rab and Rab-associated proteins to target membranes, or tethering of transport vesicles to target membranes. We reasoned that these factors, while not toxic at the levels expressed during infection, would impact the growth of yeast upon overexpression, especially during conditions of cellular stress. Therefore, to identify bacterial proteins that disrupt yeast cellular functions, we tested the effects of chlamydial ORF expression on yeast growth at various temperatures (15°C, 30°C and 37°C) in solid and liquid media or in the presence of cellular stresses (0.8 M NaCl and 0.8 M sorbitol) (Fig. 2A–C). Sensitivity to temperature, osmotic and salt stress can highlight a range of overlapping yet distinct defects in cellular pathways. For example, sensitivity to heat stress (37°C) correlates with impaired Mitogen-activated protein kinase (MAPK) signalling pathways, cytoskeletal function and membrane transport (Hampsey, 1997; Levin, 2005). In yeast, a subset of temperature-sensitive mutants can be rescued by addition of an osmotic stabilizer (e.g. sorbitol). This subclass of mutants is defective either in a protein kinase C (PKC)-dependent MAPK pathway or in ER/early Golgi functions (Verna *et al*., 1997; Levin, 2005). Similarly, osmosensitivity (e.g. NaCl) can be symptomatic of defects in a different MAPK signalling pathway (the HOG pathway), nuclear transport, RNA transcription and translation, and a subset of endosomal functions (Warringer *et al*., 2003). In contrast, cold sensitivity correlates with defects in membrane transport in the late secretory pathway and in the assembly of multiprotein complexes (Hampsey, 1997).

The growth of recombinant yeast strains was quantified by monitoring the reduction of a tetrazolium-based dye in liquid media with an automated plate reader (Biolog Systems) (Fig. 2A) and by plating serial dilutions on galactose-supplemented agar plates (Fig. 2B). Colony formations on solid agar media can sometimes amplify subtle differences in growth kinetics, especially under

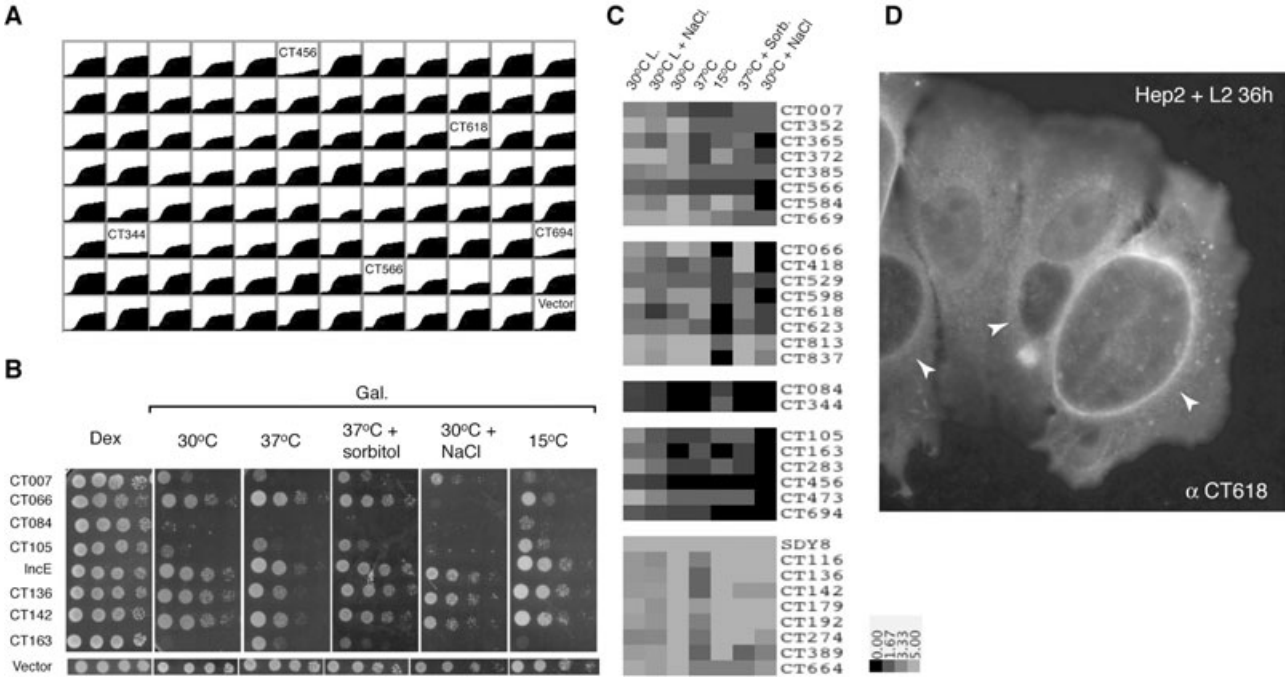

Fig. 2. Phenotypic characterization of yeast strains expressing chlamydial proteins.
A. Growth kinetics of yeast strains expressing *C. trachomatis* proteins. Recombinant yeast strains were maintained in 96 well plates. To induce chlamydial protein expression, cells were diluted 1:100 into 2% galactose-containing liquid media in the presence of a tetrazolium-based redox dye (Biolog Systems). Dye oxidation was measured at 15 min intervals in an automated Omnilog plate reader (Biolog systems) for 72 h to generate kinetic plots of yeast growth and cellular respiration during expression of chlamydial proteins. A representative plate is shown outlining cytotoxic chlamydial proteins.
B. Examples of stress-induced growth defects in yeast strains expressing various *C. trachomatis* proteins. Yeast strains were grown to late log in 2% dextrose and 10-fold dilutions spotted onto galactose agar plates or galactose agar plates supplemented with 0.5 M NaCl or 0.8 M sorbitol and incubated at the shown temperatures.
C. Phenotypic profiles of recombinant yeast strains. The growth defects of recombinant strains were quantified either by intensity of dye reduction in liquid media (A) of by comparing growth on solid agar (B). The relative growth defects were normalized to the growth of yeast strains transformed with the empty expression vector and presented graphically as a grey-scale map (5 = wt growth, 0 = no growth). Full details of these phenotypic profiles are available in Table S1. Yeast strains with similar phenotypic profiles upon expression of *C. trachomatis* proteins were grouped by k-Means clustering algorithms (Cluster 3.0 HGC, University of Tokyo) and displayed as grey-scale maps with Java Treeview. The grouping is shown for clarity of data presentation and does not necessarily imply similarity in function.
D. Immunolocalization of CT618 in infected cells. Antibodies were raised to a CT618-derived peptide and used to localize this protein by immunofluorescence microscopy. CT618 was detected at the IM exclusively in late-staged infected cells (> 36 h). Arrowheads denote inclusions.

stress conditions. The results of all phenotypic assays, expression levels and subcellular localization screens (see below) are available in Table S1.

We identified 32 *C. trachomatis* ORFs and ORF fragments that significantly impacted yeast growth (Fig. 2C). Expression of 13 ORFs in particular led to severe growth defects (Table S1). The cytosolic domains of the IM-associated proteins Cap1 (CT529) (Fling *et al.*, 2001) and the putative Inc protein CT618 caused rapid growth arrest. In addition, six *Chlamydia*-specific ORFs (CT163, CT456, CT566, CT578, CT623 and CT694) and a predicted HKD-PLD-like protein (CT084), severely impacted yeast growth under all conditions tested. In contrast, CT007, CT105, CT283 and the putative haemolysin (CT473) were most cytotoxic in solid agar media. The remaining strains exhibited milder growth defects that were exacerbated at higher temperatures (37°C). However, the temperature-sensitive growth defects of yeast strains expressing IncE/CT116, CT136, CT142, CT179, CT192, CT274 and the predicted GTPase CT418, were suppressed by growth at 37°C in the presence of an osmotic stabilizer (0.8 M sorbitol). In contrast, five ORFs (CT365, CT372, CT385, CT389 and CT669) induced an osmo-sensitive phenotype (NaCl and sorbitol sensitivity), while four ORFs (CT066, CT203, CT584 and CT598) caused extreme salt but not sorbitol sensitivity. Finally, two ORFs (the putative Inc protein CT813 and CT837) only displayed cold-sensitive growth defects.

Of the 35 potential modulators of eukaryotic cellular functions identified by sequence homologies (e.g. eukaryotic-like kinases, phosphatases and phospholipases) the PLD CT084, the putative HIT domain hydrolase CT385, the TTSS-associated ATPase CT669 and the lysophospholipase CT136 caused growth defects in yeast. In addi-

tion, a putative member of the Lon family of proteases (CT344) was extremely toxic to yeast cells.

The expression of the cytoplasmic domain of only one putative Inc protein, CT618, was toxic to yeast cells. Because the hydrophobic patch in CT618 is at the end of the ORF and it deviates from classical bi-lobal hydrophobic motif seen in other Inc proteins, there is no consensus for its classification as an IM protein (Bannantine *et al*., 2000; Shaw *et al*., 2000). We raised antibodies to a synthetic peptide derived from CT618 and determined by immunofluorescence that this protein resides at the IM of infected cells late in the infectious cycle (> 30 h) (Fig. 2D).

The mechanism of growth arrest ranged from cell lysis to the accumulation of large unbudded cells and cytokinetic defects. Because these latter phenotypes are characteristic of cells with impaired cytoskeletal functions (Solomon, 1991), we monitored actin structures by staining formaldehyde-fixed cells with rhodamine-conjugated phalloidin. The yeast actin cytoskeleton consists of actin cables for the delivery of secretory cargo along the mother–bud axis and cortical actin patches, which are the sites of active endocytosis (Engqvist-Goldstein and Drubin, 2003). Most cells expressing toxic chlamydial ORFs that led to severe growth defects had intact cortical actin patches although the normal accumulation of these patches to sites of polarized growth was lost (data not shown). This is most likely a secondary response to general cell stress and thus it is unlikely to represent a direct effect of the bacterial protein on the actin cytoskeleton. However, expression of one chlamydial protein (CT456) led to the collapse of actin cables and cortical patches into an actin-rich patch that colocalized with GFP-tagged CT456 (Fig. 3A). The collapse of actin patches inhibited actin-dependent functions such as endocytosis, as assessed by the uptake of the fluorescent lipophylic tracer FM4-64 (Fig. 3B). Hackstadt and colleagues have recently shown that CT456 (renamed Tarp for translocated actin-rearrangement protein) is translocated into mammalian cells during infection and is sufficient to induce actin polymerization when expressed ectopically in mammalian cells (Clifton *et al*., 2004; 2005).

Phenotypic characterization of Inc protein domains expressed on yeast endosomes

Because Inc proteins are the most attractive candidates as modulators of host cell functions, we were surprised that the expression of relatively few (5/33) cytoplasmic domains of Inc proteins led to any observable phenotype in yeast. We considered the possibility that the activity of these proteins requires the bi-lobal hydrophobic domains. However, our attempts at expressing full-length GFP-tagged versions of Inc proteins (IncA, IncD-F, CT229 and CT288) were unsuccessful. Because we cannot rule out potential non-specific toxic effects associated with the expression of the bi-lobal hydrophobic motif in yeast, we are unable to reach any conclusions regarding the function of full-length Inc proteins.

Despite our inability to express intact Inc proteins in yeast, we reasoned that we could uncover a biochemical function for their cytoplasmic domains, especially in re-routing endosomal traffic, by expressing these protein fragments on the surface of endosomes. We designed an expression system to artificially anchor proteins to the cytoplasmic face of yeast endosomes by using endosomal localization signals present in the endosomal Soluble NSF-sensitive Attachment Receptor (SNARE) protein Pep12p. Pep12p is targeted to endosomes through sorting signals within the transmembrane (TM) and localization (L) domains (Black and Pelham, 2000). Because SNAREs are inserted post-translationally into membranes via their C-termini, these chimeric proteins should not

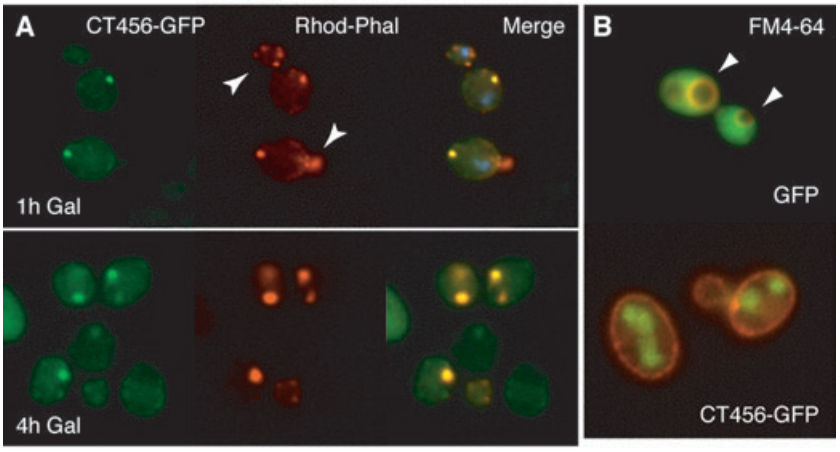

Fig. 3. CT456/Tarp inhibits actin-dependent functions in yeast.
A. Filamentous actin colocalizes with CT456-GFP. Yeast cells were grown in 2% raffinose and CT456-GFP expression was induced by addition of galactose (0.5%). Cells were fixed at 1 h and 4 h post induction and stained for filamentous actin with rhodamine-phalloidin. Arrows indicate cortical actin patches.
B. CT456 expression inhibits endocytosis. Yeast cells expressing GFP (pSDY-8 vector control) and CT456-GFP were induced with 0.5% galactose for 3 h, followed by a 30 min incubation with 10 μM of the lypophilic dye FM4-64 (red) and a 30 min chase with unlabelled media. The membrane impermeable FM4-64 intercalates into the plasma membrane and is transported to the yeast vacuole in cells by endocytosis. In CT456-expressing yeast cells, FM4-64 remained at the plasma membrane.

transit through the ER lumen where they might be subjected to glycosilation and misfolding (Borgese *et al*., 2003). We constructed a galactose-inducible expression vector (pSDY-1) to create Inc protein fusions to the endosome localization signal and transmembrane domains of Pep12p (Black and Pelham, 2000) and a GFP tag (*LEU2* P_{GAL} *GFP-PEP12*$_{L\text{-}TM}$) (Fig. 4A). The cytoplasmic domains of Inc proteins were cloned into pSDY-1 by homologous recombination and the resulting yeast strains were subjected to phenotypic assays similar to those used to characterize soluble *C. trachomatis* ORFs (see Table S2 for details). Expression of Inc proteins on endosomal surfaces significantly increased the toxicity of CT179 and CT192 over that observed for the soluble versions of these proteins. In addition, CT195 was found to severely restrict yeast growth only when expressed as a membrane bound form (Fig. 4B). The phenotypes induced by expression of other Inc proteins were not affected by their attachment to endosomal membranes.

Identification of chlamydial proteins with tropism for eukaryotic organelles

We hypothesized that important clues as to the potential function of chlamydial proteins translocated during infection could be obtained by determining their subcellular tropism for eukaryotic organelles. For example, chlamydial proteins involved in the modification of host membranes or lipid acquisition would contain the targeting information required for the desired localization (e.g. Golgi apparatus, endosomes).

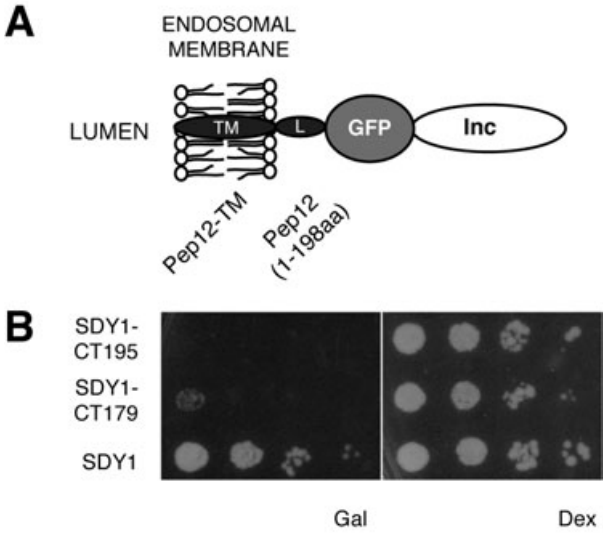

Fig. 4. Phenotypic characterization of yeast strains expressing endosome-anchored Inc proteins.
A. Schematic of endosomal display of the cytosolic domains of Inc proteins. Bacterial proteins were targeted to the cytoplasmic side of yeast endosomal compartments by fusing the soluble domains of Inc protein to the TM and endosomal localization domains of the yeast SNARE Pep12p.
B. Phenotypic analysis of membrane-anchored Inc proteins. The growth phenotypes of yeast strains expressing endosome-anchored Incs were determined as shown in Fig. 2B (see Table S2 for details).

We screened the array of yeast strains to identify GFP-tagged chlamydial proteins whose localization differed from the expected default cytoplasmic distribution (Fig. 5A and B). The majority of strains (70%) showed GFP-tagged ORFs that were either cytosolic, cytoplasmic aggregates or expressed below detection levels. In addition, chlamydial proteins were found in association with the nucleus (3%), the cell surface (4%) and punctate structures (11%). Most chlamydial proteins that impacted yeast cellular functions when expressed as soluble forms (15/32) were either cytoplasmic or formed aggregates. In addition, a significant number of cytotoxic ORFs displayed a punctate localization (11/32) which can be characteristic of a wide range of yeast organelles including the Golgi apparatus, the *trans*-Golgi network, endosomes, mitochondria, peroxisomes and cortical actin patches (Huh *et al*., 2003). To determine which organelles were targeted by these GFP-tagged chlamydial proteins, selected yeast strains expressing red fluorescent protein (RFP)-tagged proteins that mark major intracellular organelles (Huh *et al*., 2003) were transformed with chlamydial expression vectors and the colocalization between GFP and RFP-tagged proteins was assessed. In this manner, we have determined the subcellular localization of four cytotoxic chlamydial proteins that displayed a punctate localization (Fig. 5C–E). The putative PLD CT084 localizes to linear and punctate structures that are coincident with a yeast mitochondrial matrix marker Su9 (Rojo *et al*., 1995) (Fig. 5C), suggesting that CT084 is tropic for mitochondria and that toxicity may be linked to mitochondrial function. CT105 localized to the lumen of deformed yeast endosomes and vacuoles (lysosomes) (Fig. 5E). However, because yeast mutants in vacuolar transport are viable under optimal conditions (Bankaitis *et al*., 1986), it is unlikely that CT105 toxicity is linked to the loss of vacuolar function. CT163 and CT473 (not shown) displayed strong tropism for yeast lipid droplets (Fig. 5D). Because lipid droplets are involved in lipid storage and transport (Zweytick *et al*., 2000), we hypothesize that the toxicity of these proteins is linked to the disruption of lipid homeostasis.

In addition, two cytotoxic ORFs, CT283 and CT694, localized to the plasma membrane, but their expression in yeast did not affect filamentous actin structures (data not shown). However, CT694 expressing cells were specifically impaired in their ability to endocytose FM4-64 (Fig. 5F), suggesting a defect in bulk endocytosis.

Lastly, two chlamydial proteins (CT066 and CT418) localized to the yeast nucleus. Because there are no pre-

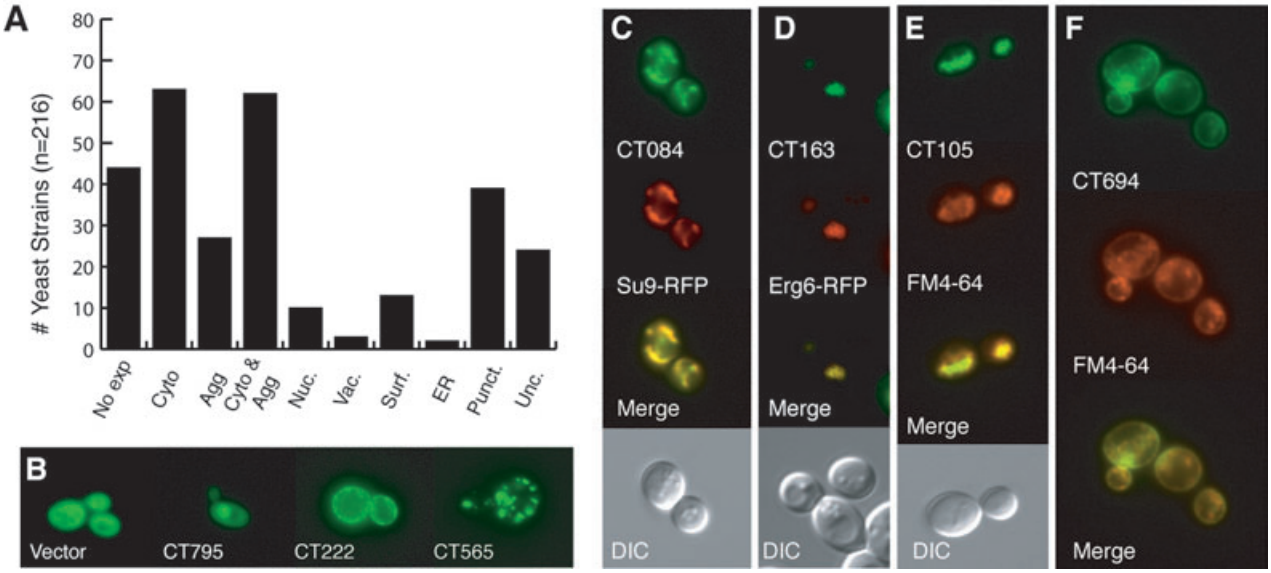

Fig. 5. Subcellular tropism of *C. trachomatis* proteins for eukaryotic organelles.
(A) Association of *C. trachomatis* proteins with distinct subcellular structures. Yeast strains were induced overnight on 1.5% raffinose/0.5% galactose agar plates and imaged live by fluorescence microscopy. Strains were placed in the following categories according to the localization of GFP-tagged proteins: No expression (No exp), cytoplasmic (Cyto), aggregates (Agg), nuclear (Nuc), vacuole (Vac), cell surface (Surf), endoplasmic reticulum (ER), punctate (Punct) and other/unclassified (Unc). In some instances chlamydial proteins exhibited localization to more than one subcellular structure (see Table S1). (B) Examples of nuclear, cell surface and punctate localization patterns in yeast strains expressing various GFP-tagged chlamydial proteins. Association of *C. trachomatis* proteins with distinct subcellular organelles, including a mitochondrial matrix marker (C), the lipid droplet marker Erg6p (D) and the endosomal tracer FM4-64 (E). (F) Impaired endocytic functions in yeast cells expressing the cell surface-tropic ORF CT694. CT694-GFP expression was induced for 4 h by addition of galactose (0.5%). Endocytic structures were monitored by incubation with 10 μM FM4-64 (see Fig. 3B).

vious reports of chlamydial proteins transported to the host cell nucleus, we were surprised that 10 bacterial proteins were nucleotropic (Table S1). To determine if the tropism of these proteins for eukaryotic organelles is conserved in mammalian cells, we expressed eight of the nucleotropic chlamydial proteins in Hep2 cells. In transient transfections, three chlamydial proteins (the carboxyl terminus of CT005, and full-length version of CT066 and the predicted GTPase CT418) accumulated in the nucleus of mammalian cells (Fig. 6A). The remaining chlamydial proteins were either expressed poorly in Hep2 cells (CT398, CT702) or were not tropic for the mammalian nucleus (CT155, CT663, CT845).

Because full-length CT005 is a predicted Inc protein yet the cytoplasmic domain of this protein accumulates in the nucleus when expressed separately, we hypothesize either that CT005 is cleaved during infection to yield a nucleotropic fragment or that CT005 on the IM binds to proteins destined for nuclear transport. Interestingly, when Hep2 cells expressing the cytoplasmic domain of CT005-EGFP were infected with *C. trachomatis*, the soluble CT005-EGFP was recruited to the surface of the inclusion (Fig. 6B). These results suggest that the carboxyl terminus of CT005 binds to a host factor on the IM or to another bacterial IM-associated protein.

Identification of chlamydial proteins secreted during infection

The screens described above are dependent on the expression of a chlamydial protein with intact biochemical activities, organelle-targeting signals and on the functional conservation of its cellular target in yeast. However, the utility of yeast expression arrays can be extended to applications that are independent of the expression of a functional protein. For example, we modified an immunological-based methodology developed by Hackstadt and colleagues to identify IM proteins (Scidmore-Carlson *et al.*, 1999). In brief, Hep2 cells were infected with *C. trachomatis* and IMs were separated from intact bacteria by density gradient ultracentrifugation (Fig. 7A and B). This purified membrane fraction was enriched for known Inc proteins and devoid of Golgi, ER and bacterial cytoplasmic (EF-Tu) and outer membrane protein (OMP-2) (Fig. 7B). We used this membrane fraction to immunize rabbits and the resulting antiserum was used to identify immunoreactive chlamydial proteins. The anti-IM antisera recognized and immunoprecipitated only four proteins from EB lysates. We postulate that these proteins may represent chlamydial factors pre-stored in EBs for secretion into the plasma membrane and early inclusions.

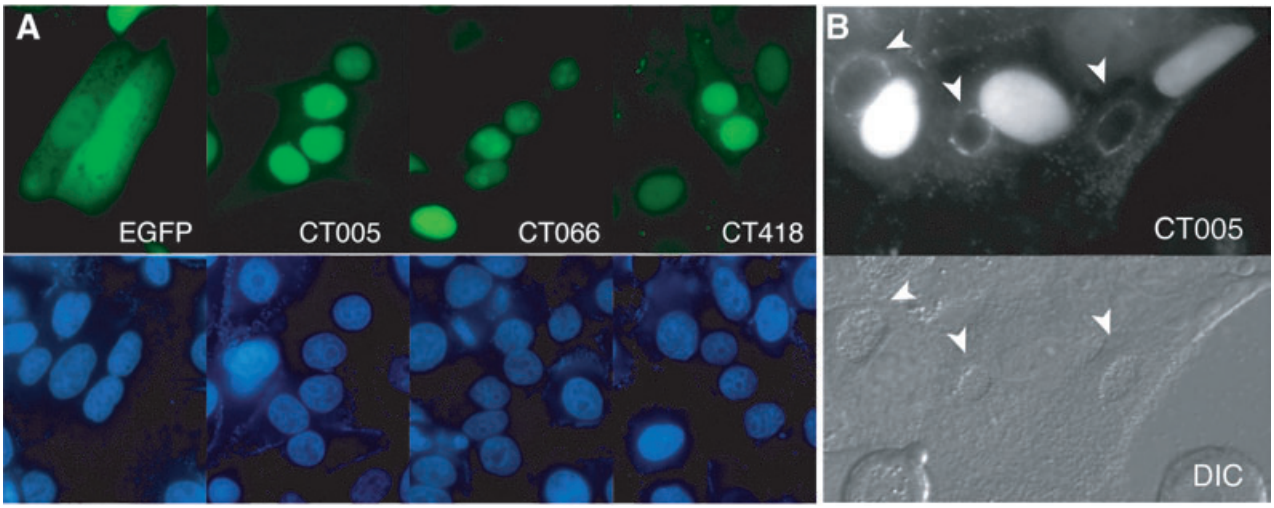

Fig. 6. Nuclear tropism of *C. trachomatis* proteins.
A. A subset of *C. trachomatis* proteins is tropic for the nucleus of mammalian cells. EGFP and EGFP-tagged forms of the cytoplasmic tails of CT005, and full-length CT066 and CT418 were transiently expressed in Hep2 cells, fixed in 4% paraformaldehyde, stained with the DNA dye Hoechst 33258 and imaged by fluorescence microscopy.
B. The cytoplasmic tail of CT005 is recruited to the surface of the *C. trachomatis* inclusion. Hep2 cells transiently transfected with an EGFP-tagged form of the cytoplasmic tail of CT005 were infected with *C. trachomatis* serovar L2 for 20 h. Note recruitment of CT005-EGFP to the surface of inclusions (arrowheads).

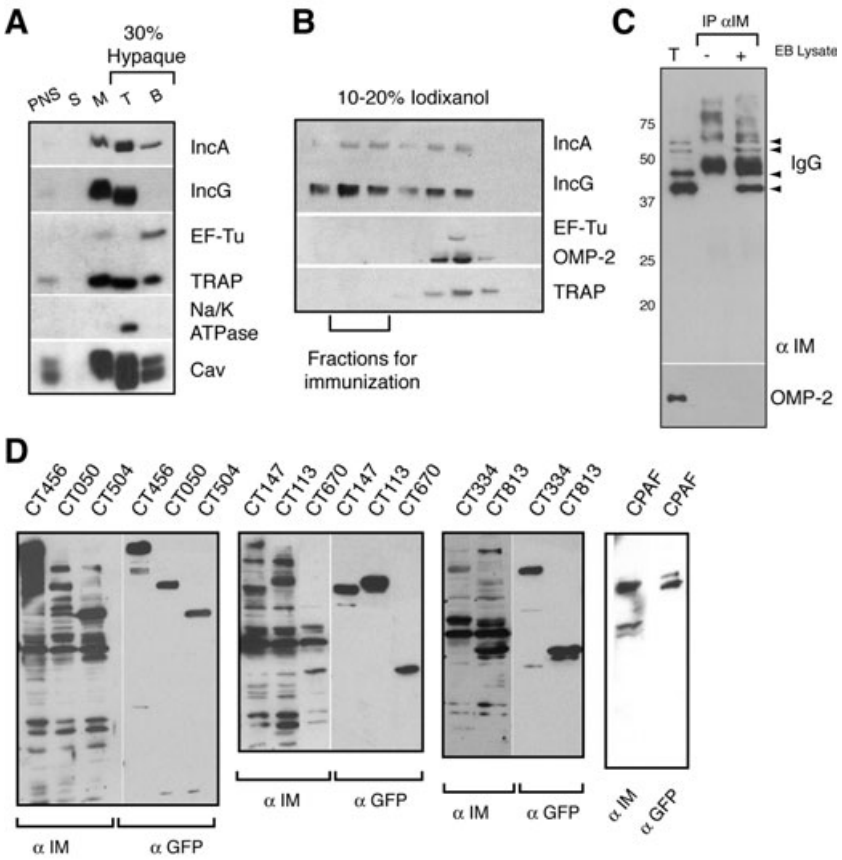

Fig. 7. Identification of *C. trachomatis* proteins exported during infection.
A. Purification of IMs. PNS of *C. trachomatis*-infected Hep2 cells were centrifuged to generate a soluble (S) and a membrane (M) fraction. The M fraction was resuspended in HBSS and centrifuged through a 30% Hypaque cushion to separate IM-enriched fractions (T) from intact bacteria (B).
B. Density gradient purification of IMs. The 'T' fraction was overlaid over a 0–25% Iodixanol step gradient and centrifuged to equilibrium. Fractions containing IM proteins were pooled and used for immunization. Fractionation markers: IM proteins (IncA, IncG), chlamydial cytoplasm (EF-Tu), chlamydial outer membrane (OMP-2), plasma membrane/Golgi (Caveolin – cav), ER/Golgi (TRAP), plasma membrane (Na/K ATPase).
C. Recognition of EB proteins by anti-IM antisera. Solubized EB proteins were incubated with 0.5 ml of crude anti-IM antisera bound to protein A sepharose beads. Immunoprecipitated (IP) material was blotted with anti-IM antisera and anti-OMP-2 antibodies. Anti-IM antisera specifically immunoprecipitated four EB proteins (arrowheads) but not OMP-2 or EF-Tu (not shown). T: 1% total lysate.
D. Representative *C. trachomatis* proteins recognized by anti-IM antibodies. Yeast strains transformed with *C. trachomatis* ORF expression constructs were induced in 2% galactose and total protein samples analysed by immunoblots with anti-Inc membrane antisera. Blots were stripped and re-probed with anti-GFP antibodies.

Neither OMP-2 (Fig. 7C) nor EF-Tu (not shown), proteins that are abundant in EBs and RBs, was immunoprecipitated by the anti-IM antisera, suggesting that antibodies were not generated to non-secreted chlamydial antigens.

When this antisera was used to screen lysates from our recombinant yeast strains, we identified 23 immunoreactive chlamydial proteins (Fig. 7D and Table S1). These proteins can be placed in the following categories.

Inclusion membrane proteins. Three previously described Inc proteins, CT115 (IncD), CT116 (IncE) and CT119 (IncA), were recognized by the anti-IM antisera. Similarly, CT249, CT288 and CT813, proteins with the classical Inc bi-lobal hydrophobic motif (Bannantine *et al*., 2000) were also recognized. It is unclear why the anti-IM antisera did not detect other Inc proteins. It is possible that these proteins are less immunogenic or not expressed to sufficiently high levels during infection to produce high titer-specific antibodies. In addition to Inc proteins, at least two *C. trachomatis* predicted integral membrane proteins, CT147 (Belland *et al*., 2003) and CT529 (Cap1) (Fling *et al*., 2001), localize to the IM despite the lack of a classical bi-lobal hydrophobic motif. The anti-IM antisera detected both CT147 and CT529 expressed in yeast (Fig. 7D and data not shown).

Type III secretion-associated proteins. Two proteins of unknown function, CT668 and CT670, whose genes map to a gene cluster encoding the chlamydial TTSS translocation channel, were strongly recognized by the anti-IM antisera. However, no antibodies were generated to the integral components of the translocation apparatus even though these proteins were represented (six ORFs) in our expression strains, further emphasizing that the material used for immunization was not contaminated with bacterial membrane components.

Surprisingly, Tarp – a target of TTSS – was strongly recognized by the anti-IM antisera (Fig. 7D). Tarp is translocated into infected cells within minutes of bacterial attachment to the mammalian surface but it is unclear if Tarp is translocated after the initial biogenesis of the bacteria-containing vacuole. Because membranes used for immunization were harvested late in the lytic cycle (44 h) and contained plasma membranes, we hypothesize that antibodies were generated Tarp translocated during a second round of chlamydial infection.

Proteases. Three proteins with putative proteolytic functions (CPAF, CT113 and CT344) were detected by the anti-IM antisera. The protease CPAF (CT858) is translocated into the host cell cytoplasm where it degrades transcription factors (RFX5 and USF-1) and microfilaments (Zhong et al., 2001; Dong et al., 2004a). Because, anti-CPAF antibodies were generated in response to immunization with IM, we hypothesize that a fraction of CPAF may associate with the IM and target membrane-associated eukaryotic factors.

CT113 and CT344 are homologous to the ATPase subunits and protease domains of Clp and Lon proteases respectively. Unlike CPAF, CT113 and CT344 lack secretion signals. Given the lack of bacterial cytoplasmic contaminants in our membrane fractions, it is unclear how antibodies were generated against these putative bacterial cytoplasmic proteins. Nonetheless, given the important role the chlamydial protease CPAF plays in modulating eukaryotic cellular functions, it would not be surprising if additional bacterial proteases access the host cytoplasm during infection.

Proteins of unknown function. The remaining *C. trachomatis* proteins that reacted with the anti-IM antisera had no known functional homologues in the available protein sequence databases (Table S1). Surprisingly, only one protein, CT472, had a putative TM domain. The only unifying thread among these proteins is that four ORFs (CT043, CT049, CT050, CT504) encoded highly acidic proteins (pI 4–5). CT049 and CT050 are members of a family of paralogously related non-globular proteins that include CT051 and CT414. The genes encoding these proteins are among the most polymorphic between ocular and genital *C. trachomatis* serovars (Carlson *et al*., 2005). To test whether these proteins are secreted during infection, we raised antibodies to CT050 and localized this protein by immunofluorescence microscopy. CT050 was found at the IM at RB attachment sites (Fig. 8), strongly suggesting that CT050 and its paralogues constitute a new family of IM-associated chlamydial proteins.

Because many of the proteins recognized by anti-IM antisera did not display any obvious phenotypes when expressed in yeast, we speculated that their biochemical function may require an association with membrane surfaces. To address this, we expressed endosome-anchored versions of these immunoreactive chlamydial proteins in yeast and monitored growth defects. Membrane-anchored CT142 and CT632 conferred severe temperature-sensitive growth defects that were not apparent in the empty vector controls (data not shown). Surprisingly, expression of membrane-anchored CPAF, unlike the soluble CPAF, was highly toxic to yeast cells (Table S1). Immunoblot analysis of soluble and membrane-anchored CPAF indicated that this protein was not proteolytically processed (data not shown) as has been observed during infection (Dong *et al*., 2004b). These results suggest that the difference in toxicity between these two versions of CPAF is not the result of preferential activation of CPAF, but rather interference with the function of eukaryotic cellular target(s) that are restricted to membrane surfaces.

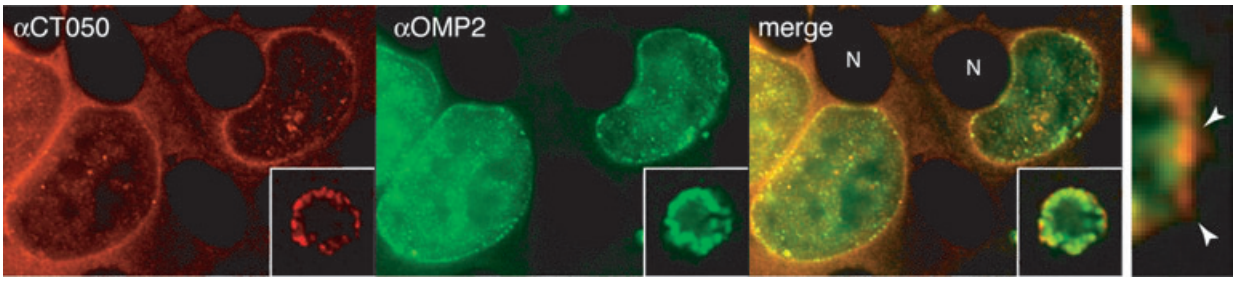

Fig. 8. Immunolocalization of CT050 in infected cells. Hep2 cells were infected with *C. trachomatis* L2, processed for immunofluorescence and labelled with anti-CT050 antibodies (red) and anti-OMP-2 to detect chlamydia (green). Serial images were acquired and deconvolved with Axiovison (v3.1) 4D software. CT050 was present at the IM both in late (32 h) and early (18 h) inclusions (inset). N, host nuclei. Right panel: close-up of RBs (green) attached to the IM. Arrows indicate contact points with localized CT050 secretion.

Discussion

We have generated an ordered array of yeast strains overexpressing chlamydial ORFs to identify proteins that met at least two of the following criteria: modulation of yeast cellular functions, tropism for well-defined eukaryotic subcellular organelles and export from the inclusion. For example, we analysed CT456 (Tarp) because of its cytotoxic properties and recognition by anti-IM antisera. Expression of Tarp in yeast inhibited actin-dependent functions and caused the accumulation of a large actin-rich patch (Fig. 3). During *C. trachomatis* invasion, Tarp is phosphorylated at tyrosine residues upon translocation into mammalian cells (Clifton *et al*., 2004). However, consistent with the lack of a role for tyrosine phosphorylation in Tarp's actin nucleation activity in mammalian cells (Clifton *et al*., 2005), the rearrangement of the actin network in yeast, which lacks homologues of the mammalian tyrosine-specific kinases, was independent of tyrosine phosphorylation. Therefore, by focusing on multiple function-based screens in yeast, we have identified one of the few chlamydial proteins known to interfere with a eukaryotic cellular process and elucidated basic aspects of its function. In the sections below, we outline the features of a selected set of chlamydial proteins that, given the phenotypes they induce in yeast and/or their subcellular localization during infection, are strong candidates as modulators of mammalian cellular functions.

Phenotype-based screens: disruption of eukaryotic cellular function and organelle tropism

Among ORFs with homology to putative virulence factors, only the putative HKD-PLD (CT084) displayed any cytotoxic effects in yeast. CT084 is conserved among the Chlamydiacea, suggesting that it may perform an important function related to intracellular growth. *C. trachomatis* encodes five additional genes encoding putative HKD-PLD proteins. Four of these genes (Ct154, Ct155, Ct157 and Ct158) are clustered in a region of high genetic diversity within the *C. trachomatis* chromosome termed the 'Plasticity Zone' (PZ) (Read *et al*., 2000; Carlson *et al*., 2004). Expression of CT154 and CT155 did not lead to any visible growth defect in yeast although CT155 ectopically expressed in mammalian cells was tropic for mitochondria (data not shown). Given that CT155 and CT084 have tropism for eukaryotic mitochondria, we speculate that these proteins may act as modulators of mitochondrial function.

The PZ also encodes ADP-ribosylating toxins, enzymes for the biosynthesis of tryptophan and the cytotoxic ORF CT163 (Read *et al*., 2000; 2003; Carlson *et al*., 2004). Surprisingly, CT163 was tropic for yeast lipid droplets (Fig. 5D). Lipid droplets are ubiquitous eukaryotic organelles involved in the regulation and storage of neutral lipids. However, recent proteomic analysis in yeast and mammalian cells indicates that these organelles may participate in a range of additional cellular functions including signalling and lipid transport (Martin and Parton, 2005). The finding that an additional cytotoxic chlamydial protein (CT473) was also tropic for lipid droplets (not shown) suggests that these organelles play an important role in chlamydial pathogenesis. Indeed, we have recently found that the *C. trachomatis* actively recruits lipid droplets to the surface of the inclusion and modulates their function (Y. Kumar and R.H. Valdivia, unpublished).

Two chlamydial proteins, CT283 and CT694, appear to inhibit cellular functions at the plasma membrane. However, unlike Tarp, expression of these proteins did not disrupt the morphology of yeast cortical actin patches. Nonetheless, expression of CT694 led to endocytic defects (Fig. 5F). Because various cellular processes in yeast (e.g. sterol homeostasis) are required for proper endocytosis (Heese-Peck *et al*., 2002), it is possible that CT694 may inhibit endocytosis via an actin-independent mechanism.

Inc proteins and other TTSS substrates are the best candidate proteins to act as modulators of eukaryotic cellular functions. In a recent study, Subtil *et al*. (2005) identified a set of non-Inc chlamydial TTSS effector proteins

that can be secreted by *S. flexneri*'s TTSS. Among these proteins, CT203, CT365, Cap1 (CT529), CT566 (secreted in *C. pneumoniae*) and the carboxyl terminus of CT578 (CopB) were identified in our phenotypic screens. Whether CT203, CT365 and CT566 are secreted during infection remains to be determined. Similarly, Tarp, which also induces a strong cytotoxic phenotype in yeast, is secreted in a TTSS-dependent manner (Clifton *et al*., 2004). Interestingly, other highly cytotoxic chlamydial ORFs identified in our screens [Cap1/CT529 (Fling *et al*., 2001), CopB/CT578 (Fields *et al*., 2005), CT618 (Fig. 2D) and CT163 (Y. Kumar and R.H. Valdivia, unpublished)] also localize to the IM, suggesting that a significant proportion of chlamydial proteins with strong phenotypes in yeast may be translocated across the inclusion.

Because expression of the bi-lobal hydrophobic motif of Inc proteins was toxic in yeast, we were not able to assign a putative function to full-length Inc proteins based on yeast phenotypes. To address this problem, we overexpressed the predicted cytosolic domains of 33 putative Inc proteins, with the assumption that these domains would be sufficient to modulate host cellular functions. In these assays, only the cytosolic domains of CT618 and CT813 showed a strong phenotype in yeast. However, because membrane localization can increase the local concentration of proteins, provide access to essential substrates/cofactors and enhance activity by providing a hydrophobic surface, we considered the possibility that the activity of Inc proteins is dependent on proximity to a membrane. To mimic the 'natural' context of Inc proteins, we anchored the soluble domain of Inc proteins to endosomal membranes by fusing them to the TM domain of the yeast endosomal SNAREs. Under these conditions, CT179, CT192 and CT195 displayed strong growth defects (e.g. Fig. 4B), suggesting that tethering the cytoplasmic domains of Inc proteins to endosomal membrane can influence the function of a subset of these proteins. Proximity to a membrane can also significantly influence the activity of other *C. trachomatis* proteins. For example, CPAF expression displays a growth phenotype in yeast only when attached to membranes (Table S2), suggesting that one of its eukaryotic targets is associated with membranes.

The lack of distinct phenotypes in yeast upon expression of most Inc proteins may indicate that these proteins are part of multiprotein complexes, perform functions (e.g. transporters, structural stability of the IM) unrelated to the modulation of host cellular functions or that their targets are not conserved in yeast.

An unexpected finding from subcellular localization screens was that three proteins (CT066, CT418 and the cytosolic domain of CT005) accumulated in yeast and mammalian nuclei (Fig. 6A). Because these nucleotropic proteins lack canonical nuclear localization signal (NLS), they either contain sequences that act as alternative NLS or bind to host proteins with a NLS. For CT005, a putative Inc protein, the latter possibility could provide a mechanism to modulate nuclear functions. Interestingly, when the cytoplasmic domain of CT005 is expressed exogenously in infected HeLa cells, this fragment was recruited to the surface of the IM, especially in mid-early inclusions (< 20 h) (Fig. 6B). These results suggest that CT005 binds to host or bacteria-derived proteins that transiently localize to the IM.

In contrast to CT005, the nucleotropic *C. trachomatis* proteins CT066 and CT418 did not contain any obvious TM regions. CT006 is a small, basic protein (pI 11.4) and CT418 is a putative GTP binding protein of the OBG family of GTPases (Pandit and Srinivasan, 2003). The functional significance of this nuclear tropism remains to be determined. Because HeLa cells have been shown to modulate the transcription of genes involved in transcription, translation, signal transduction and metabolism in response to *C. trachomatis* infection (Lad *et al*., 2005), it is possible that chlamydial proteins may influence gene expression, as has been shown for CPAF-1 (Zhong *et al*., 2001), by interfering with the nuclear cycling and/or activation of eukaryotic transcription factors. Alternatively, as *Chlamydia* possesses multiple, presumably redundant, mechanisms to ensure that programmed cell death is not engaged during bacterial replication (Fan *et al*., 1998; Xiao *et al*., 2004; Dong *et al*., 2005; Tse *et al*., 2005; Ying *et al*., 2005), these nucleotropic proteins may act to protect nuclear factors from pro-apototic signals.

Immunological-based screens: identification of C. trachomatis *secreted proteins*

We generated polyclonal antibodies to partially purified inclusion and plasma membranes from infected cells to identify immunoreactive proteins among our *C. trachomatis* expression array. As expected, antibodies were generated to eight known or predicted IM proteins (Table S1). Interestingly, antibodies were also generated to Tarp. Tarp is pre-stored in infectious EBs and translocated into mammalian cells during *C. trachomatis* invasion (Clifton *et al*., 2004). Although new Tarp transcription begins as early as 8 h after entry (Belland *et al*., 2003), there is no evidence suggesting that newly synthesized Tarp is secreted after inclusion biogenesis. Because Tarp localizes to the plasma membrane during bacterial invasion (Clifton *et al*., 2004), we hypothesize that the anti-IM antisera recognize a pool of plasma membrane-associated Tarp. This suggests that a subset of protein recognized by the anti-IM antisera may represent additional chlamydial effectors proteins translocated during invasion.

Surprisingly, antibodies were generated to 10 proteins (including three proteases) with no obvious TM anchoring motifs. Two of these proteins (CT049 and CT050) are members of a paralogously related family of polymorphic *Chlamydia*-specific proteins that include CT051 and CT414 (Carlson *et al*., 2005). We have confirmed that one of these proteins, CT050, is translocated to the host cytoplasm and localizes to RB attachment sites (Fig. 8). Because the anti-IM antisera preferentially recognized known IM proteins, failed to recognize membrane and soluble components of the TTSS and could not immunoprecipitate chlamydial OMPs (Fig. 7C), it is likely that a significant fraction of the proteins recognized by the anti-IM antisera are exported during infection.

In summary, we have devised a system for the high-throughput cloning of bacterial genes in yeast to assemble comprehensive, ordered protein expression arrays. This expression system provides a versatile platform to explore the function of pathogen-specific proteins by (i) taking advantage of the conservation of basic cellular processes among eukaryotic cells and (ii) rapidly and inexpensively expressing recombinant proteins for biochemical assays or for the identification of immuno-dominant antigens. Based on these functional screens, we have created a 'short list' of chlamydial proteins that are attractive candidates as modulators of eukaryotic cellular functions (Table 1), including functions that may have been previously overlooked in chlamydial biology (e.g. co-option of lipid droplet metabolism). The importance placed on a phenotype in yeast as a criterion for further analysis of putative chlamydial virulence factors is based on the tremendous potential of applying standard yeast genetic and genomic tools to identify affected eukaryotic cellular pathways (reviewed in Valdivia, 2004). Indeed, future work will concentrate on determining the localization and function of chlamydial proteins with the strongest phenotypes in yeast.

Although all eukaryotic cells share a basic cellular architecture, chlamydial virulence factors that target pathways required for intercellular communication, tissue specialization, cell death regulation or innate/adaptive immunity may not be necessarily well modelled in yeast. Similarly, proteins that are post-translationally processed in the bacterium, inserted into a membrane by a bacterial-encoded apparatus (e.g. TTSS), or are part of a multiprotein complex, may not retain biological activity when ectopically expressed in a eukaryotic cell. Nonetheless, as shown in this study, yeast can provide a powerful expression system to initiate a functional analysis of pathogens that are difficult to genetically manipulate, grow in culture or are too dangerous for routine analysis in the laboratory. Given the long-term coevolution of obligate intracellular pathogens with their eukaryotic hosts, we expect that these pathogens will reveal novel and unexpected insights into basic cell biological processes of eukaryotic cells.

Experimental procedures

Yeast expression vectors and construction of C. trachomatis *protein expression array*

The vector pSDY-1 was generated from p425 (2μ *LEU2* P_{GAL}) (Mumberg *et al*., 1994) by cloning a GFP-Pep12$_{L\text{-}TM}$ gene fusion from pMB336 (Black and Pelham, 2000). pSDY-8 was generated by excising P_{GAL} *-GFP* from pSDY-1 and inserting it in pRS426 (2μ *URA3*). SpeI and HindIII sites upstream of GFP allow for the directional cloning of in frame gene fusions to GFP and GFP-Pep12$_{L\text{-}TM}$. Bacterial ORFs were amplified from density gradient purified *C. trachomatis* serovar D (R. Stephens U.C. Berkeley, CA) EBs by PCR. Oligonucleotides (Sigma Genosys) where designed such that the 3′ end is specific to the gene of interest and the 5′ end is complemen-

Table 1. Features and conservation of putative chlamydial modulators of host cellular functions.

ORF	*C. muridamun*	*C. pneumoniae*	*P. acanthamoeba*	Phenotype in yeast[a]	Organelle tropism[a]	Reactivity to α IM antisera[a]	Predicted function
CT066	Tc0336 (97%)	Cpn0350 (86%)	Pc0253 (54%)	Y	Nuclear	N	–
CT084	Tc0357 (77%)	Cpn0329 (40%)	Pc0688 (26%)	Y	Mitochondria	N	Phospholipase
CT105	Tc0381 (49%)	Cpn0405 (22%)	–	Y	Endosome	N	–
CT142	Tc0419 (71%)	Cpn0255 (34%)	–	Y	Cytoplasmic	Y	–
CT163	–	–	–	Y	Lipid droplets	N	–
CT283	Tc0556 (82%)	Cpn0434 (50%)	Pc02778/9 (24%)	Y	Plasma membrane	N	–
CT344	Tc0623 (98%)	Cpn0027 (85%)	Pc0462 (65%)	Y	Punctate	Y	Protease
CT418	Tc0699 (95%)	Cpn0544 (76%)	Pc0219 (54%)	Y	Nuclear	Weak	GTPase
CT456	Tc0741 (54%)	Cpn0572 (44%)	–	Y	Aggregate	Y	Actin nucleator
CT473	Tc0758 (72%)	Cpn0592 (54%)	Pc1368 (74%)	Y	Lipid droplets	N	DUF37
CT529	Tc0816 (58%)	Cpn0648 (35%)	–	Y	Punctate	Y	TSS effector
CT578	Tc0867 (83%)	Cpn0809 (49%)	–	Y	Punctate	N	TSS effector
CT618	Tc0908 (64%)	Cpn0753 (22%)	–	Y	Punctate	N	Inc protein
CT694	Tc0066 (52%)	–	–	Y	Plasma membrane	N	–
CT813	Tc0199 (50%)	–	–	Y	Aggregate	Y	Inc protein

a. See Table S1 for details.
TSS, type III secretion. Percentages next to ORFs indicate homologies by amino acid identity. Predicted function and protein features were determined with protein analysis tools from the Expert Protein Analysis System (Expasy, http://us.expasy.org).

tary to the sequences flanking the SpeI–HindIII sites on pSDY-1/pSDY-8 (a list of oligonucleotides used is available in Table S3). Expand High Fidelity PCR system (Roche Diagnostics, IN) was used for all DNA amplifications as instructed by the manufacturer. SDY1/SDY8 were digested with SpeI and HindIII (NEB, MA), treated with calf intestinal alkaline phosphatase (Roche), and transformed with the PCR amplified *C. trachomatis* ORF into the S288c-derived yeast strain DLY1554 (MATa *ura3Δ leu2Δ his3Δ lys2Δ*) by the lithium acetated method (Brown and Tuite, 1998). The 5′ end of the PCR products provides the substrate for efficient splicing of the two DNA molecules by homologous recombination. Typically, omission of the PCR product led to a >1000-fold reduction in the recovery of URA^+ colonies. Plasmids were recovered from recombinant yeast strains as previously described (Brown and Tuite, 1998), sequenced to confirm the correct splicing of the amplified *C. trachomatis* gene and re-transformed into yeast. Yeast cells were grown in complete synthetic medium (CSM) dropout mixes (Bio 101 Laboratories) supplemented with dextrose, raffinose or galactose as indicated in the text. All yeast methods were based on protocols outlined in Brown and Tuite (1998). For immunoblot analysis chlamydial proteins were induced by growth at 30°C for 16–20 h in CSM media supplemented with 2% galactose. Cells were harvested (2–3 OD_{600}) and lysed by agitation with glass beads in 1× SDS sample buffer supplemented with 10 mM PMSF. Protein samples were resolved on 11% SDS polyacrylimide gels, transferred to nitrocellulose and probed with anti-GFP monoclonal antibodies (StressGen, CA). HRP-conjugated secondary antibodies (Amersham Biosciences) and Supersignal chemiluminescence kits (Pierce) were used for immune detection.

Phenotypic characterization and quantification of yeast growth defects

Recombinant yeast strains were maintained in CSM-uracil (pSDY-8) or CSM-leucine (pSDY-1) and 2% dextrose in 96 well plates. To determine the effect of *C. trachomatis* expression on yeast metabolic health, 10-fold serial dilutions of late-log phase cells were spotted on 2% galactose CSM-ura or CSM-leu agar plates supplemented with 0.5 M NaCl or 0.8 M sorbitol. Plates were incubated at 15°C, 30°C or 37°C for 3–10 days. The relative growth was compared with that of yeast strains expressing pSDY-1 or pSDY-8 alone and quantified in a scale of 0 (no growth) to 5 (wt growth). Growth defects in liquid media were determined by using the Biolog systems' tetrazolium-based cell respiration redox dyes and quantified on an Omnilog PM Automated System as detailed by the manufacturer (Biolog, Hayward, CA). Maximum dye reduction was normalized to that of yeast strains expressing the empty vector pSDY-8 and presented in a 0–5 scale.

Yeast cell biology techniques and microscopy

Subcellular localization screens. Yeast strains were spotted on CSM-uracil plates supplemented with 1.5% raffinose/0.5% galactose and incubated for 18 h at 30°C. Cells were scrapped from the plates, resuspended in phosphate-buffered saline (PBS) and imaged live. To confirm nuclear localization of GFP-tagged proteins, cells were fixed in 3.7% formaldehyde in PBS and stained with 10 μg ml^{-1} Hoechst 33258.

Rhodamine-phalloidin stains. Yeast cells were grown in 2% raffinose to mid log and induced to express bacterial proteins by addition of galactose to 1% for 1–4 h. Cells were fixed in 3.7% formaldehyde, washed thoroughly and incubated with 200 nM rhodamine-phalloidin (Molecular Probes, OR) in PBS for 30 min to stain filamentous actin.

FM4-64 uptake assays. Raffinose-grown cells were induced as described above. Cells were spun down, resuspended in media supplemented with 10 μM FM4-64 (Molecular Probes), incubated on ice for 30 min and shifted to 30°C for 0.5–2 h. Cells were imaged live.

Organelle identification. Yeast cells expressing RFP-tagged lipid body marker Erg6p (Huh *et al*., 2003) or the mitochondrial matrix marker Su9 (Frederick *et al*., 2004) were transformed with GFP-tagged chlamydial proteins expression vectors and imaged live after a 4 h induction of the recombinant proteins. All images were acquired with a Zeiss Axioscope epifluorescence microscope equipped with a Hamimatsu CCD camera and processed with Axiovision v3.0 imaging software.

Antibody generation

Glutathione S-transferase (GST) fusions to CT050 were generated and ~0.5 mg of protein was used to immunize rabbits (Covance Research Products). Mouse polyclonal antibodies to a CT618-derived synthetic peptide (QLD PVSQQRTLL-SPLSLLC) were generated by SACRI Antibody Services (U. Calgary, Canada). The specificity of antibodies was determined by monitoring immunoreactivity to proteins expressed in yeast. Proteins were localized in infected cells by indirect immunofluorescence. Briefly, cells were fixed in either 4% paraformaldehyde/0.25% glutaraldehyde (CT618) or methanol (CT050). Cell were permeabilized with 0.1% Triton X100, blocked with 5% serum albumin in PBS and incubated with 1:50 dilution of antisera followed by incubation with anti-mouse or -rabbit Alexa555 labelled secondary antibodies (Molecular Probes). *C. trachomatis* was detected with a mouse monoclonal (clone 502) against OMP-2 (RDI, NJ).

Mammalian expression vectors and transfections

Eight chlamydial proteins identified as nucleotropic in the yeast subcellular localization screens were subcloned into pEGFPN1 (Clontech BD, CA) to generate EGFP-tagged versions of these *C. trachomatis* proteins. Hep2 cells were transiently transfected with Lipofectamine 2000 (Invitrogen, CA) as specified by the manufacturer. After 16 h, transfected cells were fixed with 4% paraformaldehyde in PBS and stained with 10 μg ml^{-1} Hoechst 33258. For infections with *C. trachomatis* serovar L2, Hep2 cells were transfected with nucleotropic EGFP-tagged *C. trachomatis* expression vectors and infected at a moi of ~5 for 18–36 h. Fluorescent images were acquired as described above.

Generation of anti-IM antibodies and screening of chlamydial protein expression arrays

Hep2 cells (~10 confluent T175 flasks) were infected with *C. trachomatis* serovar L2 for 44 h and harvested in ~20 ml of Hank's balanced salt solution (HBSS) containing a cocktail of protease inhibitors (Sigma). The cell suspension was sonicated at 50 W (3 × 30 s) and post-nuclear supernatants (PNS) were centrifuged at 30 000 *g* for 40 min. The pelleted material (membranes and bacteria) were resuspended in ice-cold HBSS, overlaid on 8 ml of 30% Hypaque and re-centrifuged for 40 min at 30 000 *g*. The floating membrane fraction (seen as a band on top of the 30% Hypaque) was diluted in HBSS and sedimented at 100 000 *g* for 45 min. Membrane pellets from parallel gradients were pooled in 2 ml of 1× TNE (Tris-HCl pH 8.0, 150 mM NaCl, 0.5 mM EDTA), overlaid on top of a 0–25% Iodixanol (Optiprep) step gradient prepared with 2 ml each of 25%/20%/15%/10%/5% (in 1× TNE) and centrifuged at 100 000 *g* for 16 h in a swinging bucket SW41 rotor (Beckman Coulter). One millilitre fractions were collected from the top and membranes pelleted by ultracentrifugation. A sample of each fraction was solubilized in SDS sample buffer and the relative distribution of subcellular markers was assessed by immunoblot analysis as described above. Antibodies to the following markers were used: IncA and IncG (T. Hackstadt, NIH RML), EF-Tu (XY. Zhang, Boston University), OMP-2 (RDI, NJ), TRAP (C. Nikita, Duke University), caveolin-1 (Santa Cruz Biotech) and Na/K ATPase (U. Iowa Hybridoma Bank). The IM enrichment procedure was scaled up 10-fold, fractions enriched for IncA and IncG were pooled and a total of 750 μg of protein was used to immunize two rabbits. Polyclonal antibody generation was performed by Covance Research Immunological services. For analysis of antibodies generated against non-secreted chlamydial contaminants in our anti-IM antisera, 1 mg of sonicated EB were solubilized in 1% SDS at 65°C, diluted to 0.1% SDS in TNE and proteins detected by immunoprecipitation with anti-IM antisera (Fig. 7C). In addition, polyclonal antisera previously generated against *C. trachomatis* infected cells (Scidmore-Carlson et al., 1999) was generously provided by T. Hackstadt (NIH, RML) and tested (see Table S1 for details). All anti-IM Antisera was diluted 1:25–1:100 for immunoblot analyses.

Acknowledgements

We thank T. Hackstadt, M. Scidmore, R. Stephens and D. Lew for strains and reagents, and J. Heitman for access to the Omnilog automated plate reader and for critical comments on this manuscript. We also thank the anonymous reviewers for helpful comments on data presentation. Sarah McGill, Preston Fulmer, Jordan Cocchiaro and Mark Vignola assisted at various stages in the construction and characterization of the chlamydial expression libraries. This work was supported by funds from the Whitehead Foundation, the Pew Scholar's Program in Biomedical Sciences and the NIH/NIAID (AI06153). Y.K. is a recipient of an ARVO/Novartis 'Fight for Sight' Postdoctoral Fellowship.

References

Bankaitis, V.A., Johnson, L.M., and Emr, S.D. (1986) Isolation of yeast mutants defective in protein targeting to the vacuole. *Proc Natl Acad Sci USA* **83:** 9075–9079.

Bannantine, J.P., Griffiths, R.S., Viratyosin, W., Brown, W.J., and Rockey, D.D. (2000) A secondary structure motif predictive of protein localization to the chlamydial inclusion membrane. *Cell Microbiol* **2:** 35–47.

Belland, R.J., Zhong, G., Crane, D.D., Hogan, D., Sturdevant, D., Sharma, J., *et al.* (2003) Genomic transcriptional profiling of the developmental cycle of *Chlamydia trachomatis*. *Proc Natl Acad Sci USA* **100:** 8478–8483.

Black, M.W., and Pelham, H.R. (2000) A selective transport route from Golgi to late endosomes that requires the yeast GGA proteins. *J Cell Biol* **151:** 587–600.

Borgese, N., Brambillasca, S., Soffientini, P., Yabal, M., and Makarow, M. (2003) Biogenesis of tail-anchored proteins. *Biochem Soc Trans* **31:** 1238–1242.

Brown, A., and Tuite, M. (1998) *Yeast Gene Analysis*. London: Academic Press.

Campodonico, E.M., Chesnel, L., and Roy, C.R. (2005) A yeast genetic system for the identification and characterization of substrate proteins transferred into host cells by the *Legionella pneumophila* Dot/Icm system. *Mol Microbiol* **56:** 918–933.

Carabeo, R.A., Grieshaber, S.S., Fischer, E., and Hackstadt, T. (2002) *Chlamydia trachomatis* induces remodeling of the actin cytoskeleton during attachment and entry into HeLa cells. *Infect Immun* **70:** 3793–3803.

Carabeo, R.A., Mead, D.J., and Hackstadt, T. (2003) Golgi-dependent transport of cholesterol to the *Chlamydia trachomatis* inclusion. *Proc Natl Acad Sci USA* **100:** 6771–6776.

Carlson, J.H., Hughes, S., Hogan, D., Cieplak, G., Sturdevant, D.E., McClarty, G., *et al.* (2004) Polymorphisms in the *Chlamydia trachomatis* cytotoxin locus associated with ocular and genital isolates. *Infect Immun* **72:** 7063–7072.

Carlson, J.H., Porcella, S.F., McClarty, G., and Caldwell, H.D. (2005) Comparative genomic analysis of *Chlamydia trachomatis* oculotropic and genitotropic strains. *Infect Immun* **73:** 6407–6418.

Clifton, D.R., Fields, K.A., Grieshaber, S.S., Dooley, C.A., Fischer, E.R., Mead, D.J., *et al.* (2004) A chlamydial type III translocated protein is tyrosine-phosphorylated at the site of entry and associated with recruitment of actin. *Proc Natl Acad Sci USA* **101:** 10166–10171.

Clifton, D.R., Dooley, C.A., Grieshaber, S.S., Carabeo, R.A., Fields, K.A., and Hackstadt, T. (2005) Tyrosine phosphorylation of the chlamydial effector protein tarp is species specific and not required for recruitment of actin. *Infect Immun* **73:** 3860–3868.

Dong, F., Su, H., Huang, Y., Zhong, Y., and Zhong, G. (2004a) Cleavage of host keratin 8 by a *Chlamydia*-secreted protease. *Infect Immun* **72:** 3863–3868.

Dong, F., Pirbhai, M., Zhong, Y., and Zhong, G. (2004b) Cleavage-dependent activation of a *Chlamydia*-secreted protease. *Mol Microbiol* **52:** 1487–1494.

Dong, F., Pirbhai, M., Xiao, Y., Zhong, Y., Wu, Y., and Zhong, G. (2005) Degradation of the proapoptotic proteins Bik, Puma, and Bim with Bcl-2 domain 3 homology in *Chlamydia trachomatis*-infected cells. *Infect Immun* **73:** 1861–1864.

Engqvist-Goldstein, A.E., and Drubin, D.G. (2003) Actin

assembly and endocytosis: from yeast to mammals. *Annu Rev Cell Dev Biol* **19:** 287–332.

Fan, P., Dong, F., Huang, Y., and Zhong, G. (2002) *Chlamydia pneumoniae* secretion of a protease-like activity factor for degrading host cell transcription factors required for [correction of factors is required for] major histocompatibility complex antigen expression. *Infect Immun* **70:** 345–349.

Fan, T., Lu, H., Hu, H., Shi, L., McClarty, G.A., Nance, D.M., *et al.* (1998) Inhibition of apoptosis in *Chlamydia*-infected cells: blockade of mitochondrial cytochrome c release and caspase activation. *J Exp Med* **187:** 487–496.

Fields, K.A., and Hackstadt, T. (2002) The chlamydial inclusion: escape from the endocytic pathway. *Annu Rev Cell Dev Biol* **18:** 221–245.

Fields, K.A., Mead, D.J., Dooley, C.A., and Hackstadt, T. (2003) *Chlamydia trachomatis* type III secretion: evidence for a functional apparatus during early-cycle development. *Mol Microbiol* **48:** 671–683.

Fields, K.A., Fischer, E.R., Mead, D.J., and Hackstadt, T. (2005) Analysis of putative *Chlamydia trachomatis* chaperones Scc2 and Scc3 and their use in the identification of type III secretion substrates. *J Bacteriol* **187:** 6466–6478.

Fling, S.P., Sutherland, R.A., Steele, L.N., Hess, B., D'Orazio, S.E., Maisonneuve, J., *et al.* (2001) CD8+ T cells recognize an inclusion membrane-associated protein from the vacuolar pathogen *Chlamydia trachomatis*. *Proc Natl Acad Sci USA* **98:** 1160–1165.

Frederick, R.L., McCaffery, J.M., Cunningham, K.W., Okamoto, K., and Shaw, J.M. (2004) Yeast Miro GTPase, Gem1p, regulates mitochondrial morphology via a novel pathway. *J Cell Biol* **167:** 87–98.

Grieshaber, S.S., Grieshaber, N.A., and Hackstadt, T. (2003) *Chlamydia trachomatis* uses host cell dynein to traffic to the microtubule-organizing center in a p50 dynamitin-independent process. *J Cell Sci* **116:** 3793–3802.

Hackstadt, T. (1998) The diverse habitats of obligate intracellular parasites. *Curr Opin Microbiol* **1:** 82–87.

Hackstadt, T. (1999) Cell biology. In *Chlamydia: Intracellular Biology, Pathogenesis and Immunity*. Stephens, R.S. (ed.). Washington, DC: ASM, pp. 139–169.

Hackstadt, T. (2000) Redirection of host vesicle trafficking pathways by intracellular parasites. *Traffic* **1:** 93–99.

Hampsey, M. (1997) A review of phenotypes in *Saccharomyces cerevisiae*. *Yeast* **13:** 1099–1133.

Hatch, T.P. (1999) Developmental biology. In *Chlamydia: Intracellular Biology, Pathogenesis and Immunity*. Stephens, R.S. (ed.). Washington, DC: ASM, pp. 139–169.

Heese-Peck, A., Pichler, H., Zanolari, B., Watanabe, R., Daum, G., and Riezman, H. (2002) Multiple functions of sterols in yeast endocytosis. *Mol Biol Cell* **13:** 2664–2680.

Ho, T.D., and Starnbach, M.N. (2005) The *Salmonella enterica* serovar typhimurium-encoded type III secretion systems can translocate *Chlamydia trachomatis* proteins into the cytosol of host cells. *Infect Immun* **73:** 905–911.

Horn, M., Collingro, A., Schmitz-Esser, S., Beier, C.L., Purkhold, U., Fartmann, B., *et al.* (2004) Illuminating the evolutionary history of chlamydiae. *Science* **304:** 728–730.

Huh, W.K., Falvo, J.V., Gerke, L.C., Carroll, A.S., Howson, R.W., Weissman, J.S., and O'Shea, E.K. (2003) Global analysis of protein localization in budding yeast. *Nature* **425:** 686–691.

Kalman, S., Mitchell, W., Marathe, R., Lammel, C., Fan, J., Hyman, R.W., *et al.* (1999) Comparative genomes of *Chlamydia pneumoniae* and *C. trachomatis*. *Nat Genet* **21:** 385–389.

Lad, S.P., Fukuda, E.Y., Li, J., de la Maza, L.M., and Li, E. (2005) Up-regulation of the JAK/STAT1 signal pathway during *Chlamydia trachomatis* infection. *J Immunol* **174:** 7186–7193.

Lesser, C.F., and Miller, S.I. (2001) Expression of microbial virulence proteins in *Saccharomyces cerevisiae* models mammalian infection. *EMBO J* **20:** 1840–1849.

Levin, D.E. (2005) Cell wall integrity signaling in *Saccharomyces cerevisiae*. *Microbiol Mol Biol Rev* **69:** 262–291.

Martin, S., and Parton, R.G. (2005) Caveolin, cholesterol, and lipid bodies. *Semin Cell Dev Biol* **16:** 163–174.

Mumberg, D., Muller, R., and Funk, M. (1994) Regulatable promoters of *Saccharomyces cerevisiae*: comparison of transcriptional activity and their use for heterologous expression. *Nucleic Acids Res* **22:** 5767–5768.

van Ooij, C., van Kalman, L.I., Nishijima, M., Hanada, K., Mostov, K., and Engel, J.N. (2000) Host cell-derived sphingolipids are required for the intracellular growth of *Chlamydia trachomatis*. *Cell Microbiol* **2:** 627–637.

Pandit, S.B., and Srinivasan, N. (2003) Survey for G-proteins in the prokaryotic genomes: prediction of functional roles based on classification. *Proteins* **52:** 585–597.

Read, T.D., Brunham, R.C., Shen, C., Gill, S.R., Heidelberg, J.F., White, O., *et al.* (2000) Genome sequences of *Chlamydia trachomatis* MoPn and *Chlamydia pneumoniae* AR39. *Nucleic Acids Res* **28:** 1397–1406.

Read, T.D., Myers, G.S., Brunham, R.C., Nelson, W.C., Paulsen, I.T., Heidelberg, J., *et al.* (2003) Genome sequence of *Chlamydophila caviae* (*Chlamydia psittaci* GPIC): examining the role of niche-specific genes in the evolution of the Chlamydiaceae. *Nucleic Acids Res* **31:** 2134–2147.

Rockey, D.D., Scidmore, M.A., Bannantine, J.P., and Brown, W.J. (2002) Proteins in the chlamydial inclusion membrane. *Microbes Infect* **4:** 333–340.

Rodriguez-Escudero, I., Hardwidge, P.R., Nombela, C., Cid, V.J., Finlay, B.B., and Molina, M. (2005) Enteropathogenic *Escherichia coli* type III effectors alter cytoskeletal function and signalling in *Saccharomyces cerevisiae*. *Microbiology* **151:** 2933–2945.

Rojo, E.E., Stuart, R.A., and Neupert, W. (1995) Conservative sorting of F0-ATPase subunit 9: export from matrix requires delta pH across inner membrane and matrix ATP. *EMBO J* **14:** 3445–3451.

Salcedo, S.P., and Holden, D.W. (2005) Bacterial interactions with the eukaryotic secretory pathway. *Curr Opin Microbiol* **8:** 92–98.

Schachter, J. (1999) Infection and disease epidemiology. In *Chlamydia: Intracellular Biology, Pathogenesis and Immunity*. Stephens, R.S. (ed.). Washington, DC: ASM, pp. 139–169.

Scidmore, M.A., Fischer, E.R., and Hackstadt, T. (1996) Sphingolipids and glycoproteins are differentially trafficked to the *Chlamydia trachomatis* inclusion. *J Cell Biol* **134:** 363–374.

Scidmore, M.A., Fischer, E.R., and Hackstadt, T. (2003) Restricted fusion of *Chlamydia trachomatis* vesicles with endocytic compartments during the initial stages of infection. *Infect Immun* **71:** 973–984.

Scidmore-Carlson, M., Shaw, E.I., Dooley, C.A., Fischer, E.R., and Hackstadt, T. (1999) identification and characterization of a *Chlamydia trachomatis* early operon encoding four novel inclusion membrane proteins. *Mol Microbiol* **33:** 753–765.

Shaw, A.C., Vandahl, B.B., Larsen, M.R., Roepstorff, P., Gevaert, K., Vandekerckhove, J., *et al.* (2002) Characterization of a secreted *Chlamydia* protease. *Cell Microbiol* **4:** 411–424.

Shaw, E.I., Dooley, C.A., Fischer, E.R., Scidmore, M.A., Fields, K.A., and Hackstadt, T. (2000) Three temporal classes of gene expression during the *Chlamydia trachomatis* developmental cycle. *Mol Microbiol* **37:** 913–925.

Shohdy, N., Efe, J.A., Emr, S.D., and Shuman, H.A. (2005) Pathogen effector protein screening in yeast identifies *Legionella* factors that interfere with membrane trafficking. *Proc Natl Acad Sci USA* **102:** 4866–4871.

Solomon, F. (1991) Analyses of the cytoskeleton in *Saccharomyces cerevisiae*. *Annu Rev Cell Biol* **7:** 633–662.

Stephens, R.S., Kalman, S., Lammel, C., Fan, J., Marathe, R., Aravind, L., *et al.* (1998) Genome sequence of an obligate intracellular pathigen of humans: *Chlamydia trachomatis*. *Science* **282:** 754–759.

Su, H., McClarty, G., Dong, F., Hatch, G.M., Pan, Z.K., and Zhong, G. (2004) Activation of Raf/MEK/ERK/cPLA2 signaling pathway is essential for chlamydial acquisition of host glycerophospholipids. *J Biol Chem* **279:** 9409–9416.

Subtil, A., Delevoye, C., Balana, M.E., Tastevin, L., Perrinet, S., and Dautry-Varsat, A. (2005) A directed screen for chlamydial proteins secreted by a type III mechanism identifies a translocated protein and numerous other new candidates. *Mol Microbiol* **56:** 1636–1647.

Thomson, N.R., Yeats, C., Bell, K., Holden, M.T., Bentley, S.D., Livingstone, M., *et al.* (2005) The *Chlamydophila abortus* genome sequence reveals an array of variable proteins that contribute to interspecies variation. *Genome Res* **15:** 629–640.

Tse, S.M., Mason, D., Botelho, R.J., Chiu, B., Reyland, M., Hanada, K., *et al.* (2005) Accumulation of diacylglycerol in the chlamydia inclusion vacuole: possible role in the inhibition of host cell apoptosis. *J Biol Chem* **280:** 25210–25215.

Valdivia, R.H. (2004) Modeling the function of bacterial virulence factors in *Saccharomyces cerevisiae*. *Eukaryot Cell* **3:** 827–834.

Verna, J., Lodder, A., Lee, K., Vagts, A., and Ballester, R. (1997) A family of genes required for maintenance of cell wall integrity and for the stress response in *Saccharomyces cerevisiae*. *Proc Natl Acad Sci USA* **94:** 13804–13809.

Warringer, J., Ericson, E., Fernandez, L., Nerman, O., and Blomberg, A. (2003) High-resolution yeast phenomics resolves different physiological features in the saline response. *Proc Natl Acad Sci USA* **100:** 15724–15729.

Xiao, Y., Zhong, Y., Greene, W., Dong, F., and Zhong, G. (2004) *Chlamydia trachomatis* infection inhibits both Bax and Bak activation induced by staurosporine. *Infect Immun* **72:** 5470–5474.

Ying, S., Seiffert, B.M., Hacker, G., and Fischer, S.F. (2005) Broad degradation of proapoptotic proteins with the conserved Bcl-2 homology domain 3 during infection with *Chlamydia trachomatis*. *Infect Immun* **73:** 1399–1403.

Zhong, G., Fan, P., Ji, H., Dong, F., and Huang, Y. (2001) Identification of a chlamydial protease-like activity factor responsible for the degradation of host transcription factors. *J Exp Med* **193:** 935–942.

Zweytick, D., Athenstaedt, K., and Daum, G. (2000) Intracellular lipid particles of eukaryotic cells. *Biochim Biophys Acta* **1469:** 101–120.

Supplementary material

The following supplementary material is available for this article online:

Table S1. Phenotypic profile of chlamydial proteins expressed in yeast.

Table S2. Phenotypic profile of chlamydial proteins expressed on yeast endosomes.

Table S3. Primers used for the amplification and cloning of chlamydial ORFs.

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