The *Legionella pneumophila* chaperonin - An investigation of virulence-related roles in a yeast model

by

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Dedication

For the glory of God from whom I receive inspiration, strength and the will to persevere.

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ABSTRACT

The opportunistic human pathogen *Legionella pneumophila* uses its Hsp60 homologue (HtpB) to attach to and invade host cells, and releases the protein into its replicative endosome during intracellular growth. In this study a yeast model was used to investigate HtpB intracellular function. *Saccharomyces cerevisiae* cells expressing the *htpB* gene in the cytoplasm, from the galactose-inducible plasmid pEMBLyex4 displayed pseudohyphal growth (PHG) phenotypes including cell elongation, budding in unipolar fashion, the invasion of solid medium and activation of the PHG reporter construct FG(*TyA*)::*lacZ*. An Hsp60 homologue from *Escherichia coli* (GroEL) did not induce PHG in *S. cerevisiae* when expressed from plasmid pEMBLyex4. Using genetics-based methods, it was determined that many of the known regulators of PHG in *S. cerevisiae* including Ras2p (an activator of kinase signaling), Cdc42p (a GTP-binding protein), Ste20p (a homologue of the mammalian $p65^{PAK}$ kinases), Ste11p (a MAPKKK), Ste7p (a MAPKK), and the transcription factors Ste12p and Flo8p play a role HtpB-induced PHG. These findings indicate that HtpB has a biological effect in eukaryotic cells. Using the two-hybrid method, two HeLa cell proteins, Map and CksHs1, were identified as putative interacting proteins for HtpB. These proteins are homologous to Bub2p and Cks1, respectively in *S. cerevisiae*. Bub2p and Cks1 are involved in delaying mitotic exit, which can result in yeast cell elongation, and may serve as the underlying mechanism for HtpB-induced PHG. In mammalian cells Map and CksHs1 influence actin dynamics, which could be relevant for *L. pneumophila* pathogenesis, since *L. pneumophila* entry into host cells is an actin-dependent process. A preliminary investigation into the secretion mechanism(s) for Hsp60 homologues in bacteria was also pursued. As determined by immunogold electron microscopy, GroEL was localized to the cytoplasm in enteropathogenic *E. coli*, while in two *Bordetella* species, Cpn60 (the *Bordetella* Hsp60 homologue) was found in association with the bacterial cell envelope. Furthermore, in *B. pertussis* Cpn60 surface localization was dependent on BvgS, a regulator of virulence gene expression. This study contributes to the awareness that chaperonins, aside from their role in protein folding have unique functions that may facilitate the survival of organisms in their niches.

LIST OF ABBREVIATIONS AND SYMBOLS USED

Å	angstrom
~	approximately
Δ	deletion
ABC	ATP-binding cassette
ACES	N-(2-acetamido)-2-aminoethansulfonic acid
Ade	adenine
A/E	attachment/effacement
AEC	anion exchange chromatography
Amp^r	ampicillin resistance
Arg	arginine
Asn	asparagine
Asp	aspartic acid
ATP	adenosine 5' triphosphate
ATTA	AbaAp, Tec1p, Tef-1p sequence
APC	antigen presenting cell
BCYE	buffered charcoal yeast extract
BG	Bordet-Gengou
bp	base pair (s)
BYE	buffered yeast extract
Cam^r	chloramphenicol resistance
CAP	competence and adherence associated pili

CCT	chaperonin containing TCP-1
Cdc	cell division cycle
Cdk	cyclin dependent kinase
CFU	colony forming units
CHO	Chinese hamster ovary
CKI	Cdk inhibitor
COP	coat protein complex
CR	complement receptor
Cyc	iso-1-cytochrome C
CW	cell wall
ddH_2O	deionized distilled water
DAPI	4, 6-diamidino-2-phenylindole hydrochloride
DEAE	diethylaminoethyl cellulose
Dex	dextrose
DMEM	Dulbecco's minimal essential medium
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside 5'-triphosphate
Dsb	disulfide bond
E	glutamic acid
EC	extracellular
EDTA	ethylenediamine tetra-acetic acid
EM	electron microscopy
Env	envelope

EP	exponential phase
EPEC	enteropathogenic *Escherichia coli*
ER	endoplasmic reticulum
F	phenylalanine
FITC	fluorescein isothiocyanate
FRE	filamentation response element
G	glycine
g	gravity
Gal	galactose
Gal-Nac	N-acetylgalactosamine
GAP	GTPase activating protein
GDP	guanine diphosphate
GEF	guanine-nucleotide exchange factor
GFOR	glucose- fructose- oxidoreductase
Gly	glycine
GSP	general secretion pathway
GTP	guanosine 5'-triphosphate
GTPase	protein that has the capacity to bind and hydrolyze GTP
H	histidine
His	histidine
Hly	hemolysin
Htp	heat-shock protein
ICM	intracellular multiplication

Ig	immunoglobulin
Iso	isoleucine
K	lysine
kbp	kilobase pair (s)
kDa	kiloDalton
L	leucine
LAMP1	lysosomal associated protein 1
LB	Luria-Bertani
LEE	locus of enterocyte effacement
Leu	leucine
LPS	lipopolysaccharide
M	methionine
MAb	monoclonal antibody
Map	merlin-associated protein
MAPK	mitogen-activated protein kinase
MCS	multiple cloning site
MEL	melibiose
Met	methionine
MHC	major histocompatibility complex
MIP	macrophage infectivity potentiator
MIF	mature intracellular form
mRNA	messenger ribonucleic acid
MW	molecular weight

N	asparagine
Nal	Nalidixic acid
NSF	N-ethylmaleimide-sensitive fusion protein
OD	optical density
OM	outer membrane
Omp	outer membrane protein
ONPG	*o*-nitrophenyl-β-D-glactosidase
PAb	polyclonal antibody
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PE	post-exponential phase
PEG	polyethylene glycol
Phe	phenylalanine
PHG	pseudohyphal growth
Pil	pilus
PP	periplasm
ppGpp	guanosine 3', 5' bispyrophosphate
PRE	pheromone response element
pro	proline
PrP^c	cellular prion-like protein
Ptl	pertussis toxin liberation
Ptx	pertussis toxin
S	serine

SC	synthetic complete
SD	synthetic defined
SC	Stainer-Scholte
SNARE	soluble NSF attachment receptor
STD	sexually transmitted disease
Tat	twin arginine translocation
TBS	tris-buffered saline
TCP-1	t-complex polypeptide-1
TE	tris-EDTA
TEM	transmission electron microscopy
Tet^r	tetracycline resistance
Thr	threonine
TLR	toll-like receptor
Tn	Transposon
TNF	tumor necrosis factor
TriC	TCP-1 ring complex
Trp	tryptophan
TTBS	tween 20-tris-buffered saline
Tyr	tyrosine
U	Weiss unit
Ura	uracil
V	valine
Val	valine

VC	vector control
vol	volume
WASP	Wiskott- Aldrich Syndrome Protein
wt	weight
Y	tyrosine
YAC	yeast artificial chromosome
YEPD	yeast extract peptone dextrose
YPDA	yeast extract peptone dextrose adenine

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CHAPTER 1: INTRODUCTION.

Part 1. An Overview of *L. pneumophila* Biology.

1.1. History.

The *Legionella pneumophila* bacterium gained much attention when it was identified as the causative agent for an outbreak of pneumonia among Legionnaires attending a conference held in 1976 at a hotel in Philadelphia, Pennsylvania (Fraser *et al.*, 1977). One hundred eighty-one individuals contracted pneumonia and 29 (16%) patients died (Fraser *et al.*, 1977). Interestingly many hotel workers were sero-positive for *L. pneumophila* antibodies, and yet remained asymptomatic, indicating that the bacterium was readily cleared by the immune systems of healthier individuals (Fraser *et al.*, 1977). Indeed the more serious cases of Legionnaire's pneumonia occur among immune-compromised individuals such as the elderly, smokers, patients receiving chemotherapy and transplant patients on immune-suppressive drugs (Carratala *et al.*, 1994; Marston, Lipman, and Breiman, 1994). Therefore, *L. pneumophila* is considered an opportunistic human pathogen.

Infection with the bacterium most often occurs as a result of exposure to aerosols from contaminated water reservoirs; there have been no records of human-to-human transmission. In the case of the Philadelphia outbreak contaminated water aerosols were disseminated by the hotel air-conditioning system (Fraser *et al.*, 1977). Diligent management of water supplies, including treatment with biocides such as chlorine

dioxide is the best preventative measure for the disease (Srinivasan *et al.*, 2003). Despite this knowledge, in 1999, one of the largest outbreaks of Legionnaire's pneumonia occurred at the West Frisian Flower Show, held in the Netherlands (Den Boer *et al.*, 2002), during which 188 individuals became ill, of whom, 163 were hospitalized and 21 (11%) died (Den Boer *et al.*, 2002). This outbreak was traced to whirlpools that were not treated adequately to prevent bacterial growth (Den Boer *et al.*, 2002). Since the first recorded outbreak of Legionnaires' pneumonia in 1977, an increasing number of outbreaks have occurred with each passing year, so that the *L. pneumophila* organism has been classified as an emerging human pathogen (Woolhouse, 2002). Potential factors contributing to this increase in outbreaks include the aging of our population, an increase in the number of immune-compromised individuals, greater use of artificial water reservoirs, and inadequate water management (Marston, Lipman, and Breiman, 1994).

1.2. The bacterium's natural niche and the relevance for human disease.

Since *L. pneumophila* is not transmitted from person to person, it is postulated that human infection represents a dead-end scenario for the organism, and that variants that have adapted to the human host would, for the most part, be lost from the genetic pool (Swanson and Hammer, 2000). It is the current view that the capacity for *L. pneumophila* to replicate inside human alveolar macrophages stems from its adaptation to its "natural" hosts, single-celled freshwater amoeba and ciliated protozoa (Swanson and Hammer, 2000).

Legionellae spp. are ubiquitous in freshwater environments, where they live as facultative intracellular parasites of freshwater amoeba (Rowbotham, 1986). At least 39 different species of *Legionellae* have been identified, some of which have been found in association with at least 13 species of amoeba and 2 species of ciliated protozoa (Fields, 1996). *Legionellae* most often exist in parasitic relationships, but endosymbiotic relationships have been recorded (Jeon, 1995), indicating that *Legionella spp.* are in a constant state of adaptation to and co-evolution with their natural hosts.

Recognizing that amoebae are the primary host for *L. pneumophila* has broadened our understanding of *L. pneumophila* pathogenesis. When *L. pneumophila* is introduced into mice as a co-culture with amoeba cells as opposed to pure culture, the bacterium achieves higher lung colony forming units (CFU), and causes greater lung pathology and higher mortality (Brieland *et al.*, 1996). Furthermore, *L. pneumophila* harvested from cultures of amoeba invade mammalian cells more readily than do agar-grown bacteria (Cirillo, Falkow, and Tompkins, 1994). Taken together these data indicate that growth of *L. pneumophila* within amoeba exacerbates human infectivity.

The existence of the *L. pneumophila*-amoeba relationship also presents a public health challenge. Since *L. pneumophila* can replicate to high titres inside its amoebal host (Rowbotham, 1986), the presence of amoebae in water supplies allows for the delivery of large infectious doses. In addition the association of *L. pneumophila* with its amoeba host also presents an obstacle for eradication procedures. *L. pneumophila* grown within amoebae are more resistant to biocides (Barker, Lambert, and Brown,

1993; Barker, Scaife, and Brown, 1995), and *L. pneumophila* organisms residing within amoeba cysts can withstand high chlorine concentrations (up to 50 mg/L free chlorine) (Kilvington and Price, 1990).

1.3. A developmental cycle for *L. pneumophila*: replication vs. transmission.

In 1986, Rowbotham first provided microscopic evidence for the existence of two morphologically different forms of *L. pneumophila*. He noticed that *L. pneumophila* cells exiting their amoeba host were short, thick, motile, and displayed a thicker cell wall as compared to cells replicating within the host, which were longer and less electron dense (Rowbotham, 1986). More recently, Swanson's group put forth a model to describe the regulation of *L. pneumophila* differentiation (Molofsky and Swanson, 2004). In studies involving in vitro grown exponential phase (EP) and post-exponential phase (PE) bacteria, they observed that only PE bacteria were salt-sensitive (a phenotype of virulent *L. pneumophila* strains), motile, cytotoxic, osmotically resistant, infectious and competent to escape intracellular digestion, and the authors suggested that *in vitro* grown PE bacteria represent a transmissible form of the bacteria (Byrne and Swanson, 1998). Since EP bacteria can be induced to express the virulence traits when grown in spent medium, except if the medium is supplemented with amino-acids, they reasoned that amino-acid starvation provides the cue that promotes differentiation into the "transmissible form" (Byrne and Swanson, 1998). In the presence of ample nutrients, they observed that EP bacteria suppress the expression of unnecessary virulence

determinants, and they suggest that the organism assumes a "replicative mode" under such conditions (Byrne and Swanson, 1998).

In support of the hypothesis that EP and SP reflect differentiated states of *L. pneumophila* that are relevant for the infection cycle, Swanson's group has discovered a two-component regulatory system consisting of the LetA and LetS proteins that are required for the acquisition of virulence determinants (Hammer, Tateda, and Swanson, 2002). LetA and LetS are activated in response to the accumulation of guanosine 3', 5' bispyrophosphate (ppGpp) molecules, effectors of the stringent response pathway, which in *E. coli*, is activated in response to nutrient poor conditions (Hammer, Tateda, and Swanson, 2002). In *E. coli* uncharged tRNA molecules bound to ribosomes activate the RelA protein, which is a (ppGpp) synthetase (Swanson and Hammer, 2000). An increase in ppGpp results in activation of the stationary phase sigma factor, RpoS, which effects growth arrest and entry into stationary phase (Gentry *et al.*, 1993). In the case of *L. pneumophila*, an elevation in ppGpp results in the activation of both RpoS and LetA/LetS, resulting in virulence trait expression (Hammer and Swanson, 1999; Hammer, Tateda, and Swanson, 2002; Bachman and Swanson, 2004; Bachman and Swanson, 2001). They propose that reversion to the replicative form is mediated by an antagonist of the transmissible phenotype, the CsrA global regulator, which has been shown to repress cell shortening, motility, stress resistance, infectivity, and competence to evade intracellular digestion (Molofsky and Swanson, 2003).

Consistent with Swanson's model based on in vitro grown cells, was the identification of a mature intracellular form (MIF) of the *L. pneumophila* bacterium that arises at the end of the replication cycle (three days post-infection) inside HeLa cells (Garduno *et al.*, 2002). The MIFs are described as stubby rods with laminations of internal membranes originating from the plasma membrane, an electron-dense outer membrane layer, and with a cytoplasm filled by inclusions of poly-β-hydroxybutyrate (a storage carbohydrate)(Garduno *et al.*, 2002), phenotypes similar to the form first identified by T. J. Rowbotham (Rowbotham, 1986). MIFs are similar to PE bacteria in that they are flagellated, infectious, and resistant to osmotic shock. However, the MIFs display unique protein expression profiles as seen in 2-dimensional gel electrophoresis as compared to in vitro grown PE bacteria (Garduno *et al.*, 2002; Hiltz *et al.*, 2004). Furthermore, MIFs are more infectious and more environmentally robust as compared to PE bacteria (Garduno *et al.*, 2002), indicating that perhaps there exist additional steps of differentiation in *L. pneumophila* that are only achieved in vivo.

1.4. Replication of *L. pneumophila* inside host cells.

In the freshwater environment, the intracellular milieu of amoeba cells is thought to be the preferred niche for *L. pneumophila* multiplication. The replication of free-living legionellae has been difficult to demonstrate and thus remains a controversial issue (Fields, 1996; Murga *et al.*, 2001). *L. pneumophila* also has the capacity to replicate inside human alveolar macrophages (Swanson and Hammer, 2000). However, surviving

within the intracellular environment of eukaryotic cells is not a trivial task. The amoeba's goal in taking up bacteria from the environment is to digest them as a source of nutrients, while alveolar macrophages, soldiers of the immune system, are designed to engulf and destroy invaders. Therefore, to survive in the intracellular environment of an amoeba or a macrophage, *L. pneumophila* must employ effective strategies to evade intracellular digestion and to access a nutrient pool.

After internalization into macrophages *L. pneumophila* is housed within a phagosome that is markedly different as compared to normal phagosomes in that it does not acidify or fuse with lysosomes for up to eight hours post-infection (Sturgill-Koszycki and Swanson, 2000). In contrast, phagosomes containing avirulent strains of *L. pneumophila* have been observed to fuse with lysosomes as early as 5 minutes post-infection (Roy, Berger, and Isberg, 1998). This implies that virulent strains of *L. pneumophila* have the capacity to disrupt the endocytic process inside macrophages. Also peculiar to the infectious *L. pneumophila* phagosome is that it associates with smooth vesicles of unknown origin and mitochondria within minutes of uptake (Sturgill-Koszycki and Swanson, 2000; Horwitz, 1983), and between 2-4 hours post-infection the organism is found within a ribosome studded vacuole (Sturgill-Koszycki and Swanson, 2000; Horwitz, 1983), suggestive of interaction with the endoplasmic reticulum (ER), which is discussed below.

The molecular mechanisms that govern *L. pneumophila*'s capacity to replicate intracellularly are being clarified. It is well established that a Type IV protein secretion

system, called Dot/Icm (for defective organelle trafficking/intracellular multiplication), is essential for *L. pneumophila*'s escape from intracellular digestion (Brand, Sadosky, and Shuman, 1994; Berger and Isberg, 1993). The Dot/Icm system is encoded by at least 24 genes located at two separate chromosomal loci (Segal and Shuman, 1998). The *dot/icm* genes share homology with the IncI plasmid *tra* system genes that encode a DNA conjugation system present in the *Salmonella* R64 and *Shigella* ColIb-P9 plasmids (Segal and Shuman, 1999; Christie, 2001), and the Dot/Icm system maintains the functional capacity to conjugate plasmid DNA to a recipient cell (Segal, Purcell, and Shuman, 1998), and to mobilize protein substrates from inside the bacterial cell into the host cytoplasm (Nagai *et al.*, 2002; Conover *et al.*, 2003; Luo and Isberg, 2004). It is postulated that the Dot/Icm secretion system is responsible for transporting into the host cell a fast-acting preformed protein effector that prevents fusion of the *L. pneumophila* phagosome with host lysosomes (Segal and Shuman, 1998). No Dot/Icm substrates deemed essential for arresting intracellular digestion have been identified; however, the putative effector is believed to be specifically localized to the *L. pneumophila* phagosome, since other endocytic vesicles devoid of the *L. pneumophila* bacterium still interact with lysosomal compartments in a timely fashion (Coers, Monahan, and Roy, 1999; Swanson and Hammer, 2000).

Consistent with the discovery that escape from the endocytic pathway happens early in the infection process is that the *L. pneumophila* phagosome lacks early markers of the endocytic pathway including the transferrin receptor, major histocompatibility

(MHC) proteins and the Rab5 protein (Swanson and Hammer, 2000). It is postulated that the *L. pneumophila* phagosome resides outside the endocytic network, in part, due to its early association with the ER (Swanson and Isberg, 1995). It is proposed that the *L. pneumophila* phagosome is a part of the autophagic circuit (Swanson and Hammer, 2000). The process of autophagy involves the digestion of bulk cytoplasm and organelles for the acquisition of amino acids in response to cell starvation (Yoshimori, 2004). The early autophagosome consists of a multi-membranous structure that is derived from the ER (Yoshimori, 2004). The observation that *L. pneumophila* phagosomes associate with early secretory vesicles derived from the endoplasmic reticulum (Kagan and Roy, 2002) supports the hypothesis that *L. pneumophila* replicates within an autophagosme-like compartment. *L. pneumophila* laden endosomes contain the ER specific markers calnexin and Bip but lack the Golgi-specific maker giantin (Swanson and Isberg, 1995; Kagan and Roy, 2002). Furthermore it was demonstrated that *L. pneumophila* phagosomes are labeled with the GTPase Rab1, which is known to mediate ER-Golgi specific fusion (Kagan *et al.*, 2004).

In macrophage derived cell lines, 70 % of endosomes older than 18 hours and containing replicating *L. pneumophila* become acidified and contain the lysosome-associated membrane protein 1 (LAMP-1) and the acid-hydrolase, cathepsin D (Sturgill and Swanson 2000), characteristics that are consistent with the properties of late autophagosomes, which do fuse with lysosomes (Dorn, Dunn, Jr., and Progulske-Fox, 2002). Phagosome acidification is in fact necessary for *L. pneumophila* replication as

preventing phagosome acidification through the addition of bafilomycin A1 inhibits *L. pneumophila* replication (Sturgill-Koszycki and Swanson, 2000). Perhaps *L. pneumophila* only transiently delays the fusion of its phagosome with host lysosomes to allow time for assuming a more acid-resistant and hydrolase-resistant form. Residing within a phago-lysosome would afford the bacterium access to peptides that result from the digestion of incoming particles. Autophagosomes are characterized by the presence of MAP-LC3 (Apg8 in yeast), a putative microtubule-associated protein and staining with the in vivo autofluorescent marker monodansylcadaverine (Dorn, Dunn, Jr., and Progulske-Fox, 2002; Yoshimori, 2004; Biederbick, Kern, and Elsasser, 1995). The replicative compartments of intracellular organisms such as *Brucella abortus* and *Porphyromonas gingivalis* are marked by MAP-LC3 and stain with monodansylcadaverine (Dorn, Dunn, Jr., and Progulske-Fox, 2002); it would be informative to determine whether *L. pneumophila* phagosomes acquire such markers in macrophages. It has been reported that macroautophagy is not required for *L. pneumophila* replication within the social amoeba, *Dictyostelium discoideum* (Munafo and Colombo, 2002; Otto *et al.*, 2004). *D. discoideum* mutants with deletions in several genes necessary for the autophagic process including *apg8* still support *L. pneumophila* replication (Otto *et al.*, 2004). However, it can not be ruled out that autophagy plays a role in *L. pneumophila* infection in macrophage-like cell lines.

1.5. Attachment and invasion.

In the laboratory *L. pneumophila* has been shown to adhere to and invade numerous cell types including phagocytic cells such as amoeba and macrophage-derived cell lines as well as nonphagocytic cell lines such as HeLa (Swanson and Hammer, 2000; Garduno, Quinn, and Hoffman, 1998). Due to its promiscuity, one would expect *L. pneumophila* to utilize multiple components to enter its varied hosts. Some have been identified and they include lipopolysaccharide (LPS), the major outer-membrane protein (OmpS), Type IV pili, the macrophage infectivity potentiator (MIP), RtxA, and the 60-kDa chaperonin (HtpB), which will be discussed in detail.

1.5.1. Lipopolysaccharide.

The lipopolysaccharide (LPS) found on the surface of *L. pneumophila* is linked to an unusual sugar, called legionamic acid, that is believed to confer hydrophobicity on the outer membrane (Zahringer *et al.*, 1995). This may promote non-specific binding to host cells. *L. pneumophila* may also utilize LPS phase switching for pathogenesis. An LPS mutant, which did not cross-react with a monoclonal antibody raised to the LPS of *L. pneumophila* serogroup 1 was isolated from guinea pigs along with the wild-type strain RC1 that was used for infection (Luneberg *et al.*, 1998). This indicates that LPS phase switching does indeed occur in *L. pneumophila* during the infection process, perhaps due to selective pressures, and may allow *L. pneumophila* to escape a humoral response.

1.5.2. OmpS.

OmpS is organized as a trimeric porin consisting of two 28.5-kDa subunits and a 31-kDa subunit that is linked to peptidoglycan in the *L. pneumophila* cell membrane (Butler and Hoffman, 1990; Hoffman, Seyer, and Butler, 1992). Treatment of *L. pneumophila* cells with anti-OmpS polyclonal antibodies reduces the adherence of both virulent and isogenic avirulent strains of *L. pneumophila* to HeLa cell monolayers in a dose-dependent manner (Garduno, Garduno, and Hoffman, 1998). Since blocking OmpS with antibodies reduces the adherence of *L. pneumophila* cells to HeLa cell monolayers, it is likely that OmpS promotes the binding of *L. pneumophila* to HeLa cells.

1.5.3. Type IV pili.

Type IV pili, found on the surface of bacterial species such as pathogenic *Neisseria spp.*, have been shown to mediate host cell attachment (Nassif *et al.*, 1997). The *L. pneumophila* Type IV pili, also known as CAP, for competence and adherence-associated pili, mediate adherence to protozoan and mammalian cell lines (Stone and Abu Kwaik, 1998). An insertional mutation in the putative *L. pneumophila* structural pilin gene, $pilE_L$, results in reduced adherence to *Acanthamoeba polyphaga*, the U937 mammalian monocytic cell line, and a non-phagocytic HeLa cell line, but does not affect bacterial replication (Stone and Abu Kwaik, 1998). This indicates that CAP pili play a role in promoting the attachment of *L. pneumophila* to its host cells.

1.5.4. MIP.

The *L. pneumophila* macrophage infectivity potentiator (Mip) is a 24-kDa surface-associated molecule that promotes macrophage and protozoa infectivity (Cianciotto *et al.*, 1989). A *mip* null mutant was impaired in its ability to infect U937 cells; 80-fold more *mip* mutant cells were required to establish a productive infection in the U937 macrophage-like cell-line as compared to wild-type (Cianciotto *et al.*, 1989). Consistent with this finding is that 10-fold fewer *mip* mutants were recovered from U937 cultures (Cianciotto *et al.*, 1989) and 1000-fold fewer *mip* mutants were recovered from infected protozoa cultures as compared to wild-type cells (Cianciotto and Fields, 1992) early in infection. However, later in infection there was no significant difference in the replication rate of the *mip* mutants and wild-type cells (Cianciotto *et al.*, 1989), indicating that *mip* acts only to establish infection.

MIP does not appear to play an essential role in the attachment to host cells. The opsonization of *mip* mutants and wild-type *L. pneumophila*, which allowed both strains to associate in equivalent numbers with U937 cells, did not restore the mutant's infectivity to wild-type levels (Cianciotto *et al.*, 1989); also *mip* mutants were not impaired in their ability to attach to the protozoan cells (Cianciotto and Fields, 1992). Although a mammalian or protozoan protein target for Mip has not been identified, the protein is required for optimal virulence in guinea pig infection models (Cianciotto *et al.*, 1990). Features of the Mip protein that may play a role in virulence are its peptidylprolyl-cis/trans isomerase active site (Helbig *et al.*, 2003; Wintermeyer *et al.*, 1995), which

catalyzes the cis/trans isomerization of prolyl peptide bonds in oligopeptides (Fischer *et al.*, 1992), and the protein's capacity to form homodimers (Schmidt *et al.*, 1994).

1.5.5. RtxA.

A locus designated *rtxA* was found to enhance the entry of wild-type *L. pneumophila* into monocytes and epithelial cells when over-expressed from a multicopy plasmid (Cirillo, Lum, and Cirillo, 2000). The *rtxA* gene shares homology to a domain found in toxins from *Bordetella pertussis* (agent for whooping cough) and pathogenic *E. coli* (Cirillo, Lum, and Cirillo, 2000). The shared domain mediates Ca^{2+}-dependent binding that is necessary for toxin activity and attachment to host cells (Boehm, Welch, and Snyder, 1990; Welch, 2001). An *L. pneumophila rtxA* null mutant was 50 % less adherent to monocytic and epithelial cell lines than wild-type cells (Cirillo *et al.*, 2001). In addition, the *rtxA* null mutant was less cytotoxic to monocytes and displayed a reduced ability to form pores in human monocytic and murine cell lines as compared to wild-type (Cirillo *et al.*, 2001). Whether RtxA is secreted or is a cell-surface associated molecule has not been ascertained. It is postulated that RtxA from *L. pneumophila* may bind to β_2 integrins as is the case for the Rtx proteins from other bacteria (Cirillo *et al.*, 2001).

1.5.6. HtpB.

The *L. pneumophila* 60-kDa chaperonin, called HtpB, is a surface-localized molecule (Garduno *et al.*, 1998) that mediates the attachment to and the invasion of non-phagocytic

HeLa cells (Garduno, Garduno, and Hoffman, 1998). HtpB-coated latex beads compete with virulent *L. pneumophila* for association with HeLa cells (Garduno, Garduno, and Hoffman, 1998). The pre-treatment of HeLa cell monolayers with purified HtpB, or the treatment of virulent *L. pneumophila* with anti-HtpB antibodies greatly reduces the capacity of *L. pneumophila* to invade HeLa cells (Garduno, Garduno, and Hoffman, 1998).

Consistent with the hypothesis that HtpB may play an important role in virulence is the finding that avirulent *L. pneumophila* strains display less surface-associated HtpB, as seen by immune fluorescence (Hoffman, Houston, and Butler, 1990) and trypsin degradation assays (Garduno, Garduno, and Hoffman, 1998) than do their virulent isogenic counterparts. In addition MIFs, postulated to be the transmissible form of the bacterium, are enriched for HtpB on the cell surface as determined by immune-localization studies, and are 100-fold more adherent to HeLa cell monolayers as compared to agar grown bacteria (Garduno, Garduno, and Hoffman, 1998). *L. pneumophila* releases large amounts of the HtpB protein into its phagosome inside HeLa cells (Fernandez *et al.*, 1996; Garduno *et al.*, 1998), the purpose of which is currently under investigation.

1.6. Host receptors for attachment and adherence.

1.6.1. Complement and Fc receptors.

Receptors that promote the internalization of *L. pneumophila* by monocytic cell lines *via*

opsonin-mediated endocytosis have been identified. The pre-treatment of *L. pneumophila* with either non-immune or immune sera containing the complement component C3 leads to the uptake of *L. pneumophila via* the CR1 and CR3 complement receptors (Payne and Horwitz, 1987). C3 primarily fixes onto OmpS and the C3-OmpS ligand is sufficient to mediate uptake by monocytes or macrophages (Bellinger-Kawahara and Horwitz, 1990). *L. pneumophila* treated with immune serum containing anti-*L. pneumophila* antibodies can also be internalized by Fc integrin receptors (Husmann and Johnson, 1992). However, since complement levels in the human lung are usually low and anti-*L. pneumophila* antibodies would be unavailable in the early stages of infection perhaps yet unidentified receptors play a more pivotal role in mediating *L. pneumophila* uptake (Swanson and Hammer, 2000).

1.6.2. Protozoan receptor.

In the case of the protozoan host, *Hartmanella vermiformis*, a putative 170-kDa galactose/*N*-acetylgalactosamine (Gal/Gal-NAc)-specific lectin receptor may play a key role in mediating the invasion of *H. vermiformis* cells by *L. pneumophila* (Venkataraman *et al.*, 1997). The addition of Gal or Gal-NAc inhibited the invasion of *H. vermiformis* cells by *L. pneumophila* by 83% and 75% respectively, while the addition of anti-lectin monoclonal antibodies, H85 and IG7, which are specific to the 170-kDa receptor homologue found in *Entamoeba histolytica*, at the highest concentration of antibody used in these studies, reduced *L. pneumophila* invasion into *H.vermiformis* cells by 59% and

49% respectively; this effect was dose-dependent (Venkataraman *et al.*, 1997). These results indicate that Gal/Gal-NAc-sensitive factors play role in mediating *L. pneumophila* invasion into *H. vermiformis*, and that the 170-kDa Gal/Gal-NAc lectin is, in part, responsible for *L. pneumophila* invasion into *H. vermiformis* cells (Venkataraman *et al.*, 1997). The creation of a deletion strain for the gene encoding the 170-kDa lectin to evaluate the putative lectin's role in mediating the invasion of *H. vermiformis* cells by *L. pneumopihla* would definitively clarify the essentiality of the 170-kDa protein for invasion and virulence. Uptake of *L. pneumophila* by another protozoan host, *Acanthamoeba polyphaga*, however, is not completely blocked by the addition of Gal or Gal-NAc, indicating that *L. pneumophila* may use multiple and diverse receptors to bind to different protozoan host cells (Harb *et al.*, 1998). Consistent with this finding is that *L. pneumophila* mutants that are greatly incapacitated in their ability to attach to *A. polyphaga* are only minimally affected in their capacity to bind to *H. vermiformis* (Harb *et al.*, 1998).

1.6.3. Putative receptor for HtpB.

Although a host receptor for HtpB binding or internalization has not been identified, one would imagine that it could serve as a universal receptor for entry into mammalian cell lines and protozoan hosts, since the Hsp60 homologues are proteins that are highly conserved in prokaryotes and eukaryotes (Zeilstra-Ryalls, Fayet, and Georgopoulos, 1991; Lund, 1995). A member of the Toll-like receptor family, which was originally

discovered in *Drosophila*, could be a putative receptor for HtpB. Toll-like receptors are found on the surface of antigen presenting cells (APCs) such as macrophages and dendritic cells in mammals (Singh-Jasuja *et al.*, 2001). The Toll-like receptors (TLRs) have affinities for molecules that are common to many bacterial pathogens (Medzhitov and Janeway, Jr., 2000). Recognition of pathogen-derived ligands such as LPS, peptidoglycans, and bacterial CpG-DNA occurs *via* TLR4, TLR2 and TLR9 respectively (Aderem and Ulevitch, 2000; Hemmi *et al.*, 2000). Activation of the TLRs results in the induction of pro-inflammatory cytokines and costimulatory molecules that mediate activation of APCs and adaptive immunity (Modlin, Brightbill, and Godowski, 1999; Singh-Jasuja *et al.*, 2001). Recently TLR2 and TLR4 on the surface of macrophages were identified as receptors for endogenous Hsp60 and a chlamydial Hsp60 homologue (Vabulas *et al.*, 2001).

Since Hsp60 homologues elicit a potent pro-inflammatory response in cells of the innate immune system, it would seem a counterproductive strategy for *L. pneumophila* to utilize HtpB for attachment to APCs. In fact, it is known that IFNγ activated macrophages do not support *L. pneumophila* replication (Bhardwaj, Nash, and Horwitz, 1986), and that immune-compromised individuals primarily succumb to the organism (Fraser *et al.*, 1977). Since *L. pneumophila* is not believed to have adapted to the multicellular human host, its use of Hsp60 for binding to mammalian cells may not be an evolutionarily selected process, but rather a circumstantial one.

Despite this diversity in receptors reported for *L. pneumophila* uptake, the internalization of *L. pneumophila* by both amoebae and macrophages can occur by a common process termed coiling phagocytosis (Horwitz, 1984; Bozue and Johnson, 1996) which, as the name suggests, involves the whirling of plasma membrane around the bacterial cell. Heat-killed *Legionellae*, avirulent mutants or aldehyde-fixed *L. pneumophila* cells are targeted to lysosomes, despite being taken up by coiling phagocytosis (Swanson and Hammer, 2000), suggesting that coiling phagocytosis is not a pre-requisite for the intracellular survival of *L. pneumophila.* It is postulated that coiling phagosomes result when membranes of the phagocytic pseudopodia fail to fuse thus triggering the plasma membrane to coil around the particle to be ingested (Rittig, Burmester, and Krause, 1998). Perhaps the *L. pneumophila* organism has an effector(s) that perturbs the cellular machinery required for pseudopodia fusion regardless of the bacterium's mode of entry. Whether *L. pneumophila*'s mode of entry determines its ability to evade intracellular digestion is uncertain, but seeing that *L. pneumophila* evades intracellular digestion in diverse hosts despite its use of different entry mechanisms indicates that processes may be independent.

1.7. Endocytosis and phagosome dynamics.

The plasma membrane serves to maintain the integrity of biological units, and the movement of compounds across the plasma membrane must be regulated to prevent the homogenization of biological units with the extracellular environment. While small

molecules (sugars, amino acids, ions etc.) may traverse the membrane by way of integral membrane transporters, pumps and channels, larger molecules are taken into the cell surrounded by a membrane-bound spherical vesicle called an endosome (Conner and Schmid, 2003). Endocytosis can be defined as the process of taking up macromolecules by invagination and pinching off of the plasma membrane of eukaryotic cells (Conner and Schmid, 2003). Once formed, endosomes mature as they acquire specific markers through their interaction with the endocytic pathway, to become early endosomes, then late endosomes, and culminating in their interaction with lysosomes, where incoming macromolecules are degraded by acid hydrolases (Kirchhausen, 2000). The endocytic process encompasses both the uptake of particulate matter, termed phagocytosis, or cell feeding, and the uptake of fluids, termed pinocytosis, or cell drinking (Conner and Schmid, 2003). Pinocytosis and phagocytosis differ with respect to the molecules taken up, the size of the vesicles generated, and the mechanism of uptake, but are similar in that they both are actin-dependent processes (Conner and Schmid, 2003).

1.7.1. Phagocytosis.

Phagocytosis involves the uptake by eukaryotic cells of particles that are >0.5 μm in size (Tjelle, Lovdal, and Berg, 2000). It is through this process that bacterial pathogens are taken up by APCs such as macrophages and dendritic cells of the innate immune system (Greenberg and Grinstein, 2002). Particles are internalized through the interaction of receptors on the phagocyte with ligands present on the incoming particle (Aderem and

Underhill, 1999). Common bacterial surface ligands include LPS, lipoteichoic acids, and formylated peptides, which are recognized by host receptors such as integrins and scavenger receptors (Aderem and Underhill, 1999). Alternatively, the eukaryotic cell can opsonize pathogens using complement and antibodies which serve as ligands for complement receptors and Fc receptors, respectively (Aderem and Underhill, 1999). Receptor-ligand interactions allow for: (i) a zippering effect to occur between eukaryotic plasma membrane and the bacterial cell, (ii) the polymerization of actin, (iii) the engulfment of the bacterial cell, and signal generation (iv) (Conner and Schmid, 2003). The outputs associated with receptor ligand interaction can be unique. For example, while bacterial-cell uptake often results in the activation of the adaptive immune response, the uptake of apoptotic cells by the CD14 receptor does not result in activation of the immune response (Aderem and Underhill, 1999). After internalization pathogens are housed within a membrane-bound vesicle called a phagosome that is targeted to the lysosome, which contains acid hydrolases that serve to degrade the incoming pathogen (Greenberg and Grinstein, 2002). Peptides generated from intracellular digestion are then presented on the surface of APCs in the context of MHC for the purpose of activating cell-mediated immunity (Greenberg and Grinstein, 2002).

Passage of the phagosome from the plasma membrane to the lysosome occurs via sequential fusion/fission events with early and late endosomes, which results in the biogenesis of a phagosome that resembles endocytic vesicles in terms of its membrane composition (Tjelle, Lovdal, and Berg, 2000). It is postulated that there is specificity

involved with each fusion event, such that late but not early endosomes have the capacity to fuse with lysosomes (Desjardins *et al.*, 1994b). Eukaryotic cells must maintain the integrity of their organelles, and to accomplish this, the cells must impart specificity on its fusion events. Over 200 proteins have been found to associate with phagosomal membranes, and only a few have been characterized (Desjardins *et al.*, 1994a). Some of the key players that orchestrate specificity include the Rab (Ras protein from rat brain) family of proteins, SNAREs (soluble N-ethylmaleimide-sensitive fusion protein [NSF] attachment receptors) and the coat proteins clathrin, COPI, (for coat protein complex I), and COPII.

1.7.2. SNAREs.

The SNARE family of proteins is conserved in higher and lower eukaryotes (Armstrong, 2000; Ferro-Novick and Jahn, 1994) and is responsible for promoting the docking and coalescence of lipid layers during membrane fusion (Pfeffer, 1999). The SNARE proteins work as partners to promote fusion events: v-SNAREs reside on vesicles while t-SNAREs reside on the target membranes. Cognate receptors interact via coiled-coil domains to form a thermodynamically stable structure that promotes membrane fusion (Chen and Scheller, 2001). SNARE receptors are sufficient for mediating in vitro fusion events, and impart some specificity for fusion, in that cognate SNAREs are often exclusive to one another (Pelham, 2001). However, they are not sufficient to impart complete specificity in vivo (Whyte and Munro, 2002). If cognate v- and t- SNAREs

were active at all times, organelles in the cell might become stuck together (Pfeffer, 1999). Therefore it stands to reason that there exist other proteins that regulate SNARE assembly and accessibility, such as the Rab proteins.

1.7.3. Rab proteins.

There exist over 60 genes encoding Rab proteins in the human genome (Seabra, Mules, and Hume, 2002) and 11 Rab proteins in the Baker's yeast, *Saccharomyces cerevisiae* (Gotte *et al.*, 2000). Rab proteins are small GTP-binding proteins that act as molecular switches that cycle between: (i) donor and target membranes, (ii) an "on" or active state while bound to GTP, and an "off" or inactive state while bound to GDP, and, (iii) a membrane-bound state and a cytosolic state (Gotte *et al.*, 2000). Rab activity is regulated by several proteins. The activation of the Rab proteins depends on a guanine exchange factor (GEF) (Sprang and Coleman, 1998), which catalyzes the exchange of GDP for GTP, while Rab inactivation is mediated by Rab GTPase activating proteins (Rab-GAPs) that can accelerate the very slow intrinsic rate of GTP hydrolysis inherent in the Rab proteins (Scheffzek, Ahmadian, and Wittinghofer, 1998). Rab protein in the GDP-bound state, but not the GTP-bound state can be removed from the plasma membrane and solubilized into the cytoplasm by a Rab-GDP dissociation inhibitor (Rab-GDI) (Gotte *et al.*, 2000).

The Rab proteins play key roles in mediating fusion specificity and vesicle motility during membrane trafficking and perhaps vesicle formation (Schimmoller,

Simon, and Pfeffer, 1998; Tjelle, Lovdal, and Berg, 2000). For example, the Rab5 protein specifically coordinates fusion between early endosomes, while the Rab7 protein is associated with late endosomal compartments (Desjardins *et al.*, 1994b). Rab proteins have the capacity to "recognize" specific endosomes and organelles. For example, the Rab1 protein is responsible for directing vesicles from the ER to the Golgi (Plutner *et al.*, 1991). When the Golgi is treated with the fungal metabolite brefeldin A it empties its enzyme repertoire leaving a remnant structure called GRASP (Pfeffer, 2001). Rab1 was shown to directly interact with a Golgi-specific complex made up of the *cis* Golgi matrix protein, GM130, and GRASP65 (Nakamura *et al.*, 1995; Weide *et al.*, 2001). Aside from its capacity to directly recognize specific organelle structures Rabs also recruit proteins that mediate membrane association, such as the SNAREs and membrane tethering complexes (Schimmoller, Simon, and Pfeffer, 1998). The membrane tethering complexes work upstream of the SNAREs to mediate membrane association (Pfeffer, 1999). Tethering complexes have a working distance of greater than 25 nm, which is greater than half the diameter of a vesicle from a given membrane surface, while the SNAREs are responsible for mediating fusion post membrane docking, which represents a distance of less than 5-10 nm (Pfeffer, 1999). Rabs have also been reported to interact with proteins that regulate cell cytoskeleton-dependent motility. Rab6, for example, interacts with a kinesin-like motor protein called Rabkinesin6, implying a role in microtubule associated motility (Schimmoller, Simon, and Pfeffer, 1998). In addition, Rabphilin, a Rab3a effector interacts with α-actinin, an actin bundling protein, and may

orchestrate reorganization of the local actin-based cytoskeleton (Schimmoller, Simon, and Pfeffer, 1998). Therefore it is clear that Rabs play various roles in the processes of vesicle transport and fusion.

1.7.4. Coat proteins: Clathrin, COPI and COPII.

Central to the process of endocytosis and vesicle transport are the cytosolic coat proteins that are recruited onto target membranes, where they deform the membranes to form vesicles. There are three major coat proteins reported thus far and they function at different points in the endocytic and exocytic pathways. Exocytosis involves the transport of proteins between the ER, the Golgi and *trans*-Golgi network, from where proteins are sorted to their final destination within the eukaryotic cell or exported outside the cell. This is in contrast to endocytosis which involves the transport of macromolecules from the extracellular environment to inside eukaryotic cell. The three major coat proteins are clathrin, COPI and COPII, which are thoroughly reviewed by Kirchhausen, 2000. Clathrin-coated vesicles transport cargo from the plasma membrane to endosomes, and from the Golgi to endosomes; COPI coated vesicles transport cargo from the Golgi to the ER, and between Golgi cisternae; COPII vesicles transport cargo from the ER to the Golgi (Kirchhausen, 2000) (Fig. 1).

Here, the reaction cycle for the process of vesicle formation is exemplified by the COPII assembly system, and can be divided into the following steps: cargo selection, deformation of the budding membrane, scission to detach the new vesicle, loss of coat

proteins to allow for fusion of the new vesicle to its target membrane, and the recycling of coat proteins (Kirchhausen, 2000) (Fig. 2). COPII vesicles appear to be the simplest system reported thus far for coat assembly-dependent transport. The minimal requirement for the formation of COPII vesicles includes five proteins: the Sec23-Sec24p complex, the Sec13-Sec31p complex and the small GTPase Sar1p (Barlowe *et al.*, 1994). Sar1p is recruited to the ER membrane by its guanine exchange factor, Sec12p (Barlowe and Schekman, 1993), where it mediates the association of Sec23-Sec24p with cargo proteins, and with SNARE proteins (Springer and Schekman, 1998). Sar1p also recruits, to the ER membrane, the Sec13-Sec31p complex (Barlowe *et al.*, 1994) which is thought to act as a scaffold that deforms the membrane (Kirchhausen, 2000). At the end of the reaction cycle, the hydrolysis of GTP bound to Sar1p is significantly increased through the activity of Sec23p , a GTPase activating protein (Yoshihisa, Barlowe, and Schekman, 1993). It is proposed that uncoating of the vesicle, which must precede fusion of the vesicle to its target membrane, occurs spontaneously after Sar1p-GTP hydrolysis (Kirchhausen, 2000).

1.7.5. *L. pneumophila* mediated phagosomal trickery.

Molecular evidence is mounting concerning a mechanism for *L. pneumophila*'s capacity to alter its phagosome for the purpose of promoting survival and replication. When

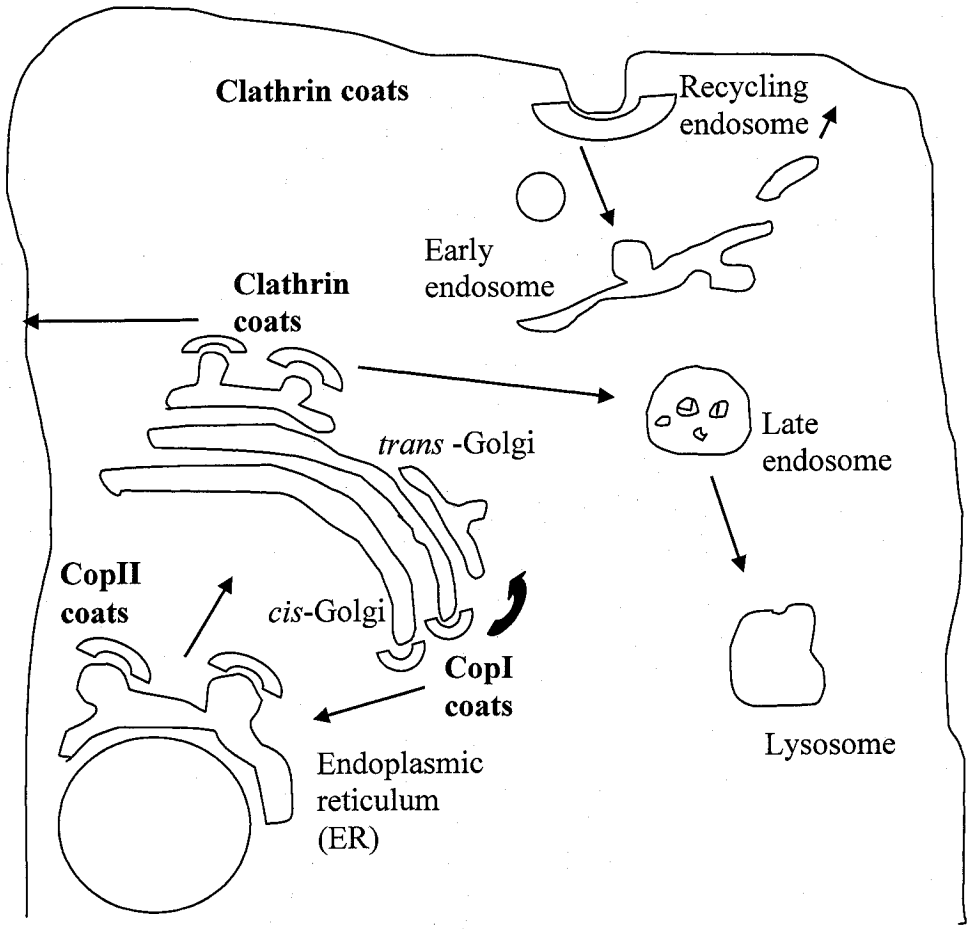


Figure 1. The main pathways for clathrin-, COPI- and COPII- mediated vesicle transport in eukaryotic cells. Clathrin-coated vesicles move along two major routes, from the plasma membrane to the ER and from the Golgi to late endosomes. COPI-coated vesicles shuttle from the Golgi to the ER and between Golgi cisternae. COPII vesicles are transported from the ER to the Golgi. This figure was adapted from Kirchhausen, 2000.

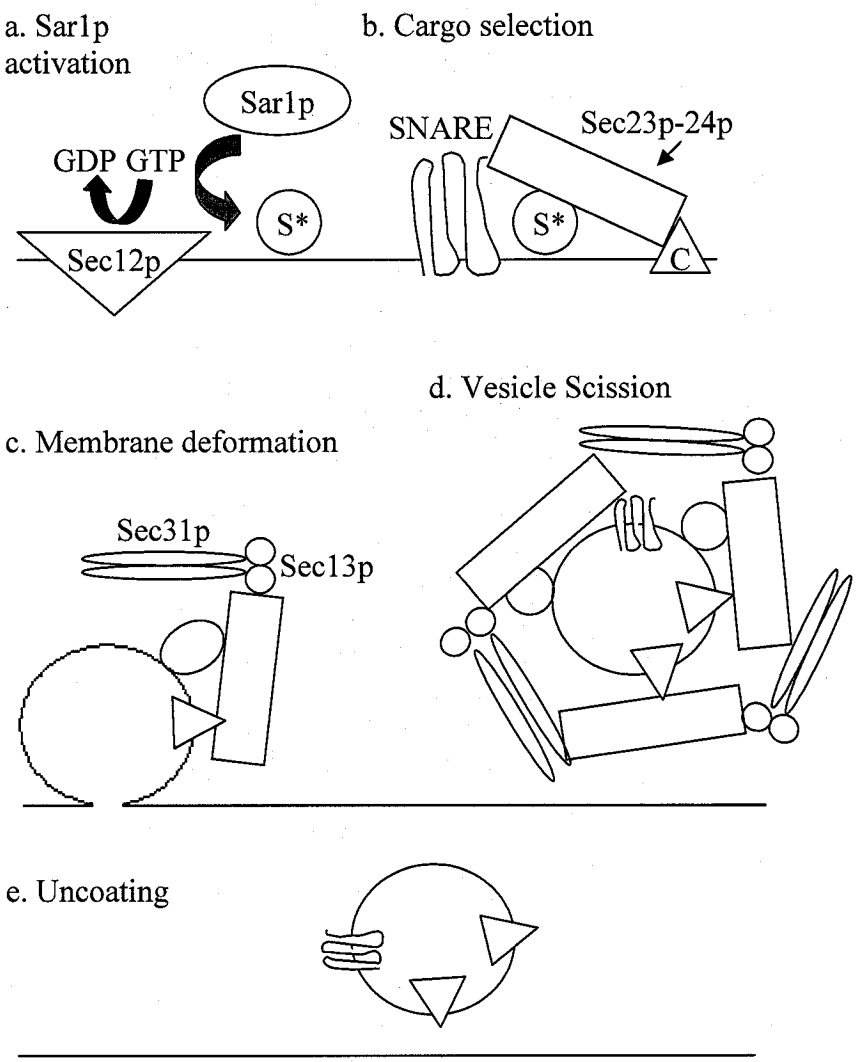


Figure 2. Key steps for COPII vesicle formation. (a) The Sar1p GTPase is recruited to the membrane and is activated by it guanine exchange factor, Sec12p. (b) Activated Sar1p recruits the Sec23p-Sec24p complex, which is required for cargo selection. (c) The Sec31p-Sec13p complex is recruited, and membrane deformation is catalyzed. (d) The coat is completed, and the vesicle buds(e) Sec23p enhances Sar1p GTPase activity, which leads to Sar1p inactivation and uncoating. C = cargo; S* = Sar1p-GTP; horizontal line = membrane. This figure was adapted from Kirchhausen, 2000.

virulent and avirulent strains of *L. pneumophila* were used to infect a HeLa cells engineered to overexpress the Rab5c isoform, virulent strains of *L. pneumophila* were housed within endosomes that contained very little or no Rab5c, for up to 8 hours post infection, as detected by electron microscopy and immunogold staining (Clemens, Lee, and Horwitz, 2000). In contrast, phagosomes containing an avirulent strain of *L. pneumophila* showed staining for Rab5c as early as 15 minutes post infection (Clemens,Lee, and Horwitz, 2000). These data suggest that virulent *L. pneumophila* has the capacity to exclude the Rab5c protein from the phagosomal membrane, thus removing the phagosome from the endocytic pathway. Perhaps a bacterial effector that is translocated by the Dot/Icm secretion system (see above) into host cells functions to exclude active Rab5c from the phagosomal membrane. A functional Dot/Icm secretion system is required during and immediately after internalization of *L. pneumophila* to facilitate a delay in phagosome-lysosome fusion but not later in the infection process (Roy, Berger, and Isberg, 1998). This time frame coincides with the exclusion of Rab5c from the *L. pneumophila* phagosomal membrane.

While Rab5c appears to be excluded from the *L. pneumophila* phagosome, the Rab1 protein, which mediates ER-Golgi vesicle transport, is recruited to the *L. pneumophila* phagosome by 30 minutes post-infection (Derre and Isberg, 2004; Kagan *et al.*, 2004). An avirulent *L. pneumophila dotA* mutant, on the other hand, did not recruit Rab1 to its phagosome, indicating that the Dot/Icm system is required for Rab1 recruitment to the *L. pneumophila* phagosomal membrane (Derre and Isberg, 2004;

Kagan *et al.*, 2004). Furthermore, it was demonstrated that Rab1 recruitment was important for *L. pneumophila* replication. Expression of the Rab1S25N dominant negative allele in a Chinese hamster ovary (CHO) cell line significantly reduced *L. pneumophila*'s capacity to replicate intracellularly (Kagan *et al.*, 2004). Rab1 activity was required for the recruitment of the ER-Golgi specific v-SNARE, Sec22b, to the *L. pneumophila* phagosome (Kagan *et al.*, 2004). The expression of either of the dominant negative alleles for GTPases that regulate COPII and COPI vesicles, Sar1H79G and ARF1T31N respectively, or use of brefeldin A, disrupts the production of ER secretory vesicles, and was shown to delay the recruitment of Sec22b to the *L. pneumophila* phagosome in CHO cells (Kagan *et al.*, 2004). Furthermore, the inhibition of Sec22b function, achieved through the overexpression of the Sec22b cognate t-SNARE, membrin, greatly reduced *L. pneumophila*'s capacity to replicate intracellularly (Kagan *et al.*, 2004). Therefore, Rab1 recruitment of Sec22b to the *L. pneumophila* phagosome appears to play an integral role in the biogenesis of a vacuole that is permissive to *L. pneumophila* replication inside eukaryotic cells. Taken together it appears that *L. pneumophila* escapes from the endocytic pathway and early lysosomal digestion by taking up residence in the exocytic pathway in association with ER-derived vesicles.

1.8. Acquisition of nutrients.

The primary source of nutrition for *L. pneumophila* inside eukaryotic cells is most likely a supply of free amino acids (Shuman *et al.*, 1998). Consistent with this is the

observation that *L. pneumophila* associates with ER derived vesicles as early as 15 minutes post infection and later resides within the ribosome-studded ER, the site of protein synthesis (Tilney *et al.*, 2001; Swanson and Isberg, 1995). By 16 hours post infection the *L. pneumophila* phagosome also associates with lysosomal compartments which may also contain peptides that result from the digestion of phagocytosed particles (Sturgill-Koszycki and Swanson, 2000). *L. pneumophila* is known to secrete proteases (Nolte, Hollick, and Robertson, 1982; Quinn and Tompkins, 1989; Keen and Hoffman, 1989; Black, Quinn, and Tompkins, 1990), which may assist in the further breakdown of peptides to be used for nutrition.

L. pneumophila requires iron for growth in vivo. *L. pneumophila* has the capacity to replicate inside naïve, but not INFγ-activated macrophages (Byrd and Horwitz, 1989). The ability of activated macrophages to restrict bacterial growth was linked to their ability to limit the availability of intracellular iron (Byrd and Horwitz, 1989). *L. pneumophila* can, in fact, replicate in activated macrophages when iron-saturated transferrin, a labile iron source, is added exogenously to cell cultures (Byrd and Horwitz, 1989). Conversely, the replication of *L. pneumophila* is inhibited in naïve macrophages that are treated with iron chelators (Byrd and Horwitz, 1989). To further support the hypothesis that iron-limitation inhibits *L. pneumophila* replication inside eukaryotic cells, by fluorescence-activated flow cytometry it was demonstrated that activated macrophages have fewer transferrin receptors, on their cell surface, as compared to naïve macrophages. This would limit the acquisition of iron-saturated transferrin, by receptor

mediated endocytosis (Byrd and Horwitz, 1989). Therefore, it would appear that activated macrophages limit iron availability as a strategy to restrict *L. pneumophila* intracellular growth.

The fact that *L. pneumophila* does bear the iron-acquisition molecule legiobactin, a non-classical siderphore, further highlights the importance of iron for its survival (Liles, Scheel, and Cianciotto, 2000). The exact role that iron plays in maintaining *L. pneumophila* viability is unclear. However, the discovery of *fur* that encodes a *L. pneumophila* homologue of Fur, a protein that represses gene transcription in the absence of iron, indicates that *L. pneumophila* gene expression may be dependent on the availability of iron (Hickey and Cianciotto, 1994). Indeed, insertional inactivation of a Fur-regulated gene, named *frgA*, was found to impair the intracellular growth of *L. pneumophila* (Hickey and Cianciotto, 1997).

1.9. Host cell death and exit from the host cell.

1.9.1. Apoptosis.

Apoptosis, also known as programmed cell death allows eukaryotic organisms to eliminate unwanted cells through a controlled process of cell destruction that is characterized by shrinkage of the cytoplasm, condensation of nuclear chromatin, nuclear fragmentation, the clustering of organelles, and the formation of ER-derived vacuoles, and is advantageous in that it does not result in the leakage of cellular components or the induction of an inflammatory response (Anderson, 1997). The caspases, a family of

cysteine proteases, are mainly responsible for causing the morphological changes that are associated with the apoptotic process (Golstein, 1997). Apoptosis may be initiated by eukaryotic cells to destroy viral or bacterial infected cells (Wyllie, 1997).

U937 and HL-60 macrophage-like cells lines undergo apoptosis when infected with virulent *L. pneumophila* strains, such that caspase-3 levels inside HL-60 cells rise quickly post-infection, and nuclear fragmentation occurs several hours post infection (Muller, Hacker, and Brand, 1996; Gao and Abu Kwaik Y., 1999; Abu-Zant *et al.*, 2005). *L. pneumophila*-induced apoptosis differs from apoptosis that takes place during the positive and negative selection of T-cells in the thymus, (Anderson, 1997) or apoptosis that is triggered in response to the pharmacological agents, saturosporine or tumour necrosis factor α (TNFα) (Abu-Zant *et al.*, 2005), in that cell destruction occurs much more rapidly for the latter stimuli (within an hour). Therefore it is plausible that *L. pneumophila* stalls the apoptotic process to allow for replication and persistence inside its host cell. Indeed other pathogens including the obligate intracellular bacteria *Rickettsia rickettsii* and *Chlamydia trachomatis* inhibit the apoptotic process in their hosts to promote intracellular growth (Clifton *et al.*, 1998; Fan *et al.*, 1998). The preactivation of apoptosis using saturosporine and TNFα, in HL-60 cell prevents *L. pneumophila* intracellular replication (Abu-Zant *et al.*, 2005), while the inhibition of caspase 3 activity in U937 macrophages and human PBMs blocks the intracellular replication of *L. pneumophila* (Molmeret *et al.*, 2004). These data indicate *L. pneumophila* infection triggers the elevation of caspase 3 levels in HL-60 cells, which may play a role in

promoting intracellular infection; however, the later stages of apoptosis appear to be inhibitory for *L. pneumophila* replication, so that the bacterium may stall the later apoptotic stages to promote intracellular growth.

1.9.2. Necrosis.

L. pneumophila can induce an uncontrolled cell death also known as necrosis (cell death due to injury) in both mammalian and protozoan host cells. *L. pneumophila* has the capacity to create pores in the membranes of macrophages, human red blood cells, and *A. polyphaga*, which can result in cell lysis (Kirby *et al.*, 1998; Gao and Abu Kwaik Y., 2000). Pore-formation and exit from host cells by *L. pneumophila* is dependent on the *icmT* locus (Molmeret *et al.*, 2002). Mutants competent to replicate inside macrophages and protozoan cells, but disabled in their ability to escape the phagosome or exit the host cell were found to have a deletion in the *icmT* gene that is encoded within *dot/icm* gene loci (Molmeret *et al.*, 2002). Since these mutants could replicate inside macrophages, it is possible that their inability to form pores is directly tied to IcmT function as opposed to a defect in the transport of proteins by the Dot/Icm system, since a functional Dot/Icm system is essential for intracellular replication. Genetic complementation of the *icmT* mutants with the wild-type gene resulted in restoration of pore-mediated egress from mammalian and protozoan host cells (Molmeret *et al.*, 2002). *L. pneumophila* possesses another protein, called the RtxA toxin, that promotes the attachment of *L. pneumophila* to host cells (discussed above), and that is also capable of forming pores in host cell

membranes (Cirillo *et al.*, 2001). Therefore exit from host cells appears to be an *L. pneumophila*-directed process, since *L. pneumophila* possesses at least two pore-forming molecules, RtxA and IcmT.

1.10. Strategies for treatment and prevention.

L. pneumophila infections in humans are successfully treated through the use of antibiotics, with erythromycin +/- rifampin as the recommended choice (Gilbert, Moellering, and Sande, 1998). A vaccine against *L. pneumophila* has not been developed. One would imagine that it would be difficult to stimulate strong immune responses in immune-compromised persons. However, individuals at high risk for Legionnaires' pneumonia, such as the elderly, could benefit from a vaccine that could tip the balance in favour of their recovery.

Since *L. pneumophila* is an intracellular pathogen, which would be shielded from antibodies circulating in the serum of infected patients, perhaps *L. pneumophila* would be most efficiently combated by eliciting cell-mediated immunity as opposed to humoral immunity. Consistent with this hypothesis, 100 % of guinea pigs (n=9) vaccinated with purified OmpS protein, which has been shown to elicit low antibody titres and strong delayed-type hypersensitivity and lymphocyte proliferation responses, were protected from challenge with *L. pneumophila*; 0 % of ovalbumin immunized guinea pigs survived a lethal challenge dose (Weeratna *et al.*, 1994). This indicates that an immunization

strategy that activates cell-mediated immunity can serve as a successful strategy to prevent infection with *L. pneumophila*.

Part2. An Overview of Chaperonin Biology.

2.1. Protein folding function.

The most recognized physiological function for the chaperonin family is its role in facilitating the folding of newly synthesized proteins, under normal physiological conditions, and denatured proteins, in response to stress (Lund, 1995). To function as protein folders, the chaperonins must assemble into multi-subunit, double-ringed complexes that form a barrel-like structure equipped with a central cavity (Sigler *et al.*, 1998). ATP-binding to the complex and ATP hydrolysis causes structural changes in the chaperonin complex, which allows the polypeptide, housed within the central cavity, to achieve a functional tertiary conformation (Sigler *et al.*, 1998).

2.2. Classification.

Based on their structure and evolutionary origin, the chaperonins are divided into group I and group II chaperonins:

Group I chaperonins (HtpB, GroEL, Cpn60, or Hsp60) are typically found in eubacteria, mitochondria and chloroplasts (Zeilstra-Ryalls, Fayet, and Georgopoulos, 1991). They form homo-oligomeric rings that consist of seven subunits of the ~60-kDa

chaperonin (Braig *et al.*, 1994). To function in protein folding, two of the seven-membered rings come together to form a 14-mer complex that then associates with a third seven-membered homo-oligomeric ring, comprised of the ~10-kDa co-chaperonin (HtpA, GroES, Cpn10, or Hsp10) (Horovitz, 1998; Xu and Sigler, 1998). This co-chaperonin ring caps the chaperonin complex in an ATP-dependent manner, and assists with the binding and releasing of protein substrates (Fenton, Weissman, and Horwich, 1996). In *E. coli* over 300 different polypeptides depend on GroEL for folding (Houry *et al.*, 1999; Houry, 2001). Group II chaperonins are typically found in archaea and in the cytoplasm of eukaryotes, where they are known as TriC (TCP-1 ring complex) or CCT (chaperonin-containing TCP-1) (Lund, 1995). They form eight- or nine-membered hetero-oligomeric rings, with subunits that may have different masses (Klumpp and Baumeister, 1998; Kim, Willison, and Horwich, 1994). Group II chaperonins do not team with co-chaperonins. Instead, they have an extended apical domain thought to cap the central cavity of the double-ringed complex (Horwich and Saibil, 1998; Fenton, Weissman, and Horwich, 1996). The Group II chaperonins are considered to be more conservative in terms of the substrates they assist in folding, although the list of known Group II substrates is on the increase (Dunn, Melville, and Frydman, 2001). The eukaryotic CCTs were previously thought to exclusively assist with the proper folding of actin and tubulin subunits, but their folding repertoire has been expanded to include cyclin E, the Hepatitis B capsid protein, myosin, luciferase, α-transducin, and the Von Hippel-Lindau tumor suppressor protein (Dunn, Melville, and Frydman, 2001).

2.3. Expression and gene organization.

Most of the characteristics that we relate to the chaperonin family as a whole have been determined based on experiments using the *E. coli* GroEL as a model. In *E. coli* a shift from 30 °C to 42 °C results in the increased expression of more than 20 proteins called heat shock proteins (Hsps) (Arsene, Tomoyasu, and Bukau, 2000). Important members of this regulon include GroEL (Hsp60), and GroES (Hsp10), the chaperone DnaK (Hsp70), and its two co-chaperonins, DnaJ (Hsp40) and GrpE, as well as, a group of ATP-dependent proteases (Arsene, Tomoyasu, and Bukau, 2000). The heat shock-induced GroEL chaperonin functions to assist denatured proteins to reassume a functional conformation; hence eubacterial chaperonins have been designated as 60-kDa heat-shock proteins or Hsp60s (Zeilstra-Ryalls, Fayet, and Georgopoulos, 1991). The *E. coli* heat shock response is positively controlled by the heat-shock promoter-specific, σ subunit of RNA polymerase, σ^{32} (Yura and Nakahigashi, 1999; Arsene, Tomoyasu, and Bukau, 2000). Due to rapid turn-over, σ^{32} levels are very low in *E. coli* cells at 30°C (10-30 copies/cell), which limits the heat-shock response (Arsene, Tomoyasu, and Bukau, 2000). The DnaK chaperone plays a pivotal role in regulating σ^{32} levels (Yura and Nakahigashi, 1999). When DnaK molecules are present in excess, they associate with σ^{32} subunits, and indirectly target them for destruction in a negative feedback loop (Yura and Nakahigashi, 1999). It is postulated that under heat-shock conditions, DnaK is prevented from targeting σ^{32} for degradation due to its association with the large volumes of misfolded proteins that arise as a result of increased temperatures (Yura and Nakahigashi,

1999).

The *L. pneumophila* chaperonin HtpB is encoded within a two-gene operon that also contains the co-chaperonin HtpA (Hoffman, Houston, and Butler, 1990) (Fig. 3). The *htpA* gene (GenBank accession no.: M31917) is located 5' to *htpB* (GenBank accession no.: M31918), within the operon, which contains a single consensus heat shock promoter region upstream of *htpA* (Hoffman, Houston, and Butler, 1990) (see Fig. 1). This gene organization is commonly found in Gram-negative bacteria including *E. coli* (Segal and Ron, 1996). The *htpAB* operon is transcribed as a single ~2-kb polycistronic mRNA molecule that codes for HtpA, a 96 amino acid polypeptide with a molecular weight of ~15-kDa, and HtpB, a 548 amino acid polypeptide with a molecular weight of ~ 62-kDa (Hoffman, Houston, and Butler, 1990). A rho-independent transcription termination site is present immediately downstream of the *htpB* gene sequence, and the N-terminal Met-Ile-Met in HtpB is post-translationally cleaved to yield the mature protein (Hoffman, Houston, and Butler, 1990).

2.4. Structure of the GroEL chaperonin monomer.

The 60-kDa GroEL monomer is composed of 547 amino acids, which fold into three flexible functional domains (Sigler *et al.*, 1998) (Fig. 4a): (i) The apical (A) domain displays great mobility and a poorly ordered secondary structure. It surrounds the mouth of the chaperonin ring (Fig. 4a), and mediates hydrophobic interactions with protein substrates. (ii) Connected to the apical domain is the slender intermediate (I) domain that

links the "A" domain to the equatorial (E) domain of the protein, and that functions as a hinge that allows conformational movements to occur. (iii) The "E" domain provides most of the residues required for inter-subunit interaction and for ATPase activity, and forms the interface at which the two seven-membered rings associate. Each seven-membered ring forms its own separate protein folding cavity, such that each chaperonin complex contains two cavities (Fig. 4b). However, only one ring, referred to as the *cis* ring, is activated for protein folding at a given time. The ring unoccupied during the folding process is referred to as the *trans* ring. The C-terminal tails of the seven 60-kDa subunits serve to close off the two cavities, so that they are kept separate, at the equatorial interface of the *cis* and *trans* rings. Based on crystal structure information, each cavity is calculated to hold 85, 000 $Å^3$, equivalent to an approximately 70-kDa polypeptide.

2.5. The GroEL protein folding cycle.

The GroEL protein folding cycle can be divided into four phases (Sigler *et al.*, 1998) (Fig. 5): (i) Hydrophobic residues exposed, on the apical domains of the GroEL monomers in the *cis* ring, facilitate binding to the hydrophobic residues exposed on the surface of the polypeptide intermediate. At the point of polypeptide recognition, ATP and GroES are not found in association with the GroEL complex, and the two rings that make up the complex appear symmetrical. (ii) The binding of GroES and ATP to GroEL monomers of the *cis* ring results in the release of the protein intermediate into the central cavity. The GroES ring binds to the apical face of the GroEL *cis* ring, and seven ATP

nucleotides bind to the equatorial domains of GroEL monomers also in the *cis* ring. A drastic conformational change in the chaperonin complex, visualized as a loss of symmetry in the *cis* and *trans* rings results. This change serves to secure seven ATP nucleotides to the "E" domains, to expand the GroEL *cis*-ring cavity, and to convert the physiological character of the *cis*-ring. Amino acids located on the apical face of the *cis*-ring are rearranged so that the polypeptide is dislodged and released into the *cis*-ring cavity, where hydrophobic residues, once exposed along the inner wall of the chaperonin complex, become hidden between subunit interfaces, and are replaced with hydrophilic and polar residues. The polypeptide, now housed within the hydrophilic confines of the chaperonin cavity, folds in such a manner as to hide its exposed hydrophobic residues to achieve a proper tertiary structure. (iii) Hydrolysis of the *cis*-bound ATPs results in decay of the high-energy *cis*-state, which primes the complex for polypeptide and GroES release. (iv) The binding of ATP moieties to the equatorial domains found in GroEL monomers in the *trans* ring triggers GroES dissociation from the complex, and expulsion of the polypeptide from the *cis*-ring. A protein may undergo several rounds of this reaction cycle until it assumes its proper tertiary conformation.

2.6. Chaperonin complex assembly.

While many advances have been made in determining the mechanism for chaperonin-mediated folding, the steps involved in chaperonin complex assembly remain more

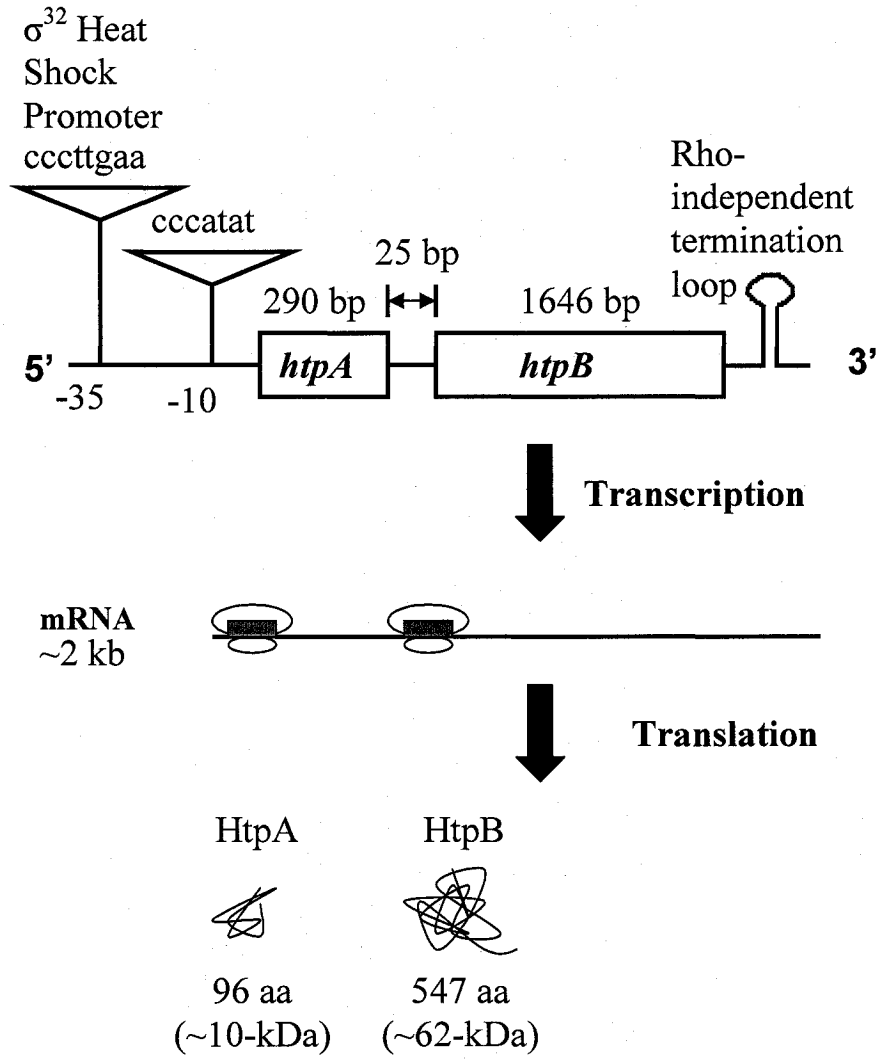


Figure 3. The *L. pneumophila htpAB* operon. The *htpA* gene is located upstream of *htpB*. The two genes are transcribed as a single mRNA transcript, approximately 2 kb in size, from a single σ^{32} heat shock promoter located upstream of *htpA*. Other promoter sequences are not shown in this figure. Translation initiation occurs from distinct sites for *htpA* and *htpB*, and results in the production of 96 aa and 547 aa protein products, respectively. Reported in Hoffman, Houston, and Butler, 1990.

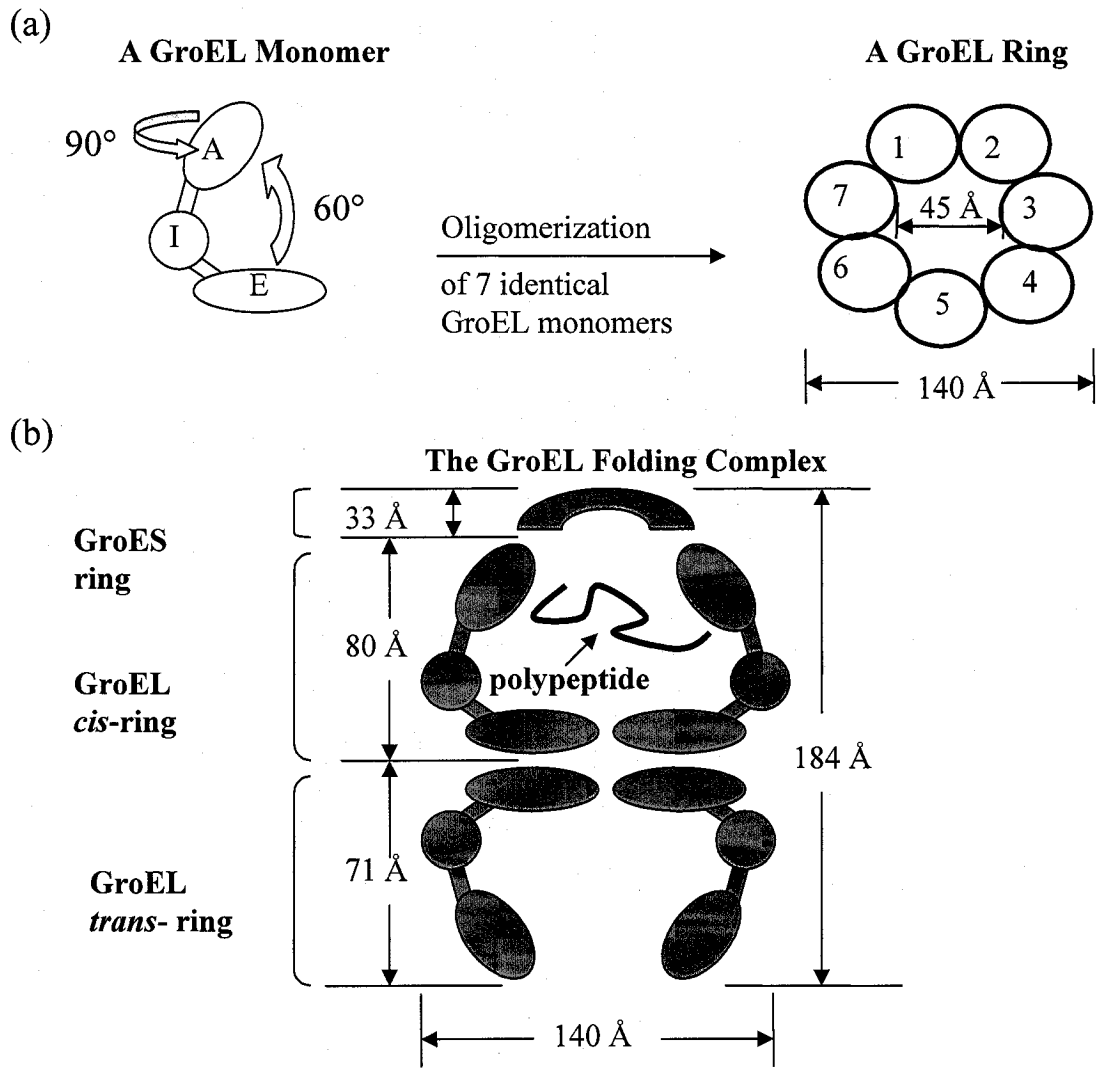


Figure 4. The GroEL chaperonin complex. (a) The GroEL monomer (~60-kDa) is comprised of an apical (A), an intermediate (I) and an equatorial (E) domain. Seven identical GroEL monomers oligomerize to form the GroEL ring, with an internal diameter of 45 Å and an external diameter of 140 Å. (c) The chaperonin folding complex (presented here in cross-section) is comprised of two seven-membered GroEL rings capped by a GroES ring. The GroES ring is comprised of seven, identical, 10-kDa subunits. Adapted from (Braig *et al.*, 1994; Xu, Horwich, and Sigler, 1997).

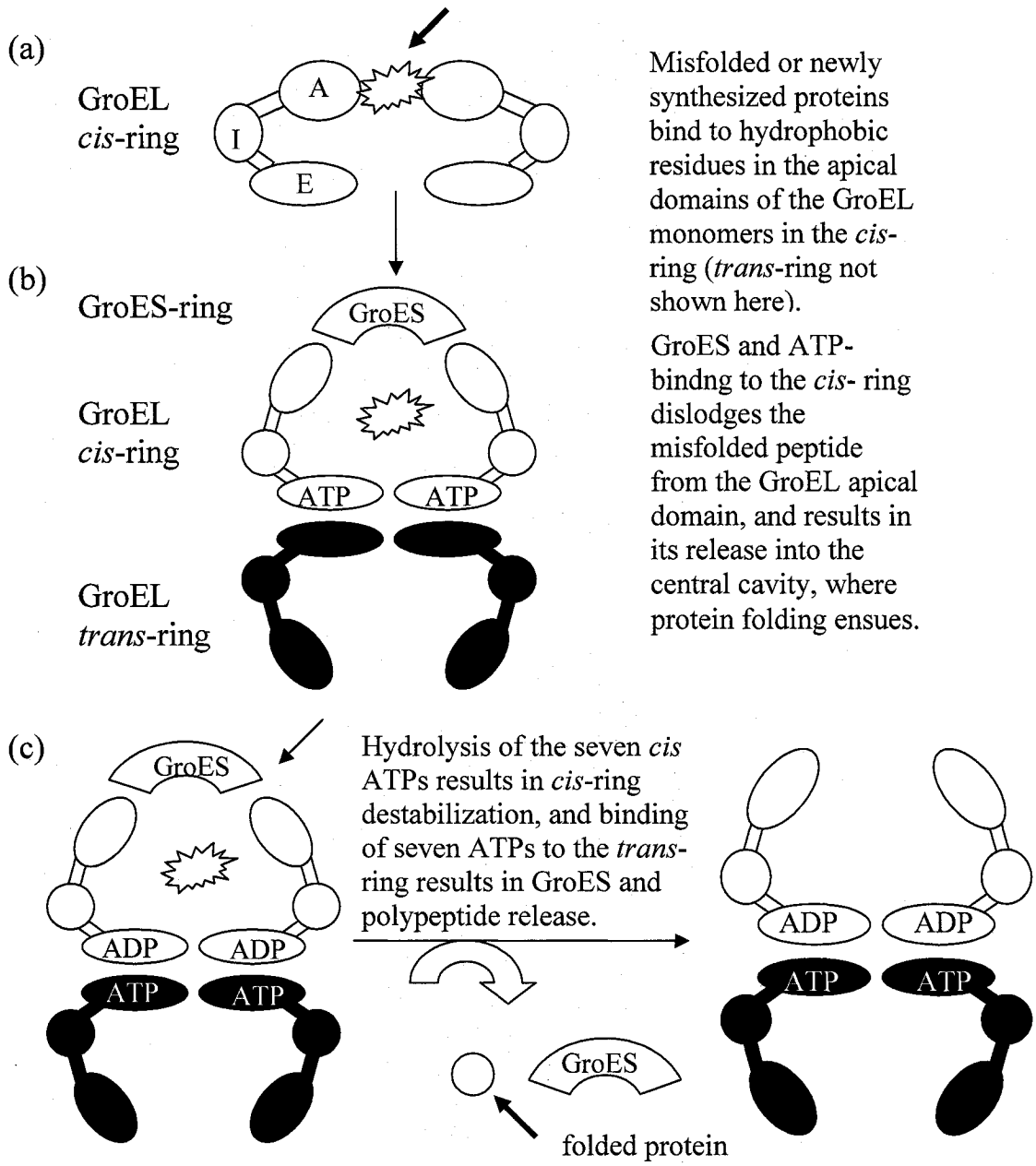


Figure 5. GroEL protein folding. (a) Misfolded proteins bind to the apical domains of GroEL *cis*-ring monomers and (b) are released into the *cis*-ring cavity upon ATP and GroES binding to the *cis* ring; protein folding ensues. (c) GroES is destablilized from the complex upon *cis*-ATP hydrolysis and the binding of ATP to the *trans*-ring. The protein is released from the complex and assumes a tertiary conformation. Adapted from Sigler *et. al.*, 1998.

elusive. The majority of data describing the assembly of the chaperonin complex come from in vitro studies done with purified chaperonin complexes and denaturing agents. GroEL monomers, denatured in 4M urea, spontaneously reassemble into tetradecamer complexes, upon removal of the denaturant, and the addition of the Mg^{2+}-ATP co-factor (Kusmierczyk and Martin, 2001). The assembly process is stimulated by the addition of already assembled tetradecamer complexes, and the GroES co-chaperonin (Kusmierczyk and Martin, 2001). It is postulated that the Mg^{2+}-ATP co-factor primes the GroEL monomers to assume an assembly-competent conformation that can then be chaperoned by already assembled complexes to form new tetradecameric structures (Kusmierczyk and Martin, 2001). Perhaps the binding of Mg^{2+}-ATP to GroEL stabilizes the equatorial domain to produce a more folding-competent form (Kusmierczyk and Martin, 2001). Alternatively, or concurrently, Mg^{2+}-ATP may promote monomer dimerization, setting the stage for cooperativity in the recruitment of other monomer subunits (Kusmierczyk and Martin, 2001).

2.7. Unique characteristics of bacterial chaperonins.

Recent studies show that bacterial chaperonins can deviate from the GroEL paradigm such that the expression of some chaperonins can be regulated independent of heat-shock, not all chaperonins are limited to a cytoplasmic location, and chaperonins have been shown to boast other functions in addition to protein folding (Table 1). In *Bradyrhizobium japonicum* (Fischer *et al.*, 1993), an endosymbiotic nitrogen fixing

Table 1. Unique characteristics of bacterial chaperonins.

Organism/Description	Membrane Location	Interesting Facts and Sources
Bordetella pertussis (causative agent for whooping cough)	Env	localizes Hsp60 to the cell surface, in a BvgS-dependent process (this study)
Bradyrhizobium japonicum (nitrogen fixing bacterium)	?	expresses five Hsp60 homologues, one of which has σ^{54}-specific promoter sequences, and is coexpressed with nitrogen fixation genes (Fischer *et al.*, 1993)
Brucella spp. (zoonosis that causes spontaneous abortion in wild and domestic animals and brucellosis manifested as arthritis, meningitis and endocarditis in humans)	Env	may use Hsp60 for intracellular survival, through its association with a cellular prion-like protein (PrP^c), that forms a tail-like structure, necessary for entry and survival inside host cells; Hsp60 is localized to the cell surface, in a VirB-dependent manner (Watarai *et al.*, 2003)
Buchnera aphidicola (symbiont of aphids)	EC	Hsp60 homologue has histidine kinase activity and circulates in the aphid hemolymph (Morioka *et al.*, 1994; van den Heuvel, Verbeek, and van der Wilk, 1994)
Chlamydiae (*C. pneumoniae*: causative agent for pneumonia and bronchitis; *C. trachomatis*: causative agent for blindness and STDs)	OM (*C. pneumoniae*)	*Chlamydiae* express three Hsp60 homologues; for *C. trachomatis* only one Hsp60 homologue is heat-shock regulated; the other two are constitutively expressed (Karunakaran *et al.*, 2003; Melgosa, Kuo, and Campbell, 1993; Kalman *et al.*, 1999b)
Helicobacter pylori (causative agent for chronic gastric disease)	PP, OM, EC	Hsp60 homologue mediates adherence to the gastric mucosa (Eschweiler *et al.*, 1993; Phadnis *et al.*, 1996; Yamaguchi *et al.*, 1998)
Legionella pneumophila (causative agent for pneumonia in the immune-compromised)	PP, OM, EC	Hsp60 homologue is an adherence and invasion factor for HeLa cells (Garduno *et al.*, 1998; Garduno, Garduno, and Hoffman, 1998); and activates a conserved eukaryotic signaling cascade (this study)
Mycobacterium leprae (causative agent for tuberculosis in mice)	Env	Hsp60 homologue has protease activity (Portaro *et al.*, 2002)

EC extracellular; Env., envelope; OM, outer membrane; PP, periplasm; STDs, sexually transmitted diseases; BvgS, virulence-associated sensor kinase; VirB, component of type IV secretion.

bacterium, and in *L. pneumophila* (Fernandez *et al.*, 1996), high-level Hsp60 expression is achieved independent of heat-shock. Furthermore, approximately half of the HtpB in *L. pneumophila* is extracytoplasmic, i.e. membrane-associated, periplasmic, or cell surface-associated (Garduno *et al.*, 1998). Therefore it is tempting to speculate that these Hsp60 homologues may have functions in addition to protein folding. Indeed the Hsp60 of *Buchnera aphidicola* (an endosymbiont of the aphid *Acrytisiphion pisum*) acts as a histidine kinase (Morioka *et al.*, 1994), that of *Enterobacter aerogenes* (a symbiont found in the saliva of *Myrmeleon* larvae) constitutes a potent insect toxin (Yoshida *et al.*, 2001), and that of *L. pneumophila* acts as an invasin and attachment factor to eukaryotic cells (Garduno, Garduno, and Hoffman, 1998). These observations illustrate that a view of eubacterial chaperonins solely as cytoplasmic protein folders is an underestimation of their functional capabilities.

Part 3. An Overview of Protein Secretion in Gram-negative Bacteria.

3.1. Background.

Extracellular secretion is essential for bacterial pathogenesis. It involves the active transport of proteins across the bacterial cell envelope. The Gram-negative bacterial cell envelope is a compartmentalized structure that consists of two distinct membranes, the cytoplasmic (inner) membrane and the outer membrane, which are separated by the periplasmic space and a peptidoglycan layer (Andersen, 2003). Since membranes form a

permeability barrier for hydrophilic compounds, specialized systems are required for protein transport into and out of the cell (Andersen, 2003). Gram-negative bacteria have devised numerous mechanisms to transport proteins across their cell envelopes. Here, type I, general secretion, type II, type III, type IV, type V and TAT secretion pathways are briefly reviewed.

3.2. Type I secretion.

Type I or ATP-binding cassette (ABC) transporters are ubiquitous and are involved in various physiological processes such as nutrient uptake, multi-drug resistance, and the secretion of toxins and signaling molecules (Schmitt and Tampe, 2002). In *E. coli* for example, there exit at least 80 ABC transporters (Andersen, 2003). Type I secretion substrates can range from ions to large molecules (Schmitt and Tampe, 2002). General features of type I secretion are exemplified by the *E. coli* hemolysin (Hly) secretion system (Thanassi and Hultgren, 2000). The Hly system transports the 110-kDa α-hemolysin, directly from the *E. coli* cytoplasm, across the inner and outer bacterial membranes, with no periplasmic intermediates (Andersen, 2003; Thanabalu *et al.*, 1998). The type I transporter is relatively simple, and is comprised of three components. The structural protein, HlyD, and the ATPase, HylB, form a translocation channel across the inner and outer bacterial membranes, and that provides the energy for protein transport (Andersen, 2003). The TolC protein, the third component, trimerizes to form a conduit-like structure through which the protein is transported outside the bacterium (Thanabalu

et al., 1998; Andersen, 2003; Thanassi and Hultgren, 2000). Substrate recognition by the Hly system requires a 60 amino acid signature motif located at the carboxyl terminus of the substrate (Thanassi and Hultgren, 2000). The signature motif contains glycine rich repeats also called RTX motifs for repeats in toxin (Thanassi and Hultgren, 2000). Binding of the substrate to the inner membrane translocase triggers the trimerization of HlyD and subsequent formation of a bridge with the TolC trimer (Thanabalu *et al.*, 1998). ATP hydrolysis is required for substrate release from the *E. coli* cell, but not for assembly of the Hly secretion system (Thanabalu *et al.*, 1998). In *L. pneumophila* genes encoding components of a putative Type I secretion system have been identified (Jacobi and Heuner, 2003).

3.3. General secretion pathway and Type II secretion.

The general secretion pathway (GSP) is widely used by pathogenic and non-pathogenic Gram-negative bacteria. Proteins such as extracellular toxins, adhesins, invasins and proteases are secreted by this pathway (Stathopoulos *et al.*, 2000). Secretion by the GSP occurs in two steps (Sandkvist, 2001): First, proteins are transported across the bacterial inner membrane and into the periplasm, where they form semi-folded intermediates; second, transport from the periplasm, across the outer membrane, and into the extracellular milieu is mediated by one of the many terminal branches of the GSP.

The *E. coli* GSP system exemplifies basic functionality of GSPs in general (Stathopoulos *et al.*, 2000): First, protein targets are recognized by a specific signal

sequence. The signal sequence is essential for translocation and consists of three domains: the N-terminal domain contains positively charged amino acids that mediate association with the inner membrane; the hydrophobic domain mediates protein insertion into the inner membrane; and the C domain contains a cleavage site, which allows for release of the protein from the inner membrane. The SecB chaperone is responsible for transporting the protein moiety to a channel, in the inner membrane, which is formed by Sec- Y, E and G. Protein translocation across the channel in the inner membrane and into the periplasm is facilitated by SecA, an ATPase. Once in the periplasm, the protein is transported to the outer membrane by one of the terminal branches of the GSP.

The main terminal branch of the GSP, also referred to as type II secretion, is responsible for transporting a large proportion of GSP intermediates, including exoenzymes and toxins, across the bacterial outer membrane (Sandkvist, 2001). General features of type II secretion are exemplified by the *Pseudomonas aeruginosa* Xcp system, which is required for the formation of extracellular proteins (Filloux, Michel, and Bally, 1998). Twelve genes encode the Xcp machinery *xcpA* and *xcpP-xcpZ*. Protein translocation via the Xcp system occurs as described in (Filloux, Michel, and Bally, 1998): In the periplasm, protein intermediates mature in various ways: they may oligomerize and/or undergo modifications mediated by oxidoreductases and disulfide bond isomerases (mediated by the Dsb family) (Sandkvist, 2001; Stathopoulos *et al.*, 2000). It is proposed that the folded proteins are then mobilized through a pore in the outermembrane formed by XcpQ, also referred to as a secretin (Filloux, Michel, and

Bally, 1998). The pseudopilins, Xcp T, U, V, W and X, after processing by the prepilin peptidase, XcpA, are believed to form a pilus-like structure that acts as a piston that threads the protein to be secreted, through the secretin pore (Filloux, Michel, and Bally, 1998). This process is dependent on the ATP binding protein, XcpR, which is found in association with XcpY, an inner membrane protein, with a large N-terminal domain that protrudes in to the cytoplasm (Filloux, Michel, and Bally, 1998). XcpP and XcpZ are inner membrane proteins with large C-terminal domains that protrude into the periplasm, and that may function in the assembly and stabilization of the secretion apparatus (Gerard-Vincent *et al.*, 2002; Robert *et al.*, 2002). XcpS is a transmembrane protein that is anchored in the inner membrane, with loops extending into the cytoplasm and periplasm; its function is not known (Filloux, Michel, and Bally, 1998).

The recognition sequences that are required for type II secretion are unknown. Primary amino acid sequence similarities or a common secondary structure have not been found among the multiple substrates mobilized by type II secretion (Izard and Kendall, 1994). It is postulated that three dimensional recognition domains found in the folded protein demarcate transport by type II secretion systems (Izard and Kendall, 1994).

Closely related to type II secretion is the biogenesis of type IV pili. Also referred to as long pili, these structures are located at bacterial poles and are utilized by bacterial pathogens for adherence to host cells and for twitching motility (Nunn, 1999). The pilus subunits, PilA and PilE, share some homology especially at the N-terminal region with the pseudopilins, Xcp- T, U, V, and W of *P. aeruginosa* (Nunn, 1999). Also the prepilin

peptidase, XcpA (also known as PilD), cleaves PilA and PilE at their N-termini (Russell and Darzins, 1994; Nunn, 1999). In addition to the requirement for PilD/XcpA, in both type II secretion and type IV pilus biogenesis, and the homology shared with the pseudopilins and PilA, three other genes required for pilin biosynthesis share homology with components of the Xcp system (Nunn, 1999): PilB and XcpR (nucleotide-binding proteins), PilC and XcpS (transmembrane proteins postulated to interact with XcpR), and PilQ and XcpQ (secretins that oligomerize to form pores in the outer membrane). These data indicate that the secretion system components are versatile in function. Many of the protein components that are required for type II and type IV pili biogenesis have been found in *L. pneumophila*.

In *L. pneumophila* the following proteins involved in type II secretion and (or) the biosynthesis of type IV pili have been identified: homologues to the PilD peptidase, the PilB nucleotide binding protein, the PilC transmembrane protein, the $PilE_L$ type IV pilin subunit, the *Legionella* secreted proteins: LspD (an ATPase), LspE (a secretin), and LspF-K (pseudopilins) (Hales and Shuman, 1999; Liles, Viswanathan, and Cianciotto, 1998). As with *P. aeruginosa* (see above), in *L. pneumophila* PilD is required for both type II secretion and type IV pilus biogenesis (Liles, Edelstein, and Cianciotto, 1999). A *pilD* mutant is greatly impaired for intracellular replication in the amoeba, *Hartmanella vermiformis*, and the U937 macrophage cell line (Rossier and Cianciotto, 2001). It is of interest that neither type IV pili nor type II secretion appear essential for replication in macrophages. Indeed $pilE_L$ or *lspDE* null mutants showed only a 10-fold reduction

in *L. pneumophila*'s capacity to replicate inside macrophages (Rossier and Cianciotto, 2001). In contrast the mutants showed a 1000-fold reduction for replication inside *Hartmanella vermiformis* as compared to wild-type (Rossier and Cianciotto, 2001). Taken together these data indicate that while type II secretion plays an important role in the intracellular infection of amoeba, it plays a lesser role during replication inside human macrophages. Furthermore, a PilD-dependent mechanism, independent of type IV pilus biogenesis and type II secretion, does appear to play a major role in macrophage infection.

3.4. Type III secretion.

Type III secretion systems are renowned for delivering bacterial virulence factors that mediate the survival of bacterial pathogens inside eukaryotic hosts (Cornelis and Van Gijsegem, 2000). Type III secretion substrates include bacterial-derived receptors that mediate the attachment to and the invasion of eukaryotic cells, as well as, biologically active proteins that alter host signaling cascades and processes such as phagocytosis, cytoskeleton rearrangement, and inflammation (Cornelis and Van Gijsegem, 2000). Type III secretion activation is dependent on host cell contact, and occurs in a one step process, in which, virulence effectors are injected, from the cytoplasm of the bacterial cell, across the bacterial cell envelope, across the host cell membrane, and into the cytoplasm of the host cell (Cornelis and Van Gijsegem, 2000).

General features of type III secretion are exemplified by the *Yersinia pestis* type III secretion system (Ysc), as many of the components involved in type III secretion s are highly conserved among Gram-negative bacteria (Cornelis, 2000). The function of only some of the over 20 components necessary for type III secretion are known, as described by Cornelis and Van Gijsegem, 2000. Like in type II secretion, there exists a secretin homologue for type III secretion that is encoded by the *yscC* gene, and that oligomerizes in the outer membrane, to create a pore for protein translocation. YscW is a lipoprotein that is thought to stabilize the insertion of YscC into the outer membrane. The YscN protein, which contains signature Walker box motifs for ATP-binding is thought to supply energy for type III secretion. YscO and YscP are thought to be a part of the apparatus external to the outer membrane. YscD, R, S, T, U, and V are designated as inner membrane proteins and YscJ is a lipoprotein that spans both the inner and outer membranes. YscC, YscJ, and another lipoprotein are postulated to be a part of needle complex that extends away from the surface of the bacterial cell, and is thought to function as a conduit, through which, effector molecules are delivered from the bacterial cytoplasm, to the cytoplasm of the bacterial host cell, in a one step process (Cornelis and Van Gijsegem, 2000; Kubori *et al.*, 1998; Kimbrough and Miller, 2002).

While the genes that make up the structure for the type III apparatus are conserved in many organisms, the effectors which they secrete are diverse, and a signature motif necessary for substrate translocation has not been clearly identified. It is known that the first 15 amino acids in most *Yersinia spp.* outer proteins (Yops) are

required for their translocation by type III secretion (Ramamurthi and Schneewind, 2003). However, it is unclear whether the translocation signals are dependent on the mRNA or amino acid sequence (Ramamurthi and Schneewind, 2003).

3.5. Type IV secretion.

Type IV secretion systems are defined by their relatedness to DNA conjugation systems found in Gram-positive and Gram-negative bacteria (Ding, Atmakuri, and Christie, 2003). Type IV secretion systems have evolved to transport DNA and/or protein molecules across the envelope of bacterial cells in an ATP-dependent manner (Sagulenko *et al.*, 2001; Sexton *et al.*, 2004; Gomis-Ruth *et al.*, 2004; Ding, Atmakuri, and Christie, 2003). The type IV secretion systems have been classified into two groups, type IVA and type IVB, dependent on whether they are most related to either the *Agrobacterium tumefaciens* VirB system (required for T-DNA transfer to plants) or the DNA transfer (*tra*) locus found in the IncI plasmid family (required for the conjugation of plasmids between bacterial cells, respectively (Ding, Atmakuri, and Christie, 2003).

The *A. tumefaciens* VirB system is comprised of 12 genes, *virB1-virB11* and *virD4* (Ding, Atmakuri, and Christie, 2003; Christie and Vogel, 2000). VirB1, B2 and B5 are important for the attachment of *A. tumefaciens* to its host cell (Christie and Vogel, 2000). VirB2 and VirB5 are the major and minor subunits respectively that make up the bacterial pilus, which initiates physical coupling between *A. tumefaciens* and its host cell (Christie and Vogel, 2000). VirB3, B6, B7, B8, B9, and B10 span the bacterial cell

envelope and form a channel across which the T-DNA-protein complex is transported, and VirB4, B11 and D4 are ATPases (Christie and Vogel, 2000). Examples of type IVA members that share homology with the *A. tumefaciens* VirB system include the pertussis toxin liberation (ptl) system in *B. pertussis* (Weiss, Johnson, and Burns, 1993), the *Legionella vir* homologues (Lvh) system (Segal, Russo, and Shuman, 1999) and the *Brucella sps.* VirB system (O'Callaghan *et al.*, 1999; Sieira *et al.*, 2000) (Fig. 6).

The IncI plasmid *tra* system can be found in the *Salmonella* R64 and *Shigella* ColIb-P9 plasmids, which have loci that mediate DNA conjugation (Komano *et al.*, 2000; Segal and Shuman, 1999). The R64 plasmid is a 121-kb plasmid that has a DNA transfer locus that spans 54 kb and that is comprised of 47 genes. Genes and DNA regions within the transfer locus are required for thin-pilus formation (*pilO-V*), DNA transfer (*traA-Y, trbBC* and *oriT*), single-stranded DNA production (*rci*, *nuc*, and *nikAB*), and plasmid stability (*pnd*) (Komano *et al.*, 2000). Other genes within the locus include *sog* (suppression of the *E. coli dnaG* mutation that substitutes for *dnaG* primase activity in discontinuous DNA replication) (Narahara *et al.*, 1997) and *exc* (responsible for inhibiting the transfer of plasmids between cells harboring the same or closely related plasmids) (Furuya and Komano, 1994; Komano *et al.*, 2000). Some type IVB systems that share homology with R64 *tra* and *trb* genes include the *L. pneumophila* Dot/Icm system (Berger and Isberg, 1993; Segal and Shuman, 1998; Marra *et al.*, 1992) and the *Coxiella burnetii* Dot/Icm system (Zamboni *et al.*, 2003; Zusman, Yerushalmi, and Segal, 2003).

In the case of *L. pneumophila*, the *dot/icm* genes are found within two DNA regions (Segal and Shuman, 1997; Segal and Shuman, 1998). Ten of the 25 genes that make up the two *dot/icm* loci share homology with R64 *tra* and *trb* genes; these include *dotA*, *B*, *C*, *D*, *icmB*, *L*, *K*, *O*, *P* and *T* (Komano *et al.*, 2000) (Fig. 7). The functions of all the *dot/icm* gene products are not fully clarified. It is known that each of the genes is essential for the intracellular survival of *L. pneumophila* inside macrophages (Segal and Shuman, 1998). It is has been demonstrated that IcmT is required for pore formation in macrophages (Molmeret *et al.*, 2002) and that DotB has ATPase activity (Sexton *et al.*, 2004). Based on sequence data, the subcellular location of the protein products encoded by the *dot/icm* loci have been predicted: DotB, Icm B, Q, R, S, W to the cytoplasm, DotA, K, IcmB, C, D, E, F, G, J, L, M, O, P, T, and TphA to the innermembrane, Dot C, DotD, and DotK to the outermembrane, and IcmK to the periplasm and outermembrane (Segal and Shuman, 1998).

It is evident that the type IV secretion systems are an eclectic group in terms of their gene constitution and organization. Likewise they also display variability with respect to their mechanism for DNA and protein transport. For example the *A. tumefaciens* VirB system is thought to transport single-stranded DNA associated with protein in a one-step process directly across the bacterial inner and outermembranes and into the extracellular matrix via a channel that spans the bacterial cell envelope (Christie, 1997). In contrast, *B. pertussis* mobilizes pertussis toxin (ptx) across the bacterial envelope in a two step process. Ptx is transported across the inner membrane and into the

periplasm via a Sec-mediated process, and then across the outer membrane and into the extracellular milieu via the pertussis toxin liberation (PTL) type IV secretion system (Christie, 2001).

Type IV secretion systems can also display cooperativity, with other secretion systems. For example, in *L. pneumophila* there exist two distinct type IV secretion systems, the Lvh system, a type IVA system, and the Dot/Icm system, a type IVB system (Segal, Russo, and Shuman, 1999). Deletion of the entire *lvh* locus, which includes 11 genes, results in a reduced (10-fold reduction), but not abolished capacity to conjugate DNA, indicative of the existence of another secretion system involved in conjugation (Segal, Russo, and Shuman, 1999). In an *icmE-lvh* multilocus mutant of *L. pneumophila*, the capacity to conjugate is completely abolished, indicating that the Dot/Icm and Lvh type IV secretion systems are responsible for *L. pneumophila*'s capacity to conjugate DNA (Segal, Russo, and Shuman, 1999). Interesting is that, in a *dotB-lvh* mutant, the capacity to conjugate is dramatically reduced, but not abolished (Segal, Russo, and Shuman, 1999), indicative of the possibility that other proteins allow for a functional Dot/Icm type IV secretion system in the absence of DotB. At least two other *dotB* homologues, which encode ATPases have been found, and could be responsible for the residual ability to conjugate DNA seen in the *dotB-lvh L. pneumophila* mutant (Segal, Russo, and Shuman, 1999). The Lvh and Dot/Icm type IV secretion systems are, however, not completely redundant and boast exclusive functions. For instance, while *icmE* or *dotB* deletion mutants cannot replicate intracellularly, the deletion of the entire

lvh region has no effect on intracellular growth (Segal, Russo, and Shuman, 1999). It is evident that multicomponent type IV secretion systems are very complex and diverse in terms of their protein interactions and mechanism of action.

3.6. Type V secretion (autotransporters).

Autotransporters as the name implies are proteins that have the inherent capacity to transport themselves from inside the bacterial cytoplasm across the bacterial envelope. They are composed of three domains: (i) the N-terminal signal sequence may either be similar to sequences found in Sec-transported substrates or may differ markedly such that they contain an extended n-domain consisting of a conserved signature (MNKIYSLKY) followed by a second motif of conserved aromatic and hydrophobic amino acids (GLIAVSELAR) (Henderson, Navarro-Garcia, and Nataro, 1998; Fernandez and Weiss, 1994) (ii) the passenger domain is located downstream to the N-terminal sequence and may either remain associated with the cell envelope or may be released into the extracelluar environment (Desvaux, Parham, and Henderson, 2004); it contains an intramolecular chaperone domain that holds the passenger domain, in an unfolded state, until the protein arrives at the bacterial cell surface (Oliver *et al.*, 2003)., and the (iii) the C-terminal translocation unit is thought to form a β-barrel structure consisting of anti-parallel β-sheets that form a pore in the outermembrane through which the passenger domain can be transported to the extracellular milieu (Desvaux, Parham, and Henderson, 2004; Henderson, Navarro-Garcia, and Nataro, 1998) .

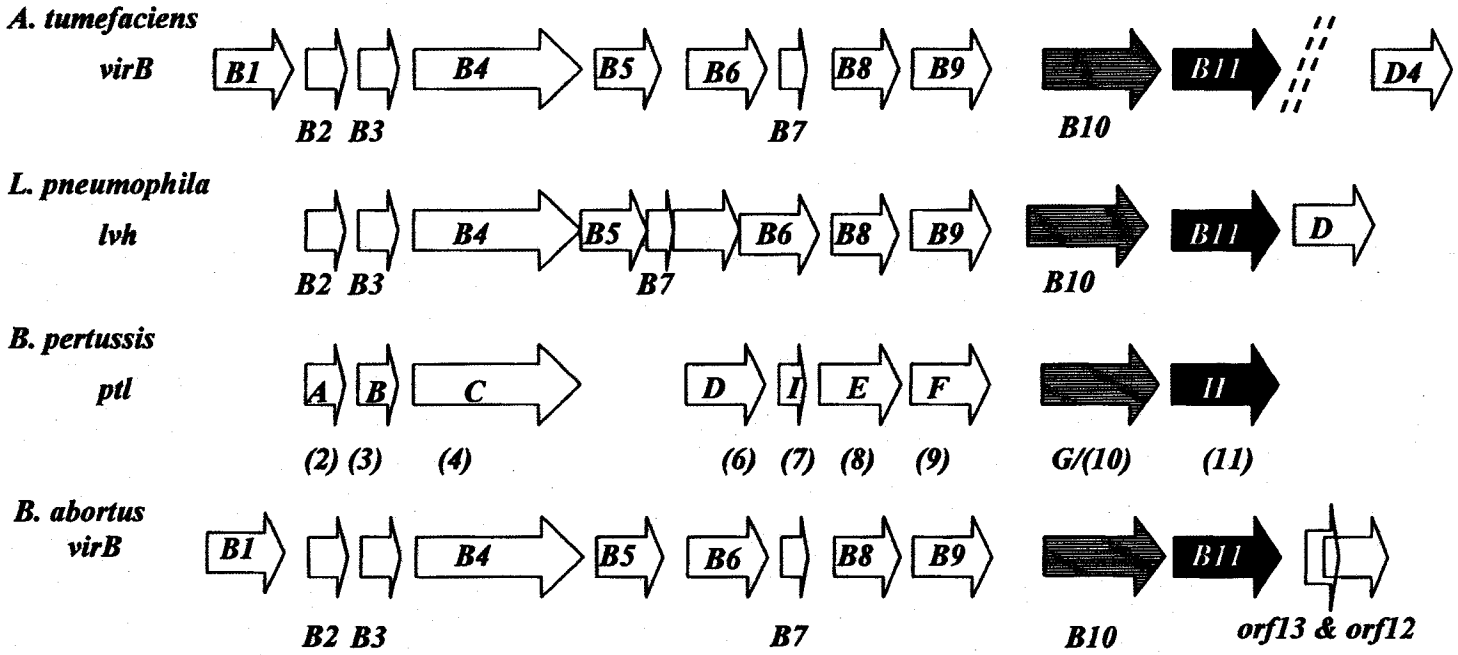


Figure 6. Gene homologies shared between the *A. tumefaciens virB* loci, the *L. pneumophila lvh* locus, the *B. pertussis ptl* locus and the *B. abortus virB* locus. Gene homologies for *virB* and *lvh* are denoted by similar labeling; gene homologies shared by *ptl* and *virB* are denoted by similarity in arrow size, arrow placement and bracketed numbers; double diagonal lines demarcate loci; shaded arrows indicate genes that share homology with the *L. pneumophila dot/icm* loci and the IncI R64 transfer locus (see Figure 7). The data represented in this figure were compiled from Ding, Atmakuri and Christie, 2003 and Christie and Vogel, 2000. orf = open reading frame.

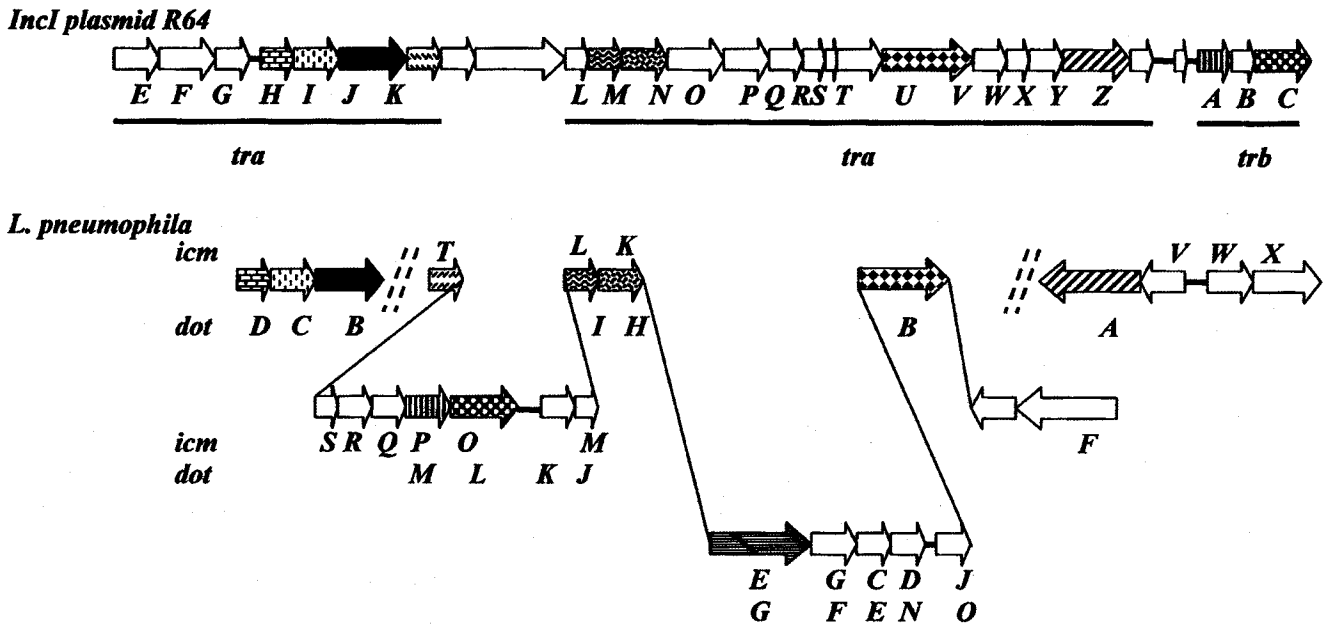


Figure 7. Gene homologies shared between the IncI plasmid R64 and the *L. pneumophila dot/icm* loci. Gene linkages for the R64 plasmid and for the *dot/icm* loci were maintatined in this diagram; however, the placement of *dot/icm* loci was reorganized to facilitate the comparison of R64 and *dot/icm* genes. Arrows represent genes; shared patterns represent homologies; double diagonal lines indicate different gene loci; thin lines represent the continuity of sequence; thick lines indicate intervening DNA sequence between genes; note that *dotB* and *dotG/icmE* share homology with genes in the *virB* system, *virB10* and *virB11* respectively, (see Figure 6). Data represented in this figure were compiled from Komano *et al.*, 2000 and Segal and Shuman, 1998.

3.7. Tat secretion.

The Tat system can transport already folded proteins along with their bound co-factors across the bacterial cytoplasmic membrane (Palmer and Berks, 2003). Substrates are recognized by a characteristic N-terminally located "twin arginine" SRRxFLK motif and a more hydrophilic N-terminal region as compared to residues found in Sec signal peptides (Berks, 1996). The twin arginine translocase (Tat) system in *E. coli* is composed of four proteins: TatA, TatB, TatC and TatE (Berks, Sargent, and Palmer, 2000). TatA, B and E share varying homology with the Hcf106 component of the ΔpH-dependent thylakoid import pathway of maize chloroplasts (Berks, Sargent, and Palmer, 2000). TatA is thought to form a channel in the bacterial cell membrane, while TatB and TatC are thought to be involved in substrate recognition (Palmer and Berks, 2003). TatE can substitute for TatA function (Palmer and Berks, 2003). Energy for protein translocation is postulated to be supplied by the transmembrane proton electrochemical gradient (Palmer and Berks, 2003). Based on analysis of the *E. coli* genome, only 26 gene products appear to have the characteristic Tat translocation motif (Hatzixanthis, Palmer, and Sargent, 2003). The Tat system, therefore, appears to be a specialized transport system. When a known Tat substrate of *Zymomonas mobilis*, glucose-fructose oxidoreductase (GFOR), was expressed in *E. coli*, GFOR was not transported into the *E. coli* periplasm (Blaudeck *et al.*, 2001). Exchange of the *Z. mobils* TAT motif for the *E. coli* motif resulted in transport, indicating that Tat transport systems are dedicated systems (Blaudeck *et al.*, 2001). *In L. pneumophila* homologues to TatA, B, and C have

been identified (De Buck *et al.*, 2004), and it has been demonstrated that TatB contributes to *L. pneumophila* virulence in eukaryotic cells (Rossier and Cianciotto, 2005).

Part 4. A Brief Overview of Type III and Type IV Secretion in *Bordetella spp.*

Bordetella pertussis is a Gram-negative coccobacillus that causes whooping cough in humans (Mattoo *et al.*, 2004). Its virulence factors include various toxins (pertussis toxin, adenylate cyclase toxin, and demonecrotic toxin), adhesion factors (filamentous hemagglutinin, pertactin, LPS, and tracheal colonization factor), and BrkA, which is involved in serum resistance (Mattoo *et al.*, 2004). *B. pertussis* modulates the expression of its virulence determinants in response to environmental conditions. At body temperature, for example, *B. pertussis* expresses its various virulence determinants; in contrast, maintenance at room temperature, or exposure to $MgSO_4$ ($\geq$ 40 mM), or nicotinic acid ($\geq$ 10 mM), prevents virulence gene expression (Mattoo *et al.*, 2004). Modulation of virulence is attributed to an environmentally sensitive phosphorelay system comprised of the intergral membrane sensor kinase, BvgS (Bordetella virulence gene sensor encoded by the *vir-1* gene), and its cytoplasmic response factor, BvgA (Konig *et al.*, 2002). BvgA is a transcription factor that, in the phosphorylated state, binds to and activates virulence factor promoters, and, in that manner, coordinates the expression of *B. pertussis* virulence.

Another member of the *Bordetella* genus is *B. bronchiseptica* (causative agent for kennel cough in dogs and snuffles in rabbits), which also modulates the expression of its

virulence determinants via the BvgAS system (Yuk, Harvill, and Miller, 1998). One interesting difference between *B. bronchiseptica* and *B. pertussis* is that they differentially express genes encoding type III and type IV secretion systems (Mattoo *et al.*, 2004). *B. pertussis* possesses a functional type IV secretion system that is encoded by nine *ptl* (pertussis toxin liberation) genes that share homology to the *Agrobacterium tumefaciens virB* operon (Weiss and Falkow, 1984) (Fig. 4). The *ptl* locus is located directly 3' to the five genes, *ptxA-E*, that encode the subunits for pertussis toxin (Mattoo *et al.*, 2001). The Ptl secretion system is responsible for mobilizing pertussis toxin from the periplasm to the outside of the bacterium (Weiss, Johnson, and Burns, 1993). *B. bronchiseptica*, on the other hand, does not have a functional type IV secretion system, but has a functional type III secretion system that is located within the 22 gene *bcs* (*Bordetella* secretion) locus (Mattoo *et al.*, 2001). The *bsc* locus encodes type III secretion machinery, its secreted proteins and chaperones. The *bscN* gene, located within this locus, shares homology with the *Yersinia yscN* gene, which is the type III secretion energy supply molecule (Yuk, Harvill, and Miller, 1998). Although both *B. pertussis* and *B. bronchiseptica* encode the genes necessary for both type IV and type III secretion, mutations in the promoter regions of the genes encoding the secretion machinery results in each species having only one of the two secretion machinery in a functional state as reviewed in (Mattoo *et al.*, 2001). In addition both type III and type IV secretion is positively regulated by the BvgAS system in *Bordetella spp.* (Yuk, Harvill, and Miller, 1998; Mattoo *et al.*, 2004).

Part 5. A Brief Overview of Type III Secretion in Enteropathogenic *E. coli.*

Enteropathogeneic *E. coli* (EPEC) is a major cause of infantile diarrhea, especially in the developing world (Vallance and Finlay, 2000). EPEC is different from commensal *E. coli* strains, found in the gut, in that it harbors a 35-kb pathogenicity island called the locus of enterocyte effacement (LEE) (Vallance and Finlay, 2000). Encoded within the LEE locus is a type III secretion system and its various substrates (EPEC-secreted proteins: EspA, EspB, EspD, EspF, an adhesin, initimin, and its translocated receptor called Tir) (Vallance and Finlay, 2000). The specific function of the Esps is unclear; however, they are required for the formation of a host cell derived pedestal at the site of EPEC contact (Vallance and Finlay, 2000). Pedestal formation is followed by the effacement of microvilli at the site of attachment (Vallance and Finlay, 2000). It is postulated that a loss of microvilli in the attachment/effacement (A/E) lesion causes a reduction in the absorptive capacity of the intestine, resulting in diarrhea (Vallance and Finlay, 2000). EPEC is the first pathogen reported to translocate a bacterial receptor, the Tir receptor, from inside its cytoplasm into its host cell (Kenny *et al.*, 1997). The bacterial ligand for Tir is intimin, and the Tir-intimin interaction allows for intimate contact between the bacteria and its host cell, and is essential for formation of the bacterial pedestal (Kenny *et al.*, 1997). The eukaryotic proteins, Wiskott Aldrich Syndrome Protein (WASP) and the Actin-related protein complex, Arp2/3, are recruited to the site of pedestal formation, and may be involved in mediating actin polymerization and pseudopod-like formation (Kalman *et al.*, 1999a).

Part 6. *Saccharomyces cerevisiae*: A Genetically Tractable Eukaryotic Model.

General knowledge about the biology and capabilities of *S. cerevisiae* with respect to its use as a eukaryotic model for gene expression and function studies is briefly described below. General information was obtained from general texts including: (i) "Getting Started with Yeast" by Fred Sherman (2002) and (ii) "An Introduction to the Genetics and Molecular Biology of the Yeast *Saccharomyces cerevisiae*", by Fred Sherman (1998), both located at http://dbb.urmc.rochester.edu/labs/sherman_f/, and technical methods for working with *S. cerevisiae* were obtained from (iii) Guthrie, C. and Fink, G. R. (Eds.): *Methods in Enzymology, Vol. 194, Guide to Yeast Genetics and Molecular Biology*. Acad. Press, NY, (1991).

S. cerevisiae (known as the Bakers' yeast, the Brewers' yeast, or the budding yeast) can exist stably in haploid (1N; N = a single set of chromosomes) and diploid (2N) states. Haploid yeast cells can be either of the **a**- or α- mating-type, and mating type is regulated by *MAT***a** and *MAT*α loci, so that diploids are heterozygous, *MAT***a**/*MAT*α. Since compatible haploid strains can be mated readily, in a laboratory setting to obtain diploids, and diploid cells can be made to sporulate to form haploid cells, the yeast system presents a flexible system for segregating alleles of interest, which is a useful tool for investigating gene functionality. Other properties that make *S. cerevisiae* attractive as a eukaryotic model include: rapid growth as dispersed cells, the ease of replica plating and mutant isolation, the existence of multiple plasmid libraries, a well defined genetic system, a highly versatile DNA transformation system, the ease of gene replacement, and

the existence of an annotated database of the complete genome sequence for the *S. cerevisiae* strain, S288C (http://www.yeastgenome.org/).

Genetic techniques that utilize *S. cerevisiae* have proven to be invaluable molecular biology tools, for assessing protein-protein interactions (two-hybrid screening), for cloning large DNA fragments (yeast artificial chromosome [YACs]), and for expressing heterologous proteins. Details concerning two-hybrid screening, as well as, the expression of proteins in *S. cerevisiae* are discussed further, as they constitute the experimental basis for an important part of this study.

6.1. Expression of proteins in *S. cerevisiae*.

The yeast system is particularly attractive for studying eukaryotic protein function, since yeast cells have the capacity to post-translationally modify proteins, by acetylation, myristilation, proteolytic processing, disulfide bond formation and glycosylation (Sherman, 1998; Byrne, O'Callaghan, and Tuite, 2005). It should be noted that yeast glycosylation patterns differ in some respects from the glycosylation patterns that are produced in other eukaryotic systems, which could affect heterologous protein function (Brooks, 2004). Another advantage of using *S. cerevisiae* for protein functionality studies is that cloned genes can be expressed under inducible promoters, so that gene expression can be controlled. A brief description of galactose-inducible gene expression is presented here as it was the method used in this study to regulate gene expression from regulatory sequences derived from the *GAL10* gene in *S. cerevisiae*.

Two proteins, Gal4p and Gal80p regulate the transcription of a group of yeast genes that are involved in the catabolism of galactose and melibiose including *GAL1*, *GAL2*, *GAL7*, *GAL10*, and *MEL1* (Johnston and Hopper, 1982; Hashimoto *et al.*, 1983; Nogi *et al.*, 1984; Sherman, 1998). One promoter that is commonly used to control the ectopic expression of genes from plasmids in *S. cerevisiae* is P_{GAL1}, which in *S. cerevisiae* functions as a divergent promoter from which *GAL1* and *GAL10* are transcribed. (Guarente, Yocum, and Gifford, 1982; West, Jr., Yocum, and Ptashne, 1984). The P_{GAL1} promoter contains copies of the galactose upstream activation sequence, UAS_{GAL}, to which Gal4p binds to activate transcription (Gancedo, 1998). In the absence of galactose Gal80p binds to Gal4p and inhibits its activity (Nogi *et al.*, 1984; Zenke *et al.*, 1996). In the presence of galactose, Gal4p inhibition is relieved so that Gal4p can bind to the UAS_{GAL} region in P_{GAL1} to activate transcription (Guarente, Yocum, and Gifford, 1982). The P_{GAL1} promoter is also subject to catabolite repression such that the presence of glucose in the growth medium can prevent gene expression even in the presence of galactose. In the presence of glucose, inhibitory proteins bind to regulatory sequences in the *GAL4* gene promoter, so that Gal4p levels are decreased, which results in a drop in the expression of Gal4p-activated genes (Gancedo, 1998). Glucose-induced inhibitors also bind to regulatory sequences in other *GAL* genes, which has a direct negative effect on the transcription of these genes, and ultimately results in reduced Gal protein levels (Gancedo, 1998). Therefore gene expression from the P_{GAL1} promoter

requires that yeast cells are grown in medium that contains an inducer such as galactose, and no inhibitory sugars such as glucose.

6.2. The yeast two-hybrid system.

The yeast two-hybrid system is "a genetic assay carried out in yeast to detect protein-protein interactions" (Sherman, 1998). It is commonly used for the following applications: testing known proteins for interaction, defining domains or amino acids critical for an interaction, and screening libraries for interactions with a protein of interest (Fields and Sternglanz, 1994). The yeast two hybrid assay hinges on the modular organization of transcriptional activators such as Gal4p, which can be divided into two functionally distinct domains, a DNA binding domain, and a transcriptionally active domain that stimulates RNA polymerase II (Vidal and Legrain, 1999). The two domains are normally found on the same polypeptide chain, but remain functional if they are brought into proximity with one another via non-covalent protein-protein interactions (Sherman, 1998). To determine whether two proteins interact (protein X and Y), a hybrid protein consisting of the Gal4p DNA binding domain and protein X and another hybrid protein consisting of the Gal4p transcription activation domain and protein Y are generated. If protein X interacts with protein Y, the Gal4p DNA binding and activation domains would be in proximity with one another at the promoter of a reporter gene. Alternatively, if there is no interaction the reporter gene remains off.

The major attributes that make the two-hybrid system a practical tool for

investigating protein interactions are that (i) many gene libraries derived from a broad spectrum of organisms are commercially available, (ii) the two-hybrid system is highly sensitive, (iii) new proteins identified are immediately available as cloned genes, (iv) the expression plasmids can be used to generate proteins for analysis, and (v) deletions and point mutations can be made in interacting proteins to identify the minimal domains necessary for interaction. Disadvantages to using the yeast two-hybrid system are that (i) proteins must be localized to the nucleus, and this may limit investigation with extracellular proteins, (ii) proteins must be able to maintain functionality as a fusion protein, (iii) sites for protein interactions may be occluded by the fusion protein, and (iv) false positive interactions can be obtained when transcription factors are fused to the Gal4p DNA-binding domain. In addition since the two-hybrid system is not a biochemical test, and does not give direct confirmation of protein interactions, the interactions identified must be confirmed by alternate methods. Biochemical methods such as protein affinity purification and immunoprecipitation are possible complements to the two-hybrid method, and their advantages and disadvantages are briefly outlined here based on the more extensive review in (Phizicky and Fields, 1995).

In the case of affinity purification, cell lysates are generated, and are either passed through an affinity column that consists of the protein of interest bound to a matrix or are mixed with latex beads coated with the protein of interest. Proteins within the cell lysates that have affinity for the protein of interest bind, while other proteins are washed from the column or beads. Advantages to this method are that (i) the system is highly sensitive,

and in theory its limit is within the range of the weakest physiologically relevant interactions known, (ii) all proteins in the cell lysate must compete for binding, which serves as a specificity-control, (iii) mutant derivatives of proteins can easily be compared for their capacity to bind to the protein of interest, and (iv) interactions that depend on multisubunit proteins can be tested. Challenges for affinity purification stem from the extreme sensitivity of these methods, so that other biologically relevant criteria must be used to support interactions found using this technique. Challenges include that (i) a highly purified preparation of the protein under investigation must be obtained to reduce false positives that could result due to presence of contaminating proteins (ii) third party protein interactions are possible, (iii) strong protein interactions that are not physiologically relevant can be detected due to the lack of compartmentalization in cell lysates, and (iv) the processing of proteins during purification procedures could have an effect on its conformation and its capacity to interact with other proteins.

Coimmunoprecipitation is another biochemical method that can be used to confirm two-hybrid interactions. It involves the generation of crude cell lysates, the addition of antigen and then antibody, antigen precipitation and washing, followed by antigen elution and analysis. Advantages include that (i) like affinity chromatography proteins must all compete for binding, which serve as a specificity-control, (ii) physiologically relevant multicomponent complexes can be coprecipitated, and (iii) interacting proteins are in their natural post-translationally modified states, which may be necessary for the interaction. Disadvantages include that (i) coprecipitation does not

necessarily represent direct interactions, since proteins that interact as part of complexes can also be precipitated, and (ii) the sensitivity of this method is not as high as for affinity chromatography, since the antigen is in lower concentration; however, adding excess antigen can improve specificity.

It is evident that the two-hybrid method is a powerful tool to detect protein-protein interactions, however, since this is a genetic method that reports interaction through an indirect measure, other assays that detect direct protein interactions, such as affinity chromatography, and/or coimmunoprecipitation may be used to complement two-hybrid studies. In addition other experimental data such as in vivo protein co-localization studies could be used to support the two-hybrid method.

Part 7: Thesis Objectives.

The long-term objective of the work presented in this thesis is to investigate the biological significance for the surface expression of the *L. pneumophila* 60-kDa chaperonin. Interest in HtpB is based on the following observations: (i) Avirulent strains of *L. pneumophila* express less HtpB on the cell surface as compared to virulent strains (Hoffman, Houston, and Butler, 1990; Garduno, Garduno, and Hoffman, 1998); (ii) HtpB expression is increased upon contact with eukaryotic host cells (Fernandez *et al.*, 1996); (iii) HtpB mediates the adherence to and the invasion of non-phagocytic HeLa cells (Garduno, Garduno, and Hoffman, 1998); (iv) *L. pneumophila* releases large amounts of the HtpB protein into its replicative

endosomes inside HeLa cells (Fernandez *et al.*, 1996; Garduno *et al.*, 1998); (v) HtpB homologues from other eubacteria have virulence-related roles (Table 1). Initially my objectives were two-fold, to explore the secretion mechanisms for various equatorial Hsp60 homologues, and to assess the general properties of HtpB with respect to its effects on the eukaryotic cell. Later I focused primarily on investigating the biological function of HtpB using the yeast model. As such, this thesis includes the preliminary contributions I made toward clarifying a secretion mechanism for HtpB and other Hsp60 homologues, the more focused and extensive contributions I made toward investigating the biological properties of HtpB in yeast, and the early steps taken toward identifying putative mammalian targets for HtpB.

CHAPTER 2: MATERIALS & METHODS.

Recipes for buffers, media and chemical solutions, and the sources for chemicals are detailed in the Appendix. Unless otherwise specified, chemical reagents were obtained from SIGMA-ALDRICH Canada, Ltd. (Oakville, ON).

Part 1. Strains and Growth Conditions.

Bacteria and yeast strains used and/or generated for this study are described in Table 2.

1.1. *Bordetella spp.*

Bordetella strains were grown at 37 °C in a humid incubator on Bordet-Gengou (BG) agar (Ovoid, USA) supplemented with 15 % sheep's blood (Ovoid, USA) The required antibiotic for selection was added to the BG agar as follows: *B. pertussis* BP388 (30 μg/ml nalidixic acid), *B. pertussis* BP347 (30 μg/ml kanamycin), *B. pertussis* BP3171 (30 μg/ml kanamycin), *B. pertussis* BPRA (30 μg/ml kanamycin, 200 μg/ml streptomycin); *B. bronchiseptica* WD3 (30 μg/ml kanamycin), *B. bronchiseptica* RB50 (no selection).

1.2. *Escherichia coli.*

E. coli strains were grown at 37 °C. DH5α was used for the amplification of plasmids and was grown on Luria-Bertani (LB) agar (Sambrook, Fritsch, and Maniatis, 1989) or in LB broth with vigorous shaking (180 rpm). JM109 was used to maintain the pSH16 plasmid (Table 3) and was grown on LB agar or in LB broth supplemented with ampicillin at a concentration of 100 μg/ml. XL10-Gold was used to amplify pEMBLyex4::*htpBΔ1197* (Table 3) for site-directed mutagenesis. XL10-Gold was grown on LB agar or with vigorous shaking (180 rpm) in NZY^+ broth (Stratagene, La Jolla, Ca). KC8 stocks were maintained on M9 minimal medium (Sambrook, Fritsch, and Maniatis, 1989). The KC8 strain, which is auxotrophic for leucine due to a *leuB*600 mutation, was cultured on M9 minimal medium lacking leucine, when used for isolating the leucine-selectable plasmid pGADT7-rec (Table 3) from yeast cells, since this plasmid encodes the yeast *LEU2* gene which complements the *E. coli leu*B mutation (Beggs, 1978). Enteropathogenic *E. coli* (EPEC) was grown on LB medium or in LB broth, each supplemented with 100 μg/ml streptomycin.

1.3. *Legionella pneumophila.*

L. pneumophila 2064 and 2064M were grown at 37 °C in a humid incubator on ACES (*N*-[2-acetamido]-2-aminoethansulfonic acid)-buffered charcoal yeast extract (BCYE) agar or in BYE broth.

1.4. *Saccharomyces cerevisiae.*

S. cerevisiae strains were propagated at 30 °C in a humid incubator. The AH109 strain was grown on YPDA, while other yeast strains were propagated using the nutrient-rich media YM-1 broth (Guthrie and Fink, 1991) or YEPD agar (Guthrie and Fink, 1991), or using Synthetic complete (SC) medium (Guthrie and Fink, 1991) or Synthetic defined (SD) media. SD media were formulated without certain amino acid(s) or purine and pyrimidine base(s) in accordance with specific yeast strain auxotrophies, such that yeast strains could grow on these media only if supplied with the biosynthetic genes that could complement their auxotrophic markers, a process termed nutritional selection. Since yeast plasmids carry these biosynthetic genes, these plasmids can be maintained in yeast strains by nutritional selection. Please see Appendix for media recipes. To induce the expression of genes cloned into the galactose-inducible plasmid, pEMBLyex4, and its derivatives, galactose was substituted for dextrose in *S. cerevisiae* media formulations at a concentration of 2 %. To modulate the expression of the *cdc42*$^{Ala\text{-}118}$ dominant negative allele from the *GAL 1-10* promoter in pGAL-CDC42$^{Ala\text{-}118}$ (Table 3), either 3 % raffinose supplemented with 0.1 % galactose (raffinose medium) or 2 % galactose alone (inducing medium) was used as the carbon source.

Table 2. Organisms used in this study.

Strains	Description	Source/Reference
B. pertussis		AAW/(Weiss and Falkow, 1984, Arico *et al.*, 1989, Weiss *et al.*, 1983 Weiss, Johnson, and Burns, 1993)
BP347	*vir-1* :: Tn5 lac (BvgS mutant)	
BP388	Nal^r Tahoma I strain (wild-type)	
BP3171	*ptl-1* :: Tn5 lac (Ptl^- strain)	
B. bronchiseptica		JFM/(Yuk, Harvill, and Miller, 1998)
RB50	wild-type strain	
WD3	type III secretion mutant (*bscN*Δ)	
E. coli		
DH5α	*supE*44 Δ*lac*U169 (ϕ80 *lacZ*ΔM15) *hsdR*17 recA1 *end*A1 *gyr*A96 *thi*-1 *rel*A1	(Sambrook, Fritsch, and Maniatis, 1989)
EPEC	virulent and streptomycin resistant	BBF
JM109	K12-derived, F'{traD36 $proAB^+$ $lacI^q$ laZΔM15} endA1 recA1 hsdR17 (r_k^-, m_k^+) supE44 thi-1 gyrA96Δ(lac-proAB), host strain for pSH16	PSH
KC8	*hsdR leuB*600 *trpC*9830 *pyf::Tn5 hisB*463 *lac*ΔX74 *strA galU,K*	BD Biosciences Clontech, Palo Alto, CA
XL10-Gold	Tet^RΔ(mcrA)183 Δ(*mcrCB-hsdSMR-mrr*)173 *endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F' *proAB* $lacI^q$ZΔM15 *Tn10* (Tet^R) Amy Cam^R]	BD Biosciences Clontech
L. pneumophila		PSH/ (Fernandez *et al.*, 1996)
2064	human isolate, serogroup 1	
2064M	salt-tolerant avirulent mutant isogenic to 2064	
S. cerevisiae		
21R	*MAT***a** *ade1 leu2-3, 112 ura3-52*	LEM/(Johnston and Hopper, 1982)
AH109	*MAT***a** *trp1-901 leu2-3, 112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1*$_{UAS}$*-GAL1*$_{TATA}$*-HIS3 GAL2*$_{UAS}$*-GAL2*$_{TATA}$*-ADE2 URA3::MEL1*$_{UAS}$*-MEL1*$_{TATA}$*-lacZMEL1*	BD Biosciences Clontech
GRX2	*MAT***a** *thr4*	GCJ
MLD158	*MAT***a**/α *ade1/ADE1 his3-11/HIS3 leu2-3, 112/leu2-3,112 ura3-52/ura3-52*	LEM/(Murray *et. al.*, 1998)
MLD67	*MAT***a**/α *ste12Δ::URA3/ste12Δ::URA3 ade1/ADE1 his3-11/HIS3 leu2-3,112/leu2-3, 112 ura3-52/ura3-52*	LEM/(Murray *et al.*, 1998)
MLD66	*MAT***a**/α *ste12Δ::URA3/ste12Δ::URA3 sup70-65/sup70-65 ade1/ADE1 his3-11/HIS3 leu2-3,112/leu2-3, 112 ura3-52/ura3-52*	LEM/(Murray *et al.*, 1998)
S288C-BY4741	*MAT***a** *flo8::kan*^{r} *his3-11 leu2-3,112 met15 ura3-52*	GCJ/(Brachmann *et al.*, 1998)
W303-1b	*MAT*α *leu2-3 112 ura3-1 his3-11, 15 trp1-1 ade2-1*	GCJ
XJ3B	*MAT*α *met6*	GCJ/(Archambault, 1992)
Y187	*MAT*α *trp1-901 leu2-3, 112 ura3-52 his3-200 gal4Δ gal80Δ met- URA3::MEL1*$_{UAS}$*-MEL1*$_{TATA}$*-lacZMEL1*	GCJ BD Biosciences Clonetech

AAW = Dr. Alison A. Weiss, University of Cincinnati, Cincinnati, OH; BBF = Dr. Bret. B. Finlay, University of British Columbia, Vancouver, BC; GCJ= Dr. Gerald C. Johnston, Dalhousie University, Halifax, NS; JFM = Dr. Jeff F. Miller, University of California, Los Angeles, CA; LEM= Dr. Lois E. Murray, Dalhousie University; PSH = Dr. Paul S. Hoffman, University of Virginia, Charlottesville, VA.

Part 2. General Molecular Biology Techniques.

2.1. Agarose gel electrophoresis.

DNA fragments were visualized in a 1 % (wt/vol) agarose gel (see Appendix for recipe). Five microliters of each DNA sample was mixed with 1 µl 6X gel loading dye (MBI Fermentas, Burlington, ON) and was loaded into the agarose gel, which was submerged in 1X TAE in a BIO-RAD Mini Sub-CellGT® electrophoresis chamber. A BIO-RAD POWER PAC 300 was used to deliver 100 V to the agarose gel for approximately 1 hour. One-kbp or 100-bp DNA markers (MBI Fermentas) were resolved for reference.

2.2 Restriction endonuclease digestion.

Double-stranded DNA was digested with the appropriate restriction enzymes (MBI Fermentas, or New England Biolabs, Beverly, MA) using the supplied buffers and under conditions outlined by the supplier.

2.3 DNA quantification.

Double-stranded DNA fragments were quantified using an LKB BIOCHROM Ultrospec II (Pharmacia now at Pfizer Inc.) set at A_{260} and the conversion factor: 1U A_{260} = 50 mg/ml dsDNA.

2.4. Cohesive end ligation

For cohesive end ligations, the plasmid and insert to be ligated were digested with appropriate restriction endonucleases to produce compatible ends. A 10 µl ligation reaction was prepared as follows:

1 µl, 10X ligation buffer (as supplied by New England Biolabs or MBI Fermentas)

50 - 200 ng vector DNA and insert DNA with a cohesive end ratio of 1:3 respectively

1 µl T4 DNA ligase, ~6 Weiss Units/ul (New England Biolabs or MBI Fermentas)

ddH_2O, nuclease free

The reaction was either incubated at room temperature for 1 hour or at 14 °C for 18 hours. T4 DNA ligase was heat-inactivated at 65 °C for 10 minutes. Two - four microliters of the ligation mix was used to transform 40 µl of electrocompetent *E. coli* DH5α (Stratagene) or Top10 (Invitrogen) cells as described below.

2.5. "T" - "A" tailing.

The "T"-"A" tailing method was used as an alternative for cloning DNA fragments into the pBlueScript® II plasmid (pBS) (Stratagene, La Jolla, CA), and was particularly convenient for cloning PCR amplicons generated with Taq DNA polymerase, which, independent of a template, preferentially adds a dATP residue at the 3' end of double-stranded DNA fragments (Clark, 1988). Plasmid DNA to be ligated was made competent for ligation to Taq polymerase generated amplicons by "T"- tailing (Marchuk *et al.*, 1991). In this study pBS was digested with *Eco*RV, and was incubated with Taq

polymerase (1 U enzyme/μg plasmid/20 μl volume) (MBI Fermentas) in 1X buffer (MBI Fermentas) in the presence of 2 mM dTTP (MBI Fermentas) for two hours at 70 °C and was purified by spin column (QIAquick PCR purification kit, QIAGEN Inc., Mississauga, ON). In the case of amplicons generated using the proofreading polymerase, PFU polymerase (MBI Fermentas), dATP residues (MBI Fermentas) were added to the amplicons using the procedure outlined above prior to ligation.

2.6. Electrocompetent DH5α *E. coli* cells.

LB medium (80 ml) was inoculated with 1 ml of an overnight DH5α culture and the fresh culture was grown to a density of A_{600} 0.5 - 1 U with shaking at 37 °C. The culture was chilled on ice for 15 minutes, divided into two pre-chilled 35 ml centrifuge tubes, and centrifuged at 4,000 x *g* for 8 minutes at 4 °C in the JA-20 rotor and a J2-21M/E Beckman high-speed centrifuge (BECKMAN COULTER, Fullerton, CA). The cell pellets were washed three times in chilled 10 % (vol/vol) glycerol, first with 25 ml, then with 12.5 ml, and finally with 1.5 ml volumes. The cell pellets were each suspended in 80 μl 10 % glycerol and the cells were split into 40 μl aliquots that were either kept at 4°C for same day use or stored at -80 °C for up to 6 months. Each 40 μl aliquot was used for a single transformation event and frozen electrocompetent cells were thawed on ice prior to use.

2.7. Electroporation of *E .coli* cells.

Electroporation was carried out using a Gene Pulser® (Bio-Rad laboratories Inc.) set at

1.5 kV, 25 μF and 200 Ω for 1 mm gap cuvettes (VWR International, Mississauga, ON) and adjusted to 2.5 kV for 2 mm gap cuvettes (VWR International). Two to four microliters of a standard ligation reaction mix were added to a 40 μl aliquot of DH5α competent cells, which were incubated at 4 °C for 1 minute, transferred to a pre-chilled cuvette, and pulsed in the Gene Pulser®. A time-constant ranging from 4.5 - 5 milliseconds suggested successful transformation by electroporation. The cell-DNA mixture was promptly and gently bathed with 1 ml LB medium, removed from the Gene Pulser® chamber and transferred to a 15 ml tube in which it was vigorously shaken (200 rpm) at 37 °C for 1 hour. The cells were then plated on the appropriate selective medium.

2.8. Rapid plasmid isolation from *E. coli*.

Two milliliters of the transformant culture were grown overnight in selective LB broth. One and one half milliliters of the culture were pelleted in a bench-top micro-centrifuge (Eppendorf) at 16 000 x *g* for 15-30 seconds. The supernatant was discarded and the cells were suspended in 100 μl STET solution (see Appendix for recipe). Lysozyme (10 μl, freshly made at 10 mg/ml, MBI Fermentas) was added to the suspension, which was incubated on ice for 10 minutes, then boiled for 2 minutes and centrifuged for 15 minutes. Plasmid DNA from 50 μl of the supernatant was purified using the GENECLEAN® II kit (Qbiogene, Carlsbad, CA) protocol according to the manufacturer's instructions. For greater yields the QAIprep Spin Miniprep kit (QIAGEN Inc., Carlsbad, CA) was used according to the manufacturer's instructions.

2.9. Alkaline lysis technique for plasmid isolation from *E. coli.*

Three milliliters of an overnight DH5α culture were subjected to centrifugation (1.5 ml at a time) in a 1.5 ml tube in a micro-centrifuge (Eppendorf) at 16 000 x *g* for 30 seconds at 4°C. The cell pellet was suspended in 100 µl plasmid solution I (see Appendix for recipe) and was incubated for 1 minute at room temperature. Two hundred microliters of solution II (see Appendix for recipe) were added, and the tube was inverted gently three times and incubated on ice for up to 5 minutes. One hundred fifty microliters of plasmid solution III (see Appendix for recipe) were added, and the tube was inverted four times and incubated on ice for 5-15 minutes. The samples were subjected to centrifugation for 10 minutes at 16 000 x *g* in a micro-centrifuge (Eppendorf). From the supernatant, plasmid DNA was ethanol precipitated as described below, and the resulting DNA pellet was suspended in 50 µl nuclease free ddH_2O. One and one half microliters of RNase A (10 mg/ml) (MBI Fermentas) were added, and the plasmid preparation was incubated either at 37 °C for 20 minutes, room temperature for 30 minutes, or overnight at 4 °C. The DNA sample was purified by ethanol precipitation as described below and suspended in 50 µl nuclease-free ddH_2O.

2.10. Ethanol precipitation of DNA.

A 3 M solution of sodium acetate pH 5.2 was added to the DNA preparation at a final concentration of 10 % vol/vol. The solution was mixed with vigorous tapping. Two and one half volumes of 95 % ice-cold ethanol (stored at -20 °C) were added, and the solution was incubated at -70 °C for at least 15 minutes, -20 °C for at least 30 minutes or

overnight at either of the two temperatures. The samples were subjected to centrifugation for 8 minutes at 4 °C. The DNA pellet formed was washed twice with 70 % ethanol and then either vacuum dried for a couple minutes or air-dried overnight. The DNA pellet was suspended in the desired volume of ddH_2O or in 1X TE (see Appendix for recipe).

2.11. End-filling a 5' DNA overhang with Klenow enzyme.

A Klenow reaction was prepared as follows:

3 to 5 μg DNA diluted to 50 μg/ml in the final reaction volume (see below for details)

1X Klenow buffer (GIBCO® now at Invitrogen) diluted from a 10X buffer supplied

33 mM dNTPs

1 U Klenow enzyme (GIBCO® now at Invitrogen)/μg DNA

ddH_2O as required to achieve indicated reagent and DNA concentrations, as final reaction volume is determined by the starting concentration of DNA

The reaction was incubated for 15 minutes at room temperature, and terminated with the addition of 0.5 M EDTA, pH 8.0 to a final concentration of 10 mM, and heat inactivation at 75 °C for 10 minutes.

2.12. Rapid chromosomal DNA isolation from *E. coli*.

Three milliliters of an overnight culture of DH5α were pelleted at 16 000 x *g* for 20 seconds in a micro-centrifuge (Eppendorf). The pellet was suspended in 567 μl 1X TE buffer, 30 μl, 10 % SDS and 3 μl, 20 mg/ml proteinase K (MBI Fermentas), and was incubated at 37 °C for 30 minutes. A 600 μl volume of buffer saturated phenol (GIBCO®

now at Invitrogen) was added. The preparation was mixed gently by inversion for 30 minutes on a mechanical rotor, and then centrifuged at 16 000 x *g* for 10 minutes. The upper aqueous phase was harvested and to it was added a 600 µl volume of a phenol: chloroform/isoamyl alcohol mixture (see Appendix for recipe). The preparation was mixed gently by inversion for 30 minutes, and then centrifuged at 16 000 x *g* at room temperature for 10 minutes. The upper aqueous phase was carefully removed (the interface that contained cell debris was avoided) 3 M sodium acetate to a final concentration of 10 % (vol/vol). The preparation was inverted three times and to it was added 0.6 volume isopropanol. The preparation was incubated at room temperature for 5 minutes and then centrifuged at 16 000 x *g* for 5 minutes at 4 °C. The DNA pellet was washed with 70 % ethanol, air-dried and dissolved overnight at 4 °C or at room temperature in 100 µl nuclease-free ddH_2O. One to two microliters of purified chromosomal DNA were used as template in a 25 µl PCR reaction.

2.13. Gene amplification by PCR.

PCR amplification using recombinant Taq DNA polymerase (MBI Fermentas) was carried out using buffers and protocols supplied by the manufacturer. A 100 µl PCR master cocktail was prepare as follows:

10 µl, 10X PCR buffer (MBI Fermentas)

10 µl, 25 mM $MgCl_2$ (MBI Fermentas)

10 µl, dNTP mix (2 mM dATP, 2mM dCTP, 2mM dGTP, 2 mM dTTP) (MBI Fermentas)

4 µl, 10 pmol/µl forward primer (Invitrogen or IDT, Inc., Cloralville, IA)

4 µl, 10 pmol/µl reverse primer (Invitrogen or IDT, Inc., Cloralville, IA)

2 µl, 5 U/µl Taq DNA polymerase

ddH_2O as required to bring the reaction volume up to 100 µl including the template volume, which is added last.

To each individual reaction (final volume 25 µl) was added DNA template ranging from 0.005 - 0.5 ng or the equivalent volume of ddH_2O (contamination control). For PCR amplification using high fidelity PFU DNA polymerase (MBI Fermentas), the PCR mix was very similar to that of recombinant Taq DNA polymerase with the following exceptions: the 10X PCR buffer as supplied by MBI Fermentas was unique, 1mg/ml bovine serum albumin (BSA) was required, 20mM $MgSO_4$ was used in place of $MgCl_2$, and the dNTP concentration was increased to 0.32 mM (16 µl of a 2mM dNTP stock was added to a 100 µl PCR master cocktail). PCR amplification was carried out using an MJ Research PTC-200 thermocycler.

A typical program used for amplifying a 1 kb DNA fragment is as follows: Step 1: 94 °C for 2 - 4 minutes, Step 2: 94 °C (melting temperature) for 1 minute, Step 3: appropriate annealing temperature (see Appendix for formula) for 1 min, Step 4: 72 °C (elongation temperature) for 1 minute, Step 5: Repeat from Step 2, 25 times (cycling), Step 6: 72 °C for 10 minutes (extension time), Step 7: 4 °C for 10 minutes (cooling), Step 8: End. In the case of fragments larger than 1 kb, the elongation time, Step 4, was increased by a minute for every additional kb of DNA.

Part 3. Plasmids: Description, Construction and Verification.

Table 3. Plasmids used in this study.

Plasmid Name	Marker	Comments	Source/ Reference
B3081	*LEU2*	YCp (*CDC42*$^{Ala\text{-}118}$); expression controlled by a galactose inducible promoter	LEM/(Ziman *et al.*, 1991)
pBluescript KS/SK (pBs)	*Amp*^{r}	standard cloning vector	Stratagene (La Jolla, CA)
pBs::*cpn10cpn60*	*Amp*^{r}	*Cpn10cpn60* operon in pBs	RCF/ (Fernandez and Weiss, 1995)
pBs::*htpB*	*Amp*^{r}	*htpB Dra*I fragment in pBs	this study
pBs::*htpB*-2	*Amp*^{r}	*htpB*, amplified with primers, *GAL4htpB*-F2 and *GAL4htpB*-R, and cloned into pBs	this study
pEL45	*Amp*^{r}, *URA3*	*Ste20Δ::URA3* deletion construct	LEM/(Leberer et al., 1992)
pEMBLyex4	*Amp*^{r}, *URA3*	galactose-inducible yeast expression vector	GCJ/(Erhart and Hollenberg, 1983)
pEMBLyex4::*htpB*	*Amp*^{r}, *URA3*	*L. pneumophila htpB Dra*I fragment in pEMBLyex4	this study
pEMBLyex4::*htpB-1197*	*Amp*^{r}, *URA3*	codes for the HtpB^{H400G}mutant protein	this study
pEMBLyex4::*HSP60*	*Amp*^{r}, *URA3*	*S. cerevisiae HSP60* cloned into pEMBLyex4	this study
pEMBLyex4::*HSP60Δ 1-72*	*Amp*^{r}, *URA3*	*HSP60* with and N-terminal truncation in pEMBLyex4	this study
pEMBLyex4::*groEL*	*Amp*^{r}, *URA3*	*E. coli groEL* in pEMBLyex4	this study
pFG(TyA)::lacZ-HIS3	*HIS3*	transcriptional reporter for pseudohyphal growth	LEM/(Roberts and Fink, 1994)
pGADT7-rec	*LEU2*	Gal4p AD vector that bears the HeLa cell cDNA library	Stratagene

Table 3 continued.

Plasmid Name	Marker	Comments	Source/ Reference
pGBD-C1	*TRP1*	GAL4 DNA-BD vector	LEM/(James, Halladay, and Craig, 1996)
pGBKT7-53	*TRP1*	gene encoding murine p53 cloned into a GAL4 DNA-BD vector	Stratagene (La Jolla, Ca)
pLM86	*HIS3*	pPP389::*htpB* derivative with *HIS3* swapped for *URA3*	LEM
pLM87	*TRP1*	pPP389::*htpB* derivative with *LEU2* swapped to *TRP1*	LEM
pPP389	*Amp*^{r}, *LEU2*	derivative of pEMBLyex4 used for leucine selection	PPP
pPP389::*htpB*	*Amp*^{r}, *LEU2*	*htpB DraI* fragment cloned into pPP389	this study
pPP389::*HSP60*	*Amp*^{r}, *LEU2*	*S. cerevisiae HSP60* cloned into pPP389	this study
pPP389::*HSP60Δ1-72*	*Amp*^{r}, *LEU2*	*HSP60* with an N-terminal truncation cloned into pPP389	this study
pras2::*URA3*	*Amp*^{r}, *URA3*	*ras2Δ::URA3* deletion construct	LEM/ (Kataoka *et al.*, 1984)
pHL129	*Amp*^{r}	pBs.*::flo8::hisG::URA3+::Kan R::hisG*	LEM/(Liu, Styles, and Fink, 1996)
pHL135	*URA3*	pRS202::*FLO8*	LEM/(Liu, Styles, and Fink, 1996)
pRS313	*Amp*^{r}, *HIS3*	Low copy number yeast plasmid	LEM/ (Sikorski and Hieter, 1989), from LEM
pRS313::*RAS2*	*Amp*^{r}, *HIS3*	*RAS2* in pRS313, for gene complementation	this study
pRS315	*LEU2*	plasmid backbone equivalent to B3081; low copy plasmid	LEM/ (Sikorski and Hieter, 1989)
pRS426	*URA3*	plasmid equivalent to pRS202 (the pHL135 vector backbone)	LEM

Table 3 continued.

Plasmid Name	Marker	Comments	Source/ Reference
pSH16	*Amp*^{r}	*htpAB* operon in puc19	PSH/ (Hoffman, Butler, and Quinn, 1989)
pSL1077	*Amp*^{r}, *URA3*	*Ste7Δ::URA3* deletion construct	LEM/ (Stevenson *et al.*, 1992)
pSL1094	*Amp*^{r}, *URA3*	*Ste12Δ::URA3* deletion construct	LEM/ (Stevenson *et al.*, 1992)
pSL1311	*Amp*^{r}, *URA3*	*Ste11Δ::URA3* deletion construct	LEM/ (Stevenson *et al.*, 1992)
*pTRC99A::*groELS	Amp^r	E. coli groELS *operon in pTRC99A*	StressGen, Biotech-nologies Corp. (Victoria, BC)

GCJ = Dr. Gerald C. Johnston, Dalhousie University, Halifax, NS; LEM = Dr. Lois E. Murray, Dalhousie University; PPP = Dr. Pac. P. Poon, Dalhousie University; PSH = Dr. Paul S. Hoffman, University of Virginia, Charlottesville, VA; RCF = Dr. Rachel C. Fernandez, University of British Colombia; Vancouver, BC

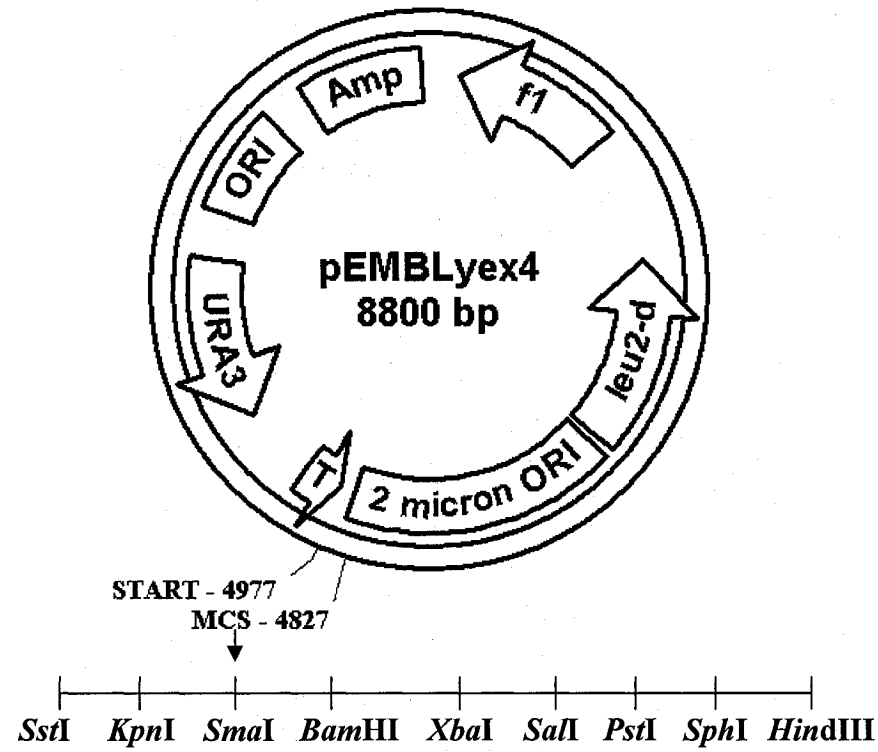
3.1. pEMBLyex4.

Recombinant proteins were expressed in *S. cerevisiae* strains from the galactose-inducible vector, pEMBLyex4 (Fig. 8). The pEMBLyex4 vector is an autonomously replicating, high copy plasmid. The plasmid consists of a pEMBL18^{+} backbone (essentially pUC18 with the phage f1 origin) (Yanisch-Perron, Vieira, and Messing, 1985), sequences from the high copy yeast 2μ plasmid that allow for autonomous replication, the *URA3* gene, the *leu2-d* gene, a multiple cloning site (MCS), a galactose inducible GAL-CYC promoter and a yeast transcriptional terminator (Cesareni and Murray, 1987). The GAL-CYC hybrid promoter consists of promoter sequences from *GAL10* and *CYC1* (encodes iso-1-cytochrome C); the hybrid promoter is regulated by Gal4p and galactose (Guarente, Yocum, and Gifford, 1982). DNA fragments cloned downstream of the GAL-CYC promoter must contain a single 5'-ATG-3' in frame with the gene to be expressed, since translation is initiated from the first 5'-AUG-3' codon encountered on the mRNA transcript.

3.2. pEMBLyex4::*groEL*.

The primers *groELpEMBL*-F and *pTrc99A*-R were used to PCR amplify from *pTRC99A::groELS* using PFU polymerase, a promoter-less *groEL* gene that was cloned

A



B

```
                    20                  40
TGATCATATGGCATGCATGTGCTCTGTATGTATATAAAACTCTTGTTTTCTTCTT

  60                  80                  100
TTCTCTAAATATTCTTTCCTTATACATTAGGTCCTTTGTAGCATAAATTACTATA

      120                 140                 160
CTTCTATAGACACGCAAACACAAATACACACACTAAATTACCGGATCAATTCGAG
                                                Sst1

          180                 200                 220
CTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTATGATCC
                                          HindIII
```

Figure 8. The pEMBLyex4 yeast expression plasmid. Features of the pEMBLyex4 plasmid including the markers, *URA3*, *leu2-d* and Amp, origins of replication, 2 micron (for replication in yeast cells), f1 and ORI (for replication in bacterial cells), GAL1-CYC transcription start sequences (START/T) and a multiple cloning site (MCS) (Panel A) are indicated. DNA sequence for a portion of GAL1-CYC region and the pEMBLyex4 MCS (Jim Murray, EMBL, Heidelberg, Germany) is presented in Panel B.

into the *Sac*I and *Hin*dIII sites of pEMBLyex4. Plasmids isolated from nine DH5α clones were screened for *groEL* inserts by restriction digest and plasmid DNA isolated from a single DH5α clone was transformed into W303-1b. A DNA sample of this plasmid was sequenced to determine the accuracy of PCR amplification. No nucleic acid substitutions were detected in the sequence information obtained (Macrogen, Korea); however, sequence information was not obtained for a gap from nucleotide positions 1107 – 1407 in the *groEL* wild-type gene. Yeast-protein extracts were tested for GroEL expression by Western blot using a commercially available anti-GroEL monoclonal antibody (referred to as *E. coli* MAb) or using an in-house cross-reacting anti-HtpB polyclonal antibody (*L. pneumophila* PAb). Recombinant GroEL function was not tested.

3.3. pEMBLyex4::*htpB*.

Plasmid pSH16 was digested with *Dra*I to yield a blunt-ended, 1.889 kb fragment containing a promoter-less *htpB* gene that was subcloned using the "T-A" cloning method into the *Eco*RV site of pBs. The pBS::*htpB* plasmid was sequenced to determine gene orientation, and the *htpB* fragment was excised from pBs with *Sal*I and *Xba*I, and then ligated into the multiple cloning site (MCS) of pEMBLyex4, so that *htpB* was placed 3' to the plasmid encoded, galactose-inducible GAL-CYC promoter. Residual pBs MCS sequences located between the GAL-CYC promoter were removed by digestion with *Sac*I and *Sma*I. Plasmid ends were filled will the Klenow enzyme, ligated and transformed into DH5α. Plasmids isolated from DH5α clones were screened for the *htpB* insert by PCR with the primers, *htpB*-F and *htpB*-R, and positive constructs were transformed into

yeast strain W303-1b. Yeast-protein extracts were tested for HtpB presence by Western blot with the in-house anti-HtpB monoclonal antibody GW2X4B8B2H6 (subsequently referred to as *L. pneumophila* MAb, Helsel *et al.* 1998).

3.4. pPP389.

Plasmid pPP389 is a derivative of the pEMBLyex4 vector, in which the *leu2-defective* (d) gene was replaced with a wild-type allele, *LEU2* (Erhart and Hollenberg, 1983). The *leu2-d* gene is a poorly expressed allele of *LEU2*, and in growth conditions that demand leucine biosynthesis can increase the stability and copy number of 2μ plasmid derivatives; however, it is a poor selectable marker for lithium-mediated plasmid transformation. Therefore, pPP389 and not pEMBLyex4 was used when leucine-selection was required for the transformation of plasmids into *S. cerevisiae*.

3.5. pPP389::*htpB*.

An *htpB* fragment excised from pEMBLyex4::*htpB* with *Xho*I and *Sal*I was ligated into the MCS of pPP389 to yield pPP389::*htpB*. HtpB expression from pPP389 was verified in a manner similar to that outlined for pEMBLyex4::*htpB*.

3.6. pPP389::*HSP60* and pPP389::*HSP60Δ1-72*.

The yeast mitochondrial Hsp60 (Hsp60p) was PCR-amplified using Taq polymerase from *S. cerevisiae* 21R genomic DNA, either with or without its N-terminal mitochondrial targeting sequence, with the primers *HSP60*F or *HSP60Δ1-72*F respectively, and the

common reverse primer *HSP60*R (Table 5). The *Hsp60* and *Hsp60Δ1-72* PCR amplicons were each digested with *Sac*I and *Bam*HI and cloned into the MCS of pPP389. Plasmids were isolated from five DH5α clones for each of the two constructs, and these 10 plasmids were screened by restriction digestion for the *Hsp60* wild-type and *Hsp60Δ1-72* alleles. Plasmids found to contain the insert of the expected size were appropriately named pPP389::*HSP60* or pPP389::*HSP60Δ1-72*. Plasmids isolated from one independent DH5α clone for each of the two constructs were transformed into W303-1b. DNA samples for these plasmids were sequenced to determine the accuracy of PCR amplification. For the *HSP60* wild-type allele five nucleic acid substitutions were introduced of which four did not lead to conserved amino acid substitutions resulting in the following changes: K247E, L326W, K329N and K448E. In the case of the *Hsp60Δ1-72* allele seven nucleic acid substitutions were introduced during PCR amplification of which 5 did not lead to conserved amino acid changes including: G74S, K147M, V210S, L460F and F522Y. Yeast-protein extracts were tested for Hsp60p and Hsp60Δ1-24p expression by Western blot with a commercially available yeast Hsp60p-specific monoclonal antibody (subsequently referred to as yeast MAb). Recombinant Hsp60 wild-type and Hsp60Δ1-24p function was not tested.

3.7. pGBD-C1::*htpB*.

The primers *GAL4htpB*-F2 (sequence start position + 10 in *htpB*) and *GAL4htpB*-R (Table 5) were used to PCR amplify from plasmid pSH16 using PFU polymerase, an

htpB gene fragment that was "T"-"A" cloned into the *Eco*RV site of pBs to create pBs::*htpB*-2. From plasmid pBS::*htp*-2, an *htpB* fragment was excised with *Eco*RI and *Sal*I and cloned into the MCS of pGBD-C1, to create pGBD-C1::*htpB*, which encodes a fusion protein consisting of the Gal4p-DNA-BD and HtpB. Three separate pGBD-C1::*htpB* plasmids isolated from DH5α were digested with *Eco*RI and *Sal*I to confirm insertion of the *htpB* gene fragment into pGBD-C1. One of these plasmid clones was then transformed into the yeast strain Y187, and protein extracts from two yeast transformants were tested for the Gal-DNA binding domain – HtpB fusion protein by Western blot using the *Legionella* MAb (Fig. 40). The *htpB* gene fragment in this pGBD-C1::*htpB* clone has not yet been sequenced to confirm accuracy of the *htpB* gene.

3.8. pEMBLyex4::*htpBΔ1197.*

The *htpB-1197* mutant allele in which histidine residue 400 in the wild-type HtpB protein is changed to glycine was generated using the QuikChange™ XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. For this study two oligonucleotides were designed to create the H400G change, encoded by the nucleotides 5'-GGT-3' (see primer sequences in Table 5) instead of 5'-CAT-3'. The oligonucleotides were purified by polyacrylamide gel electrophoresis (GIBCO® now at Invitrogen). The *htpB-1197* mutant allele was generated using the following reaction mixture:

5 µl, 10 x *PfuTurbo* reaction buffer as supplied

10 ng pEMBLyex4::*htpB*

125 ng oligonucleotide partner for the sense strand

125 ng oligonucleotide partner for the anti-sense strand,

1 µl dNTP mix

3 µl QuikSolution

ddH_2O up to a final volume of 50 µl

1 µl *PfuTurbo* DNA polymerase (2.5 U/µl) (added last)

The reaction was incubated in an MJ Research PTC-200 thermocycler as follows: Step 1: 95 °C for 1.5 minutes, Step 2: 95 °C for 50 seconds, Step 3: 60 °C for 50 seconds, Step 4: 68 °C for 26.53 minutes (2.5 minutes/kbp DNA), Step 4: Repeat from Step 2, 18 times, Step 5: 68 °C for 7 minutes, Step 6: 4 °C for 20 minutes, Step 7: End.

Once the reaction cycle was completed, 1 µl of the *Dpn*I restriction enzyme (10 U/µl) was directly added to the reaction, which was incubated at 37 °C for 1 hour, so that the parental DNA was digested. A 45 µl aliquot of the XL10-Gold ultra-competent cells was placed in a pre-chilled Falcon 2059 polypropylene tube. Two microliters of the β-ME mix supplied was added to the cells, which were incubated on ice for 10 minutes, with gentle swirling every 2 minutes. Two microliters of the *Dpn*I -treated DNA was added to the competent cell mixture, which was incubated on ice for 30 minutes. The cell mixture was heat-shocked at 42 °C for 30 seconds, and was then incubated on ice for 2 minutes. One-half milliliters NZY^+ broth, preheated to 42 °C, was added to the cells, which were incubated at 37 °C for 1 hour with shaking at 200 rpm. The transformation mixture was then plated onto LB supplemented with ampicillin at 100 µg/ml and incubated at 37 °C for >16 hours.

Table 4. Antibodies used in this study.

Antibody	Description	Source and/or Reference
B. pertussis MAb (BpHsp 4656 A5 culture supernatant)	anti-Cpn60 specific for *B. pertussis* homologue; does not cross-react with HtpB	Dr. Juan Arciniega, US FDA Laboratory of Pertussis (Bethseda, MD)
L. pneumophila PAb	anti-HtpB mouse polyclonal; cross-reacts with Cpn60 and GroEL	Dr. Rafael A. Garduno, Dalhousie University
L. pneumophila MAb (GW2X4B8B2H6 culture supernatant)	anti-HtpB mouse monoclonal; does not cross-react with the yeast Hsp60p	Dr. Paul S. Hoffman, University of Virginia/(Helsel *et al.*, 1988)
E. coli PAb (SPA-875)	anti-GroEL rabbit polyclonal; cross-reacts with GroEL, Cpn60, and HtpB	StressGen Biotechnologies Corp. (Victoria, BC)
E. coli MAb (SPS-870)	anti-GroEL mouse monoclonal; does not cross-react with HtpB	StressGen, Biotechnologies Corp.
Yeast MAb (SPA-808)	anti-Hsp60p monoclonal; does not cross-react with HtpB	StressGen Biotechnologies Corp.
alkaline phosphatase conjugated anti-rabbit IgG	secondary antibody used for detecting rabbit polyclonal antibodies in Western blot applications	CEDARLANE Laboratories Ltd. .(Hornby, ON)
pan-ras MAb (OP22)	a mouse monoclonal; cross-reacts with p21 translational products from H-, K-, and N-ras, c- and v-ras proteins from human, rat, mouse, and other mammals	Oncogene™ Research Products (Boston, MA) supplied through CEDARLANE Laboratories Ltd.
alkaline phosphatase conjugated anti-mouse IgG	secondary antibody used for detecting mouse monoclonal antibodies in Western blot applications	CEDARLANE Laboratories Ltd.
anti-rabbit IgG gold conjugate 10 nm (G3779)	secondary antibody used for detecting rabbit polyclonal antibodies in immunogold applications	SIGMA-ALDRICH Canada, Ltd. (Oakville, ON)
Trypanosome anti-tubulin (TAT)	anti- α-tubulin monoclonal	Dr. T. H. MacRae, Dalhousie University/ (Woods *et al.*, 1989)
FITC-conjugated anti-mouse IgG (green); A = 492 nm, E = 570 nm	anti-mouse IgG used for detecting the TAT monoclonal antibody	T. H. MacRae, available commercially from Jackson ImmunoResearch Laboratories Inc.(West Grove, PA)

(For commercially available antibodies cross-reactivity information was usually obtained from the supplier or were determined for this study as is the case for antibodies that were prepared in house, in which case the data are not shown but are available.)

Table 5. DNA primer sequences used for gene amplification.

Gene or gene fragment amplified	**Primers used (F = forward, R = reverse)**
groEL (1.789-kbp)	*GroELpEMBL*-F 5'CGCGAGCTCATGGCAGCTAAAGACGT3' *pTRC99A*-R 5'TCAGACCGCTTCTGCGTTC3'
htpB (1.667-kbp)	*GAL4htpB*-F2 5'GGAATTCCGTTTTGGTGATGAC3' *GAL4htpB*-R 5'GCGTCGACTATTGGATAACCGGGAG3'
htpB fragment (686-bp)	*htpB*686-F 5'AAAGTATGTGCTGTCAAAGC3' *htpB*686-R 5'AGCCATACGGGTTACTTTAG3'
htpB fragment (~1-kbp)	*htpB*-F 5'GCCATTGCTCAAGTTGGAACTAT3' *htpB*-R 5'GCGTTGAAACCGTAGTTGTCTTT3'
yeast *HSP60* (1.738-kbp)	*HSP60*-F 5'CGCGAGCTCATGTTGAGATCATCCGT3' *HSP60*-R 5'CGGGATCCATCATACCTGGCATTCCT3'
yeast *HSP60Δ1-72* (1.669-kbp)	*HSP60Δ1-72*-F 5'CGCGAGCTCATGAAAGAATTGAAATTCGGT3' *HSP60*-R 5'CGGGATCCATCATACCTGGCATTCCT3'
RAS2 (1.967-kbp)	*RAS2*full-F 5'GTGGCCGTATCAATGGATC3' *RAS2*full-R 5'GGGAAAGAGAAGCTTGTTATTC3'
RAS2 fragment (~1.5-kbp)	*RAS2*-F 5'AGGACCTTTTCATTCACCTCG3' *RAS2*-R 5'TTTACCCGGCAACCATATGA3'
FLO8 (~2.8-kbp)	*FLO8*up-S 5'GGGGTACCGATCACGATGAAGTTGTAGAG3' *FLO8*dwn-AS 5'GCTCTAGACACTTCTATCTTATCATGGAAG3'
HeLa cDNA inserts (variable sizes)	5'MATCHMAKER screening primer 5'CTATTCGATGATGAAGATACCCCACCAAACCC3' 3'MATCHMAKER screening primer 5'GTGAACTTGCGGGGTTTTTCAGTATCTACGATT3'
pEMBLyex4 *::htpB-1197* (~10 kbp)	sense strand complement 5'GCAGCGCGAGTAGCACCAAGAGCATCTTCAAC3' anti-sense strand complement 5'-GTTGAAGATGCTCTTGGTGCTACTCGCGCTGC-3'

(Restriction sites and regions of interest as indicated in the text are underlined.)

Plasmid pEMBLyex4 was tested for the presence of the *htpB1197* mutant allele by PCR with the primers, *htpB*686-F and *htpB*686-R, which flank nucleotide 1197 in *htpB*. Amplicons generated were digested with *Nla*III, since an *Nla*III restriction sequence located at position 1197 in *htpB* is present in the wild-type sequence, but lost in the mutant allele (Fig. 21).

3.9. pRS313::*RAS2*.

The *RAS2* gene was amplified by colony PCR using PFU polymerase from strain W303-1b with primers, *RAS2*full-F and RAS2full-R (Table 5). The 1967 bp PCR product generated was digested with *Xba*I at position 44, and was cloned into the *Xba*I and *Eco*RV sites of pRS313 to create plasmid pRS313::*RAS2* in *E. coli* DH5α. Presence of the ~1.9-Kbp fragment containing *RAS2* was confirmed in two plasmid clones by restriction digest with *Xba*I and *Xho*I, since the *Eco*RV site was lost, and one plasmid clone was selected for further testing. Ras2p function as expressed from this pRS313::*RAS2* plasmid was confirmed by its ability to complement a *ras2Δ* mutation in the W303-1b strain background (Fig. 24).

Part 4. Yeast Techniques.

4.1. Chromosomal DNA isolation from *S. cerevisiae*.

The steps for this procedure were carried out at room temperature unless otherwise indicated. An overnight yeast culture (usually 10 ml) was subjected to centrifugation in a

Beckman table top centrifuge at 1200 x *g*. The cell pellet was suspended in 0.5 ml ddH_2O. Cells were then transferred to a screw cap 1.5 ml tube and were pelleted in a micro-centrifuge. The supernatant was decanted and the pellet was disrupted by vortexing. The cells were then suspended in 200 µl filter sterilized breaking buffer (see Appendix for recipe). Glass beads (~200 µl vol, 425 - 625 µM in diameter) and 200 µl of a phenol/chloroform/isoamyl alcohol emulsion were added to the preparation, which was mixed using a vortex at high speed for 3 minutes. Two hundred microliters 1X TE buffer (see Appendix for recipe) was then added to the same preparation, which was mixed briefly using a vortex. The preparation was subjected to centrifugation at 16 000 x *g* in a micro-centrifuge for 5 minutes, and the aqueous layer was transferred to a clean 1.5 ml tube. To precipitate the chromosomal DNA from the aqueous layer, 100 % ethanol was added to the aqueous fraction, and the preparation was then mixed by inversion. The preparation was then subjected to centrifugation at 16 000 x *g* for 3 minutes. The supernatant was removed and the DNA pellet formed was dissolved in 0.4 ml 1X TE buffer. Thirty microliters of 1 mg/ml RNase A [MBI Fermentas] was then added to the resulting DNA solution, which was then incubated for 5 minutes at 37 °C. To precipitate the yeast chromosomal DNA, 10 µl of a 4 M ammonium acetate solution, and 1 ml of 100 % ethanol were added to the to the DNA solution, which was mixed by inversion and subjected to centrifugation at 16 000 x *g* in a micro-centrifuge for 3 minutes. The supernatant was discarded. The DNA pellet formed was suspended in 50 µl 1X TE.

4.2. Lithium acetate transformation of *S. cerevisiae*.

Yeast cells were transformed using an optimized procedure (Gietz *et al.*, 1992). A culture of *S. cerevisiae* cells was grown overnight to a density of 1 - 2 x 10^7 cells/ml in YPDA medium. Approximately 2 x 10^8 cells were harvested by centrifugation at 850 x *g* in a Beckman table-top centrifuge. The pelleted cells were suspended to 2 x 10^6 cells/ml in 100 ml pre-warmed YPDA medium (30 °C), and grown to a final cell density of 1 x 10^7 cells/ml. The freshly-grown cells (~ 10^9) were washed in 10 ml sterile dd$H_2$0, suspended in 1 ml ddH_2O and transferred to a 1.5 ml micro-centrifuge tube. The cells were then washed in 1 ml of a freshly diluted 1X TE/lithium acetate solution (see Appendix for recipe) diluted freshly from 10X TE (see Appendix for recipe) and 10X lithium acetate stock (see Appendix for recipe) solutions, and finally suspended at 2 x 10^9 cells/ml 0.5 ml 1X TE/lithium acetate solution. Fifty microliters of the yeast cell suspension was mixed with 1 µg transforming DNA and 50 µg single-stranded salmon sperm carrier DNA in a maximum volume of 20 µL. To the cell suspension was added 300 µl of a 40 % PEG 3350 solution (see Appendix for recipe). The cells were then incubated at 30 °C with agitation on a roller drum for 30 minutes. The cells were heat-shocked at 42 °C for 15 minutes and were pelleted by centrifugation at 8 000 x *g* for 5 seconds in a micro-centrifuge (Eppendorf). The cells were suspended in 1X TE and were plated onto the appropriate selective media.

4.3. Direct plasmid transfer from yeast to *E. coli* by electroporation.

The direct transfer of plasmids from diploid *S. cerevisiae* cells (AH109/Y187) to *E. coli* strain KC8 was carried out by electroporation as described in (Marcil and Higgins, 1992). Highly efficient electro-competent KC8 cells, (2 - 5 x 10^9 transformants per μg of puc18 plasmid DNA) were obtained form BD Biosciences (Palo Alto, CA). A 40 μl aliquot of the electrocompetent KC8 cells was thawed on ice. A single yeast colony was mixed with the thawed KC8 cells by gentle pipetting, transferred to a pre-chilled 2 mm gap cuvette, and kept on ice. The Gene Pulser® was set to 1.5 kV, 25 μF, and 200 Ω, and the yeast/bacteria mixture was pulsed and then incubated on ice for 30 seconds. The Gene Pulser® voltage setting was adjusted to 2.5 kV and the yeast/bacteria mixture was subjected to a second pulse. The cell mixture was then immediately bathed in pre-warmed LB broth (37 °C) and was agitated vigorously in a 15 ml tube at 37 °C for 1 hour. The cells were plated onto LB medium with the appropriate selection and the plates were incubated overnight at 37 °C.

4.4. Gene disruption in *S. cerevisiae*.

Gene disruption of *RAS2*, *STE20*, *STE11*, *STE7* and *STE12* in W303-1b was done by gene replacement with constructs *pras2::URA3*, pEL45 (*steΔ20::URA3*), pSL1311 (*ste11Δ::URA3*), pSL1077 (*ste7Δ::URA3*) and pSL1094 (*ste12Δ::URA3*). For each construct 10 μg DNA were cleaved with the appropriate restriction enzymes: for *pras2::URA3*, *Eco*RI/*Hin*dIII; for pEL45, *Xba*I/*Sal*I; for pSL1077, *Kpn*I/*Cla*I; for pSL1094, *Bam*HI/*Xho*I; and for pSL1311, *Sst*I/*Sph*I. The cleaved DNA was used for

lithium acetate transformation of yeast and the deletion mutants were selected by their ability to grow on SD medium lacking uracil (Guthrie and Fink, 1991). For each of the *steΔ* mutants, several colonies from the SD plates were tested for their ability to mate with the tester strain, GRX2-1a. On a given SD plate, if progeny did not result from the mating of *steΔ::URA3* mutants with GRX2-1a, but did result for the mating event between wild-type W303-1b (control) and GRX2-1a, the selected *steΔ::URA3* mutants were considered sterile and were accepted as having the appropriate gene disrupted. For the *ras2* deletion mutant, the disrupted locus was PCR amplified using the *RAS2*-specific primers, *RAS2*-F and *RAS2*-R, and digested with *Eco*RV to confirm presence of the *URA3* cassette (data not shown), which contains an *Eco*RV unlike the wild-type *RAS2* allele that is devoid of an *Eco*RV site.

4.5. Mating protocol for *S. cerevisiae*.

Compatible *S. cerevisiae* strains with different auxotrophies, and opposite mating types were inoculated onto the same spot on YEPD agar and cultured overnight at 30 °C, then streaked onto medium that supports the growth of diploids, but not the haploid parent strains. In one case yeast cells were streaked onto SD medium without essential amino acids, uracil or adenine. On this medium only diploid cells resulting from the mating event could grow, since only they, unlike their parents, would contain at least one functional allele for each of the genes that encode proteins required for the synthesis of essential amino acids, purine bases or pyrimidine bases.

4.6. β-galactosidase liquid assay for *S. cerevisiae*.

β-galactosidase liquid assays for *S. cerevisiae* were carried out according to the protocol outlined in Breeden and Nasmyth, 1985. Yeast cells were grown in synthetic defined medium supplemented with amino acids to maintain growth with plasmid selection. The OD_{600} of yeast cultures was measured, and the corresponding cell numbers were determined by cell counts obtained using a Beckman Coulter Counter. Approximately, 1 x 10^6 - 1 x 10^7 cells were harvested by centrifugation. The supernatants were discarded and the cells were suspended in 1 ml Z-buffer. Three drops of chloroform and 2 drops of 0.1 % SDS were added to each sample with a Pasteur pipette and the sample was mixed using a vortex at top speed for 10 seconds. The samples were preincubated at 28 °C for 5 minutes and the reaction was started by the addition of 0.2 ml of an *o*-nitrophenyl-β-D-galactosidase (ONPG) stock solution (4 mg/ml ONPG in Z buffer). Once the solution had developed a pale yellow color, the reaction was stopped by the addition of 500 µl of 1 M Na_2CO_3. The amount of time elapsed during the assay was recorded as this variable is required to calculate β-galactosidase units. Cell debris was removed by centrifugation for 10 minutes in a micro-centrifuge and the OD_{420} of each reaction was measured (see Appendix for the β-galactosidase unit calculation). Three independent isolates were tested for each variable and each experiment was performed twice.

4.7. Method for assaying pseudohyphae formation and invasive ability.

Yeast cultures were grown overnight at 30 °C with shaking at 150 rpm in SD medium supplemented with the amino acids required to maintain growth and plasmid selection ,

and containing dextrose as the sole carbon source (non-inducing medium) (Guthrie and Fink, 1991). The overnight cultures were used to inoculate fresh cultures that were grown to exponential phase, and 1 x 10^7 cells were harvested. Dilutions $1:10^3$, $1:10^4$, $1:10^5$ and $1:10^6$ were made in duplicate in both non-inducing and inducing medium (SD medium made with galactose as the sole carbon source). Yeast cells were inoculated onto the appropriate solid inducing solid medium in 100 µl drops, and placed in a humid incubator at 30 °C. Cell morphology was recorded 15 - 20 hours after inoculation, while cells were still immersed in medium, with a Nikon DIAPHOT-TMD inverted microscope using a 40X objective. To test for invasive growth, 100 µl from each cell dilution was spotted onto solid inducing medium and plates were left at room temperature (agar side up) until the drops had just adsorbed. Plates were then inverted and placed in a humid incubator at 30 °C. After two days, cells were observed using a Nikon DIAPHOT-TMD inverted microscope with a 40X objective and morphological changes in the micro-colonies were observed. After five days incubation, the yeast cells were washed from the agar surface with ddH_2O and the plates were assessed microscopically for the presence of agar invasive filaments. Photographs were taken with an attached Nikon 2000 35 mm camera using FUJI color film ASA 200 for prints. Alternatively, images were captured digitally using a Pro-series, monochrome, video camera and Image-Pro 4.0 software (Media Cybernetics Inc.).

4.8. Two-hybrid screening.

A Gal4p-based two-hybrid system (Fig. 9) was used to screen a MATCHMAKER HeLa cell cDNA library (BD Biosciences, Palo Alto, CA) for mammalian proteins that interact with HtpB. HtpB was expressed as a fusion protein with the Gal4p DNA-binding domain (Gal4p DNA-BD) from plasmid pGBD-C1::*htpB* (see Materials and Methods section 3.7), while the HeLa cell proteins were expressed as fusion proteins with the Gal4p activation domain (Gal4p AD) encoded within the pGADT7-rec vector (BD Biosciences, Clonetech, Palo Alto, CA). Therefore, HeLa-cell fusion proteins that interact with the HtpB fusion protein would bring together the Gal4p DNA-BD and the Gal4p AD, leading to the expression of reporter genes that are regulated by the Gal4p transcription factor inside yeast cells. The haploid yeast strain AH109 (*MAT***a** *trp1-901 leu2-3, 112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1*$_{UAS}$*-GAL1*$_{TATA}$*-HIS3 GAL2*$_{UAS}$*-GAL2*$_{TATA}$*-ADE2 URA3::MEL1*$_{UAS}$*-MEL1*$_{TATA}$*-lacZMEL1*) was transformed to carry plasmid pGBD-C1::*htpB* that bears the *TRP1* selectable marker and that encodes the Gal4p DNA-BD-HtpB fusion protein. AH109 is genetically engineered to express four reporter genes that are fused to three different Gal4p upstream activation sequences; the *GAL1* UAS upstream of the *HIS3* gene, which allows histidine biosynthesis; the *GAL2* UAS upstream of the *ADE2* gene, which allows adenine biosynthesis, and the *MEL1* UAS upstream of *lacZ* and *MEL1*, to facilitate β-galactosidase and α-galactosidase production respectively (BD Biosciences, 2001). The haploid yeast strain, Y187 (*MATα trp1-901 leu2-3, 112 ura3-52 his3-200 gal4Δ gal80Δ met- URA3::MEL1*$_{UAS}$*-MEL1*$_{TATA}$*-lacZMEL1*) carried the HeLa cell cDNA library (BD Biosciences, Palo Alto, CA) in the pGADT7-Rec vector that bears the

TRP1 selectable marker to create plasmids that encode Gal4p AD-HeLa cell fusion proteins.

For the two-hybrid assay the AH109 and Y187 strains, which are auxotrophic for adenine, histidine, leucine and tryptophan were mated and diploids were isolated on synthetic defined medium supplemented with amino acids to support growth, to maintain plasmid selection, and to screen for Gal4p-mediated transcription of the *ADE2* and *HIS3* genes. Since tryptophan and leucine biosynthesis are the selectable markers for the pGBD-C1::*htpB* plasmid and the pGADT7-Rec vector respectively, and histidine and adenine biosynthesis and the ability to produce α-galactosidase are the selectable markers for indicating an interaction between the Gal4p DNA-BD-HtpB fusion protein and Gal4p AD-HeLa cell fusion proteins inside the diploid yeast cells, blue yeast colonies that could grow on synthetic defined medium without adenine, histidine, leucine and tryptophan and supplemented with other essential amino acids and with X-α-gal (a chromogenic substrate that turns blue upon cleavage by α-galactosidase) were considered to have the HtpB fusion protein, and at least one HeLa cell fusion protein that interacts with HtpB. The details of this procedure are reported below.

One large (2-3 mm), fresh (2 days old) colony of AH109 transformed with pGBD-C1::*htpB* was inoculated into 50 ml SD medium lacking tryptophan, and was incubated overnight at 30 °C with shaking at 200 rpm. The cells were grown to a minimum density of 1 x 10^8 cells/ml. The 50 ml culture was centrifuged at 850 x *g* for 5 minutes in a Beckman table-top centrifuge, and 45 ml of the supernatant was decanted. A 1 ml library aliquot (BD Biosciences, Clonetech) stored at -80 °C was thawed at 4 °C.

Ten microliters of the library aliquot was stored on ice for quantification. AH109 transformed with pGBD-C1::*htpB* was combined with the library aliquot in a 2 L sterile flask, and 45 ml 2X YPDA/Kanamycin 40 μg/μl was added. Two 1 ml aliquots of 2X YPDA/Kanamycin 40 μg/μl were used to rinse the library tube and were added to the 2 L flask, and the culture was incubated overnight (24 hours) with gentle swirling (30 - 50 rpm). The mating mixture was centrifuged at 850 x *g* for 10 minutes. The mating flask was rinsed twice with 50 ml 2X YPDA/Kanamycin 40 μg/μl. The rinses were used to suspend the original yeast pellet, and the cell suspension was centrifuged as before. The supernatant was decanted and the pellet was suspended in 10 ml 0.5X YPDA/Kanamycin 40 μg/μl. The total volume of cells was measured.

Aliquots of the cell cultures (the mating mixture as well as the pure 10 μl aliquot of the library culture) were plated as follows: 100 μl each of 1:10 000, 1:1000, 1:100, and 1:10 dilutions were plated on solid SD medium lacking leucine. In addition the mating mixture was also plated onto SD medium lacking tryptophan (to check for viability of Y187 + pGBD-C1-*htpB*), and SD medium lacking leucine and tryptophan (to check for number of diploid progeny). Growth was scored on the SD/-Leu, SD/-Trp, and SD/-Leu-Trp plates and the mating efficiency was determined, so that this data could be used to calculate the total number of HeLa cell cDNA constructs that were screened (see Appendix for formulas).

The remaining mixture was plated in 200 μl aliquots onto 50 large (150 mm) plates containing either SD medium that lacked the amino acids, leucine, tryptophan and histidine (triple dropout [TDO] medium) for less stringent selection or on medium additionally lacking adenine (quadruple dropout [QDO] medium) for more stringent

selection. It was anticipated that the pre-selection of diploids onto the less stringent TDO medium would allow for the isolation of plasmids that encode Gal4p AD-HeLa cell fusion proteins that interact only weakly with the Gal4p DNA-BD-HtpB fusion protein (BD Biosciences, 2001). The cells were incubated at 30 °C for 3-8 days on TDO medium, and 8-21 days on QDO medium. Colonies that formed were maintained on SD medium that lacked leucine and tryptophan (double dropout [DDO] medium). Colonies growing on TDO medium were replica plated onto QDO medium containing the chromogenic substrate X-α-gal and incubated at 30 °C for 3 - 8 days. Blue yeast colonies that could grow on the QDO medium that lacked adenine and histidine were selected for further analysis as described below.

The AD library plasmids, from these colonies were directly transferred to the KC8 *E. coli* strain by electroporation as described above (Marcil and Higgins, 1992). Isolated plasmids that contained the *GAL4 AD-cDNAX* gene fusions were then tested to determine whether reporter gene activation was authentic, and would occur only when plasmid *pGBD-C1::htpB* was also expressed in strain AH109. AH109 [pGADT7-rec::*cDNAX*] cultures were transformed separately with the pGBD-C1 vector (negative control), pGBD-C1::*htpB* ("bait" plasmid) or pGBKT7-53 (encoding the unrelated murine p53 protein fused to the Gal4p DNA-BD, negative control), and were plated onto DDO medium containing X-α-gal to test for expression from the *MEL1* reporter gene, so that reporter gene activation was measured by the cleavage of X-α-gal, which causes yeast colonies to turn blue. Plasmids that conferred a more intensive blue pigmentation and more rapid onset when paired with the "bait" plasmid pGBD-C1::*htpB* as compared to

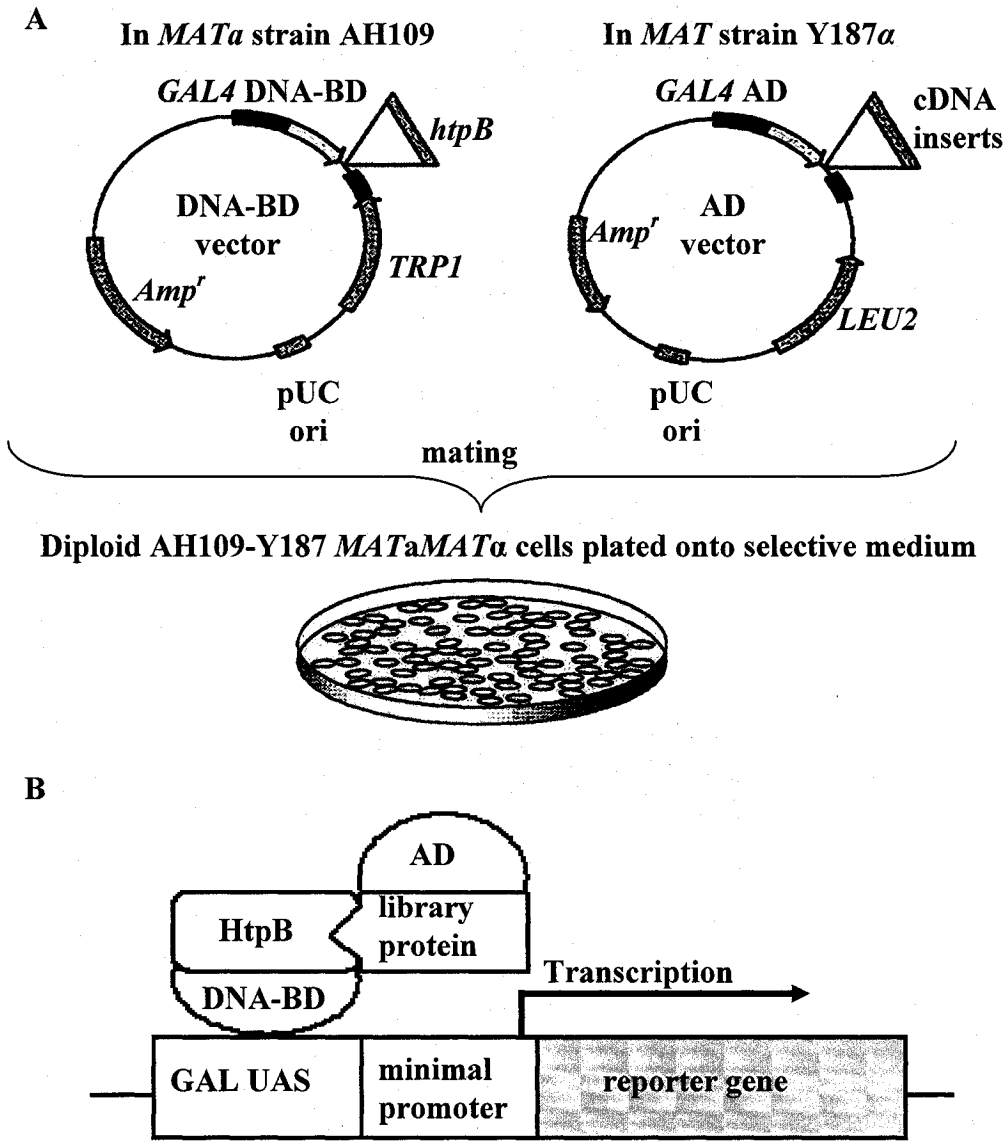


Figure 9. Screening of a HeLa cell cDNA library with HtpB in as "bait" using the yeast two-hybrid system. Diagram illustrating plasmid constructs (adapted from BD Biosciences, 2001), coding for the HtpB/GAL4 DNA-BD and cDNA/GAL4 AD fusion proteins, were transformed into the haploid AH109 and Y187 yeast strains respectively (Panel A). Diploid progeny, resulting from mating of the latter strains, were plated onto selective medium (Panel A). Diagram illustrating the two-hybrid protein interaction between HtpB/GAL4 DNA-BD and library protein/GAL4 AD at the promoter of a reporter gene (Panel B, adapted from BD Biosciences, 2001).

negative control plasmids pGBD-C1 or pGBKT7-53 were selected or further analysis (Intensity and speed of pigment acquisition was used as the selection criteria since the AH109 strain bearing no plasmids demonstrated low level *MEL1* expression). These plasmids were analyzed further to determine the approximate size of the cDNA inserts; the plasmids were digested with *Hin*dIII and restriction fragments were visualized by agarose gel electrophoresis, and in addition the inserts were amplified by PCR with 5'and 3'MATCHMAKER screening primers (Table 5) and visualized by agarose gel electrophoresis. Aliquots of the plasmids were also diluted to 100 ng/ul and sent to Macrogen (Korea) for sequencing from both the 5' and 3' ends, using the sense strand primer, T7 primer (5' - TAA TAC GAC TCA, CTA, TAG, GGC - 3'), and the anti-sense strand primer AD-antiS (5' - AGA TGG TGC ACG ATG CAC AG - 3') (Macrogen, Korea). Sequences obtained were analyzed using the Blastn analysis program (NCBI, Entrez nucleotide database).

Part 5. Protein Techniques.

5.1. SDS-PAGE and Western blotting.

One milliliter of a *L. pneumophila* cell suspension with an OD_{620} of 1.0 unit was pelleted in a micro-centrifuge (Eppendorf) at maximum speed (16 000 x *g*) for 20 seconds. The supernatant was discarded and the pellet was suspended in 100 µl of Laemmli sample buffer, boiled for 5 minutes, infused with 10 µl β-mercaptoethanol, and boiled again for 5 minutes. For yeast cells, 1 x 10^8 cells were collected by centrifugation (850 x g for 8

minutes) from an exponential phase yeast culture, suspended in 200 µl of sample buffer infused with 10 % (vol/vol) β-mercaptoethanol, supplemented with a commercially available yeast protease inhibitor cocktail (SIGMA-ALDRICH Canada, Ltd., Cat.# P8215), mixed with ~100 µl glass beads (425 µm - 600 µm in diameter), and agitated strongly using a vortex for 15 minutes at 4 °C or alternatively were broken open in a Mini Bead Beater (Cole-Parmer) at maximum speed for 30 seconds. Samples were then boiled for 5 minutes, and unbroken cells and cell wall debris were pelleted at 16 000 x *g*. Ten microliters of the cleared protein extracts were loaded into a 5 % (wt/vol) polyacrylamide stacking gel cast onto a 12 % (wt/vol) polyacrylamide resolving gel. The loaded gel was submerged in running buffer and subjected to 150 V for approximately 1 hour in a Bio-Rad Mini-protein® II electrophoresis apparatus. Gels containing the resolved proteins were either stained, with Coomasie blue or silver stain (Blum, 1987), or were blotted onto nitrocellulose membranes using a Bio-Rad Mini-protein® II Cell mini electro-transfer apparatus. For staining with Coomasie blue, the gels were submerged in Coomasie stain with agitation for 1 hour or overnight. The gel was washed for 1 hour with destain solution I (see Appendix for recipe) followed with a second wash with destain solution II (see Appendix for recipe). The gels were then immersed in gel preservation solution (see Appendix for recipe) for at least 1 hour prior to drying. Gels were dried in cellophane or on Whatman paper at 80 °C for 1 hour 15 minutes under vacuum using a Bio-Rad Slab dryer, model 443. Electro-transfer of proteins was carried out in transfer buffer in a Bio-Rad Mini-protein® II Cell mini electro-transfer apparatus set at 100 V for 1 hour. To verify efficient transfer of proteins from the gel, nitrocellulose membranes were stained

with 1X Ponceau S stain solution (see Appendix for recipe) for five minutes with agitation. Background staining was removed with ddH_2O. Once efficient transfer was confirmed, the protein bands were destained with 1X phosphate-buffered saline (PBS) (see Appendix for recipe).

The transferred proteins were then specifically labeled with the appropriate primary and secondary antibodies (Table 4) following standard procedures (Towbin, Staehelin, and Gordon, 1979). Briefly, membranes were blocked in TTBS (see Appendix for recipe) containing 1 % (wt/vol) skim milk and 1 % (wt/vol) BSA for 1 hour. Post-blocking, the nitrocellulose membrane was washed with TTBS for 10 minutes with agitation on a VWR orbital shaker, model DS-500E, and incubated with the primary antibody solution with agitation for 1 hour. Proteins were labeled with antibodies diluted in TTBS containing 0.1% (wt/vol) BSA as follows: *L. pneumophila* MAb (1:200), yeast MAb (1:1 000), *E. coli* MAb (1:1 000), *B. pertussis* MAb (1:200), an in-house *L. pneumophila* PAb (1:1000) or the commercially available pan-ras (Ab-2) MAb (2.5 µg/ml). The membrane was then washed with TTBS with agitation for 7 minutes and incubated in the secondary antibody solution for 1 hour. The secondary antibodies (alkaline phosphatase conjugate of an anti-mouse immunoglobulin G [IgG] or an anti-rabbit IgG) (Cedarlane Laboratories Ltd.) were diluted 1:5,000. The membrane was washed three times in TTBS, twice in TBS (see Appendix for recipe), and twice in developing buffer (see Appendix for recipe). The nitrocellulose membrane was immersed in developing solution (see Appendix for recipe) until the chromogenic

substrate had developed to an appropriate density. The reaction was stopped with washing in ddH_2O.

5.2. Protease sensitivity assay (trypsin assay).

L. pneumophila strains were harvested from BCYE plates and suspended in BYE broth. The cell suspensions were standardized to 1 OD_{620}/ml (~ 10^9 bacteria/ml) in BYE. Two 0.5 OD aliquots were harvested for each strain by centrifugation in a micro-centrifuge (Eppendorf) at 3000 x *g* for 1 minute. One aliquot was suspended in 100 µl pre-warmed Dulbecco's minimum essential medium (DMEM; GIBCO®) with 2.5 µg/ml trypsin, while the second aliquot, used as control, was suspended in DMEM without trypsin. The samples were incubated at 37 °C for 30 minutes. Trypsin inhibitor was added to the trypsin-treated sample at a final concentration of 25 mg/ml, and PBS was added to the control. The samples were incubated at 37 °C for 10 minutes and were centrifuged at 3000 x *g* in a micro-centrifuge (Eppendorf) for 1 minute. The supernatant was separated away from the pellet and the pellet was suspended in 100 µl 1X Laemmli sample buffer while 25 µl of 5 x sample buffer was added to the supernatants. The samples were boiled for 5 minutes and 15 % (vol/vol) β-mercaptoethanol was then added to the samples, which were boiled for an additional 5 minutes. The samples were subjected to SDS-PAGE and Western blotting using the *L. pneumophila* MAb.

5.3. Anion exchange chromatography.

5.3.1. Selection of starting conditions for anion exchange chromatography.

The test-tube method as described in (Pharmacia Biotech, 1982) was one of two methods used for determining start conditions for anion exchange chromatography (AEC). One hundred milligrams of diethylaminoethylcellulose matrix (DEAE, from Sigma Chemicals, St. Louis, Missouri, USA) was placed in each of 5 glass test-tubes to create mini-columns. The DEAE matrix was washed three times (30 min contact time per wash) with 10 ml column buffer (35 mM KCl, 25 mM NH_4Cl, 50 mM Tris-HCl pH 7.6, 1 mM DTT, 5 mM EDTA, 1 mM PMSF), adjusted to pH 7.5, 7.0, 6.5, 6.0, or 5.8. The mini-columns were left overnight to equilibrate at room temperature in 10 ml column buffer. A 75 ml overnight culture of *E. coli* DH5α carrying pBluescript::*cpn10cpn60* was grown at 30 °C. For each mini-column, bacterial pellets were harvested from 10 ml of the overnight culture, were suspended in 500 µl column buffer and were sonicated for 4 minutes (cycles of 30s pulses and 30s incubations at 4 °C). The lysed cells were clarified by centrifugation and the supernatants were added to the mini-columns. The mini-columns were mixed for five minutes at room temperature and the gel matrix was left to settle. Ten microliters of each sample was spotted onto a nitrocellulose membrane and was developed by standard Western blot methods with the *B. pertussis* αCpn60 MAb. The buffer showing the least intense substrate development was chosen as the optimal solution for the binding of Cpn60 to DEAE as indicated by the test-tube method. Column buffer at the optimal pH for binding was prepared with varying KCl molarity: 0.01M, 0.035M, 0.085M, 0.11M, 0.135M, 0.16M, 0.185M, 0.21M, 0.235M, 0.26M, 0.285M, and

0.31M. These buffers were used to prepare mini-columns and the binding assay was performed as described above. Ten microliters of the supernatant from each mini-column was spotted onto a nitrocellulose membrane and was developed as described above. The buffer showing the least intense substrate development was chosen as the optimal KCl concentration for binding, and the buffer showing the most intense substrate development was noted. A computational method that was used, in addition to the test-tube method, for determining the starting pH for AEC is described here. The amino acid sequence for the *B. pertussis* Cpn60 was retrieved from the NCBI Protein Database (Accession No. : I40331), and was pasted into the "Compute pI/MW tool" at the web address http://sosnick.uchicago.edu/pi_tool.html or the "Protein calculator" at the web address http://www.scripps.edu/~cdputnam/protcalc.html. These programs computed theoretical isoelectric points for Cpn60. A recommended starting point for binding to an anion exchange column is at least 1 pH unit above the isoelectric point of the protein of interest (Pharmacia Biotech, 1982).

5.3.2. Sample preparation for anion exchange chromatography.

Two liters of a bacterial culture (DH5α carrying the *cpn10cpn60* operon from *B. pertussis*) was grown overnight at 30 °C with agitation in LB medium containing ampicillin at a concentration of 100 μg/ml. The overnight culture was shifted to 37 °C for 1 hour to increase *cpn10cpn60* expression by heat-induction, and then chilled on ice for 30 minutes. Bacterial cells were harvested in 200 ml aliquots by centrifugation at a speed of 3000 rpm for 10 minutes at 4 °C (Varifuge 20 RS Heraeus Sepatech, Germany).

The cell pellets were combined and suspended in approximately 15 ml column buffer. Ten drops of Triton X (SIGMA Chemicals, St. Louis, MO, USA) were added to the culture, which was then subjected to 1100 psi in a French press. The cell lysate was sonicated and centrifuged at a speed of 5000 rpm for 15 minutes at 4 °C (Varifuge 20 RS Heraeus Sepatech, Germany). The supernatant was transferred to a 50 ml polypropylene tube, ten drops Triton X was added, and column buffer was added to a final volume of 20 ml.

A previously packed 50-ml DEAE (Whatman) anion exchange column (Rafael Garduno, Dalhousie University) was equilibrated in column buffer. The sample was added at a rate of 1 drop/18 seconds (~10ml/hour) by peristaltic pump and was followed with column buffer for 3.5 hours. The run-off (50 ml) was collected. The absorbance reading at 280 nm was recorded (ISCO UA-5 absorbance/fluorescence detector). The column flow rate was adjusted to 1 drop/13 seconds (~13.9 ml/hour) and was left to flow overnight. An elution gradient ranging from 200 mM KCl to 400 mM KCl in column buffer was set up using a gradient mixer (GM1, Pharmacia biotech, Uppsala, Sweden). The first 50 ml (run-off) was collected in two 25-ml aliquots, and the remaining sample was fractionated into ~5 ml aliquots using the LKB Bromma 7000 ULTTRORAC fraction collector. The run-off and fractions were visualized by SDS-PAGE, and the fractions containing the largest amount of Cpn60 were treated with $(NH_4)_2SO_4$ at a concentration of 60 % saturation (1 g / 1.3 ml sample) to precipitate proteins in the sample. The mixture was pelleted in a polycarbonate tube at a speed of 20, 000 rpm in a Varifuge 20 RS (Heraeus Sepatech, Germany) for 15 minutes at 4 °C. The pellet was

suspended in 11 ml column buffer. It was dialyzed for 48 hours in column buffer. Twenty drops Triton X and 2 ml column buffer were added to facilitate solubilization of the protein sample. The sample was stored -20 °C.

5.3.3. Procedure for washing the anion exchange column.

To remove precipitated proteins from the DEAE column, four bed volumes (200 ml) of 1 M NaOH was filtered through the column at a rate of 3 drops/2 seconds by peristaltic pump. Five bed volumes of sterile ddH_2O (250 ml) were then administered at the same flow rate through the column. The column was left bathed in sterile ddH_2O overnight. To remove strongly bound hydrophobic proteins, lipoproteins and lipids, two bed volumes (100 ml) of the nonionic detergent, Triton X, dissolved in 1 M acetic acid, was quickly filtered through the column, followed by five bed volumes (250 ml) of 70 % ethanol and four bed volumes (200 ml) of ddH_2O. For short-term storage the column was stored at 4 °C and with a steady flow of column buffer filtering through it. For long-term storage the column matrix was submerged in 0.05 % sodium azide.

5.4. Gel filtration chromatography.

Column preparation for gel filtration chromatography is described. Columns (Pharmacia, Piscataway, NJ) were washed with sterile ddH_20, then with 70 % ethanol, and were further sterilized by UV irradiation for 30 minutes. The columns were then leveled and suspended vertically using a clamp and stand in a 4 °C environmental chamber. Tubing present at the bottom end of the column was clamped.

Superfine Sephadex G200 superfine (46.6 g) with a fractionation range of 5 000 - 250 000-Da was added to 1400 ml column buffer. The slurry was heated to 90 °C for 5 hours in a water-bath and then cooled to 4 °C. The slurry was decanted with one constant flow into the sample reservoir attached to the O-ring of the leveled column, and then gently stirred to remove air bubbles. The column reservoir was capped and was attached to a peristaltic pump so that the flow rate of column buffer did not exceed 36 ml/hour. Column buffer was allowed to flow through the column (length 54 cm and diameter 5.1 cm) for 24 hours. The sample reservoir was removed from the column and the excess Sephadex was suctioned from the column so that the Sephadex bed level was just below the O-ring. Buffer was added to the top of the column O-ring and the upper 4 inches of the column was stirred gently and then left to settle for 1 hour.

The column adapter was filled with 20 % ethanol and the attached tubing was clamped. The adapter was inserted at a 45 ° angle and the adapter tightening mechanism was slackened just enough to create a sliding seal inside the column. The adapter was lowered until it made contact with the top of the gel bed and was inserted 2 mm further into the bed. The adapter was lodged into position using its tightening mechanism. The adapter tubing was connected to the buffer source and buffer was passed through the column for 24 hours as described above.

To test for homogeneity of the column and to determine the void volume of the column, a 20 ml of mixture of Blue Dextran 2000 (Pharmacia, Uppsala, Sweden, 2 mg/ml) and 30 % (vol/vol) acetone was filtered through a sterile 0.45 μm syringe filter,

and was added to the column by gravity feed. The mixture was followed with column buffer at a flow rate of 36 ml/hr.

The protein sample containing the BpCpn60 protein that was obtained after purification by anion exchange chromatography was gravity fed into the Sephadex G200 column and was followed with column buffer for 32 hours at a flow rate of 36 ml/hr. After the void volume was collected, the remaining volume eluted was fractionated using the LKB Bromma 7000 ULTTRORAC fraction collector and the respective absorbance was tracked using an ISCO UA-5 absorbance/fluorescence detector. Samples from the run-off and every third fraction collected were visualized by SDS-PAGE. Fractions containing the BpCpn60 protein were pooled and the sample was concentrated to approximately 20 ml by ultra filtration with a 62mm, PM10 membrane (Millipore), which excludes proteins greater than 10-kDa. The sample was then run through a G75 matrix (fractionation range 3 000 - 80 000-Da) packed into a column (Pharmacia Biotech, Uppsala, Sweden) measuring 95 cm in length and having an internal diameter of 2.5 cm as described above.

5.5. Lowry protein assay.

The Lowry protein assay was carried out as described in Lowry *et al.*, 1951. Protein standards containing 100, 50, 25, and 12.5 μg/ml BSA were prepared. A 1:10 and 1:100 dilution of the sample to be measured was made in a final volume of 1 ml. The blank consisted of 1 ml distilled water. Reagent A (1 ml, see Appendix) was added to each sample and the blank, which were mixed and incubated at room temperature for 10

minutes. Reagent B (0.5 ml, see Appendix) was added to the samples and blank, which were mixed immediately and allowed to incubate for 30 minutes at room temperature. The absorbance at 750 nm was determined using the Unico UV-2100 spectrophotometer. A standard curve was prepared (μg protein on the x-axis and absorbance 750 nm on the y-axis) and used to determine the concentration of unknown samples.

5.6. Bradford protein assay.

BSA standards were prepared as for the Lowry protein assay. To 800 μl of each sample was added 200 μl Bradford reagent (Bio-Rad, cat#500-0006). The solutions were mixed by inversion and the Absorbance at 595 nm was determined. A standard curve was prepared as for the Lowry protein assay and was used to determine the concentration of unknown samples.

5.7. Immunogold electron microscopy.

Samples were fixed at room temperature for 2 hours in 4% paraformaldehyde freshly depolymerized in warm cacodylate buffer. After fixation the samples were suspended in 0.1 M cacodylate buffer, and sent to the Dalhousie electron microscopy (EM) unit for dehydration in ethanol and embedding in LR white resin (Marivac, Quebec). Ultra thin sections were prepared with an LKB ultra microtome. Specimens were cut not more than 24 hours before immuno-gold labeling. Sections were placed on the shiny side of 300 or 400 mesh nickel or copper grids. Samples were etched by floating the grids (shiny side facing the solution) for 10 minutes on drops of freshly made 1 mg/ml sodium

borohydride (Fisher Scientific) in ddH_2O at room temperature. The samples were then floated on drops of 30 mM glycine in 0.1 M sodium borate buffer, pH 9.6. Samples were blocked in a 1 % (wt/vol) skim milk, 1 % (wt/vol) BSA solution in water for 40 minutes at room temperature. Samples were then washed three times by floating and agitating grids on 1-ml aliquots of wash buffer. Samples were then floated overnight on drops of the primary antibody solution at 4 °C, and then at 37 °C for 1 hour. Samples were then washed three times as indicated before, and floated on drops of secondary antibody solution for 1 hour at room temperature. The samples were washed 3 times in wash buffer for 10 minutes each and then in ddH_2O twice for 10 minutes each. Grids were fixed in 2.5 % (vol/vol) gluteraldehyde in wash buffer for 15 minutes. Grids were stained with 2 % (wt/vol) uranyl acetate and lead citrate by Mary Ann Trevors (Dalhousie University EM suite) or Dorota Wadowska (University of Prince Edward Island EM suite) as previously described (Garduno *et al.*, 1998). The sections were observed using a Philips EM300 transmission electron microscope (TEM) (Dalhousie University EM suite, Halifax, NS) at an accelerating voltage of 60 kV or a Hitachi 7500 TEM (University of Prince Edward Island EM suite, Charlottetown, PEI) at an accelerating voltage of 80 kV.

5.8. Procedure for immuno-staining tubulin and DNA in HeLa cell monolayers.

HeLa cells grown on cover-slips were obtained from Elizabeth Garduno, Dalhousie University, Halifax, NS. The cells were fixed in methanol for 10 minutes at -20 °C and then washed three times in PBS for 5 minutes each. Ten microliters of the TAT antibody (Woods *et al.*, 1989) was diluted 1:250 in PBST was spread gently on each cover-slip,

incubated in a humid chamber at room temperature for 30 - 45 minutes, then washed three times in PBS for 5 minutes each. A Fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (10 µl diluted 1:250 in PBST, Jackson ImmunoResearch Laboratories Inc.,West Grove, PA) was added to the cover-slip, which was incubated for 30- 45 minutes at room temperature. The cover-slip was washed three times in PBS for 5 minutes each. To stain the HeLa nuclear material, 10 µl of 4, 6-diamidino-2-phenylindole hydrochloride (DAPI from Molecular Probes, Eugene, OR) at a concentration of 0.001 µg/ml were added to the cover-slip, incubated at room temperature for 2 - 5 min, and rinsed in water. The cover-slips were then mounted on slides with ~25 µl vectashield® (Vector laboratories, Inc., Burlingame, CA). Samples were viewed using a Leitz Aristoplan epifluorescence microscope with filter blocks A (513678) for the DAPI stain and I3 (513683) for viewing FITC.

Part 6. Procedure for Staining Yeast Lysosomes.

Yeast cultures were grown to between 7×10^7 - 1×10^8 cells /ml. Cell pellets (1×10^8) were collected by centrifugation and was suspended gently in pre-warmed (30 °C) LysoTracker Yellow (L7527, Cedarlane Laboratories, Ltd.), a lysosome-specific probe that was diluted to a final concentration of 250 nm in the appropriate selective medium. The cells were incubated at 30 °C for 15 minutes and then pelleted and suspended in 1 ml pre-warmed medium. A sample of the cell suspension (~10 µl) was mounted onto a microscope slide with 5 µl glycerol, and was viewed using a Zeiss Axioplan II fluorescence microscope, and images were captured with a Spot II digital camera.

CHAPTER 3: RESULTS.

The intracellular bacterium, *L. pneumophila*, has a unique chaperonin, called HtpB, that deviates from the standard description of chaperonins due to its extra-cytoplasmic location (Garduno *et al.*, 1998), its constitutive expression at high levels and its poor up-regulation upon heat shock (Fernandez *et al.*, 1996; Lema *et al.*, 1988), its up-regulation when *L. pneumophila* contacts host cells (Fernandez *et al.*, 1996), and its function as an invasin (Garduno, Garduno, and Hoffman, 1998). Moreover, HtpB is abundantly released by *L. pneumophila* inside host cells (Fernandez *et al.*, 1996; Garduno *et al.*, 1998) by an as yet undefined mechanism. The functional significance of the released protein is unclear. This study includes experiments done to elucidate the biological effects of HtpB release inside eukaryotic cells, and includes preliminary investigations to identify a secretion mechanism for bacterial Hsp60 homologues.

Part 1. An Investigation of HtpB Function in the Yeast Model.

1.1. HtpB is expressed in the budding yeast, *S. cerevisiae*.

Due to its genetic tractability the budding yeast, *S. cerevisiae*, was selected as the eukaryotic model in which HtpB bioactivity was investigated. HtpB was expressed in the cytoplasm of *S. cerevisiae*. Since the *htpB* gene is of prokaryotic origin, the promoter sequences that regulate HtpB expression in *L. pneumophila* are inadequate for mediating expression in *S. cerevisiae*. As such, a promoter-less *htpB* gene (Accession No.: M31918, NCBI Nucleotide Database) was cloned into the yeast expression vector called

pEMBLyex4 or its derivative, pPP389, which contain the galactose-inducible yeast promoter, GAL-CYC and transcription termination sequences from the 2 μm *FLP* gene (Cesareni and Murray, 1987).

S. cerevisiae cells containing pEMBLyex4 or pEMBLyex4*::htpB* were inoculated into medium containing dextrose as the sole carbon source. Exponential phase *S. cerevisiae* cells were harvested and used to inoculate SD medium containing either galactose (Gal+/inducing medium) or dextrose (Dex/non-inducing medium) as the sole carbon source. Galactose-induced HtpB expression was detected by Western blot with the *L. pneumophila* MAb only in *S. cerevisiae* cells carrying pEMBLyex4*::htpB* (Fig. 10, Lane 6). The recombinant HtpB expressed in yeast migrated to a molecular weight similar to that seen in wild-type *L. pneumophila* (Fig. 10, Lane 7). A fraction of the recombinant HtpB protein appeared degraded in Western blots (Fig. 10, Lane 6). HtpB degradation could have resulted from exposure to yeast proteases released upon yeast cell lysis, during protein sample preparation for SDS-PAGE. HtpB protein was detected in *S. cerevisiae* protein extracts 10 hours after the inoculation of *S. cerevisiae* into inducing medium, and the full-length HtpB protein remained detectable up to 22 hours post inoculation into inducing medium, indicating that the HtpB protein was stably expressed inside yeast cells (Fig. 11).

Growth of yeast cells carrying pEMBLyex4*::htpB* was compared to that of cells carrying the pEMBLyex4 control vector, by means of spotting serial 10-fold dilutions of yeast cells in duplicate onto solid medium using a starting inoculum of 10^7 yeast cells.

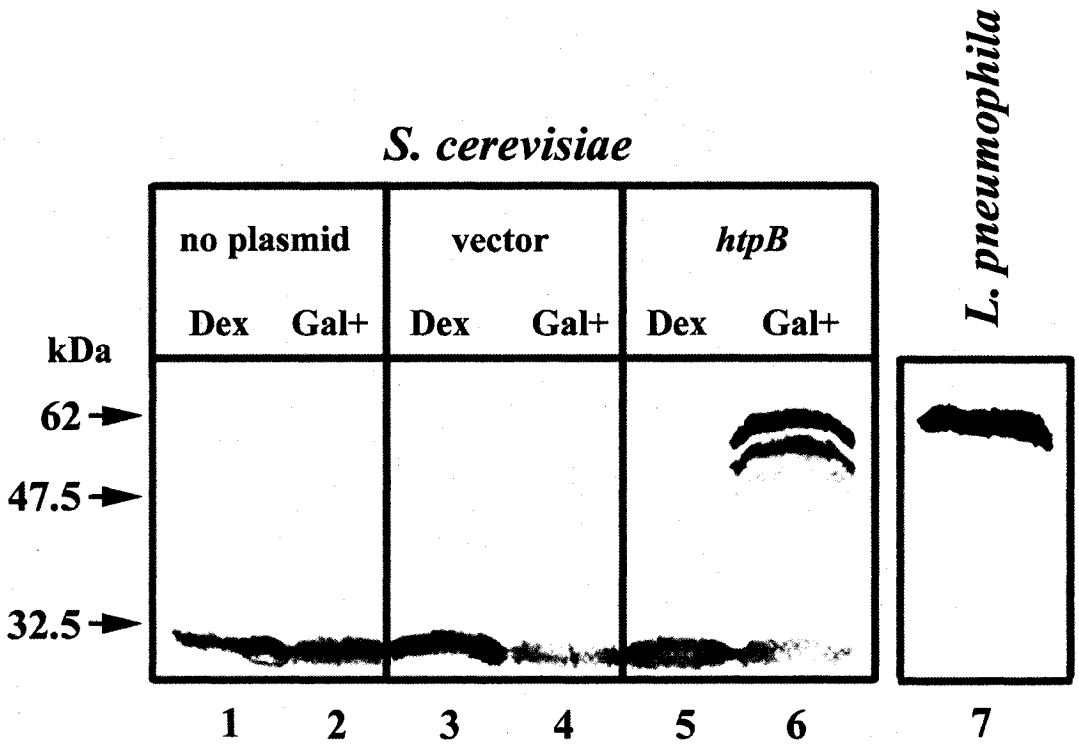


Figure 10. The *L. pneumophila* HtpB protein is expressed in *S. cerevisiae*. *S. cerevisiae* cells were grown under conditions that induced (galactose medium, Gal+) or did not induce (dextrose medium, Dex) *htpB* expression from a galactose-inducible promoter. Whole cell lysates from *S. cerevisiae* bearing: no plasmid (Lanes 1 and 2); the vector pEMBLyex4 (Lanes 3 and 4); or the plasmid pEMBLyex4::*htpB* (*htpB*) (Lanes 5 and 6), and a whole cell lysate from *L. pneumophila*, separated by SDS-PAGE, transferred to a nitrocellulose membrane, and subjected to western blotting using the *L. pneumophila* MAb, which is specific for HtpB, are depicted. Molecular weight markers (kDa) used were from New England Biolabs (Catalogue No. 7720).

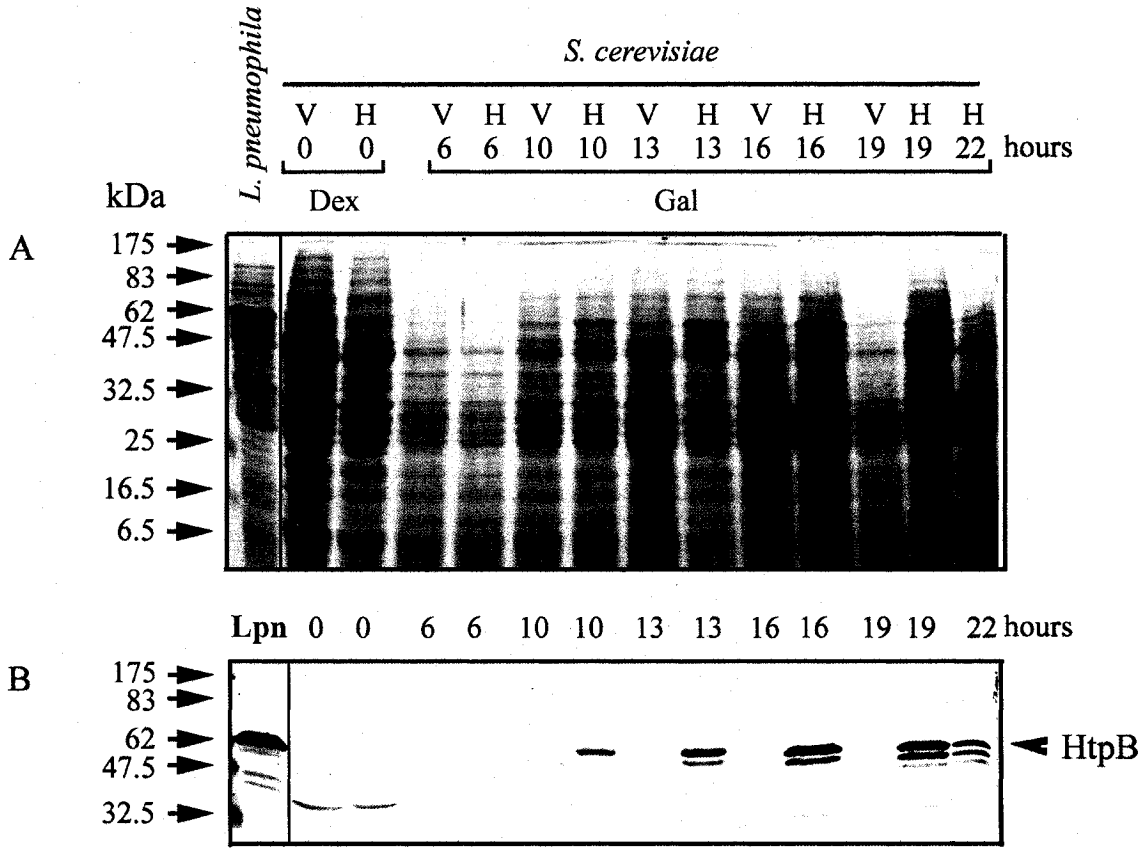


Figure 11. The HtpB protein is detected in *S. cerevisiae* for up to 22 hours post inoculation into inducing medium. *S. cerevisiae* cells carrying the vector control pEMBLyex4 (V) or plasmid pEMBLyex4::*htpB* (H) were grown in non-inducing medium (Dex) and then transferred to inducing medium (Gal). Whole cell lysates were harvested at different time points and were subjected to SDS-PAGE (Panel A) and Western blotting (Panel B) with the *L. pneumophila* MAb, which is specific for HtpB. A sample of a *L. pneumophila* whole-cell lysate was also probed (Lpn). Molecular weight markers (kDa) used are from New England Biolabs (Catalogue No. 7720). Arrowhead indicates HtpB protein.

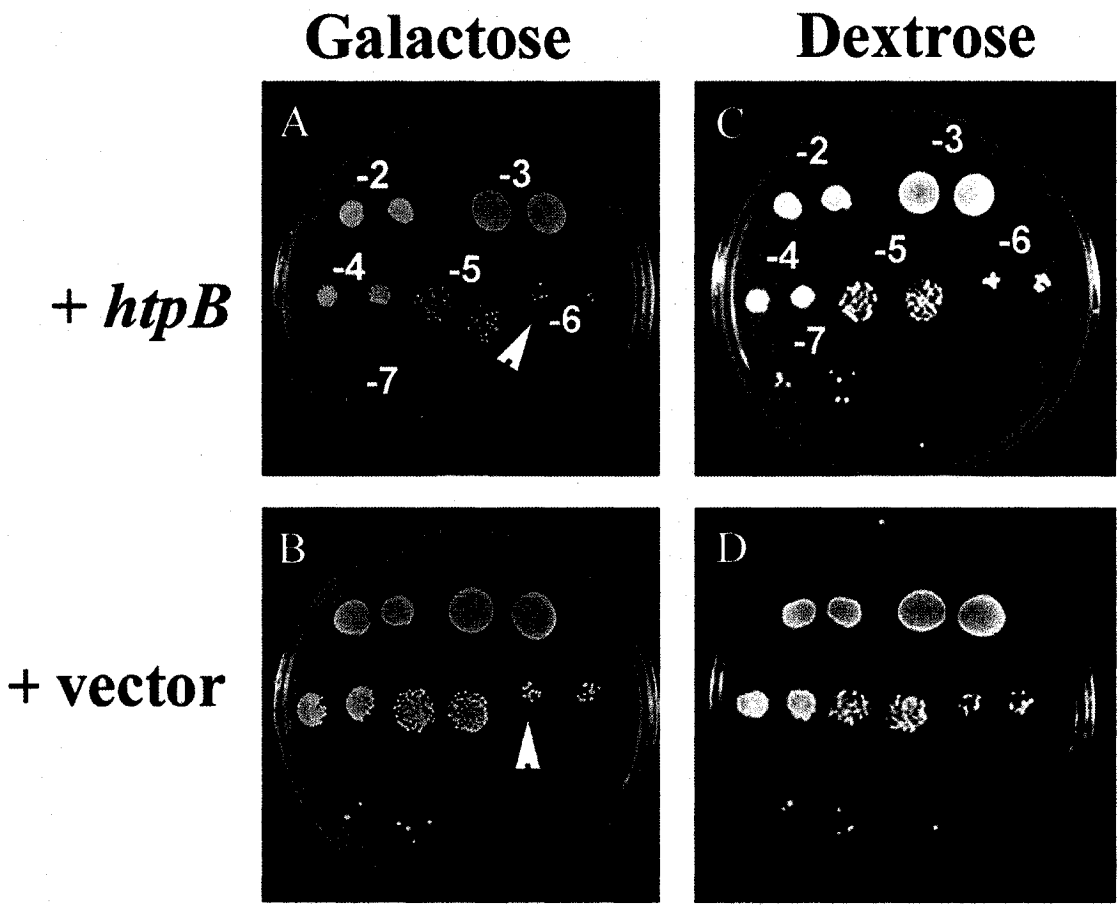


Figure 12. HtpB expression does not reduce yeast colony formation. Image depicts spots of 10-fold serially diluted yeast cells (in duplicate) bearing either pEMBLyex4::*htpB* (+ *htpB*) or pEMBLyex4 (+ vector) that were grown at 30 °C on solid medium containing either galactose or dextrose. The spotting order of the 10-fold dilutions are shown in the top panels (-2 to –7 = dilutions 10^{-2} to 10^{-7}). Arrowheads indicate duplicate spots inoculated on inducing medium with equivalent numbers of cells expressing *htpB* or carring the control vector for visual comparison.

On inducing medium at 30 °C, there was less than a two-fold drop in the number of colonies visible for cells expressing HtpB (Fig. 12, top panel A, white arrowhead) as compared to control cells carrying the pEMBLyex4 vector (Fig. 12, Panel B, white arrowhead). On non-inducing medium at 30 °C, there were virtually no growth differences between yeast cells containing pEMBLyex4 or pEMBLyex4::*htpB* (Fig. 12, Panels C and D). These results indicate that at the optimal growth temperature for *S. cerevisiae*, HtpB expression is not toxic.

1.2. HtpB expression may alter the organization of *S. cerevisiae* lysosomes.

When *L. pneumophila* infects mammalian cells, the bacterium delays the association of the mammalian lysosome-specific marker, LAMP-1, with the host-derived endosome within which *L. pneumophila* replicates (Sturgill-Koszycki and Swanson, 2000). *L. pneumophila* expresses HtpB on it cell surface and releases HtpB into replicative endosomes (Garduno, Garduno, and Hoffman, 1998; Garduno *et al.*, 1998). Therefore, the effect of HtpB expression on the organization of lysosomes in *S. cerevisiae* was assessed, as it is suspected that HtpB may play a role in delaying the fusion of *L. pneumophila* replicative endosomes with host lysosomes. When wild-type *S. cerevisiae* cells, strain W303-1b, were stained with the lysosome specific fluorescent dye LysoTracker™ (Zhang *et al.*, 1994; Diwi, Zhang, and Haugland, 1994, Invitrogen Canada, Inc., Burlington, ON), stained structures appeared as punctuate dots distributed around the yeast cell circumference in unbudded (Fig. 13A) and in budded cells (Fig. 13B). In yeast cells expressing HtpB from pEMBLyex4::*htpB*, staining with

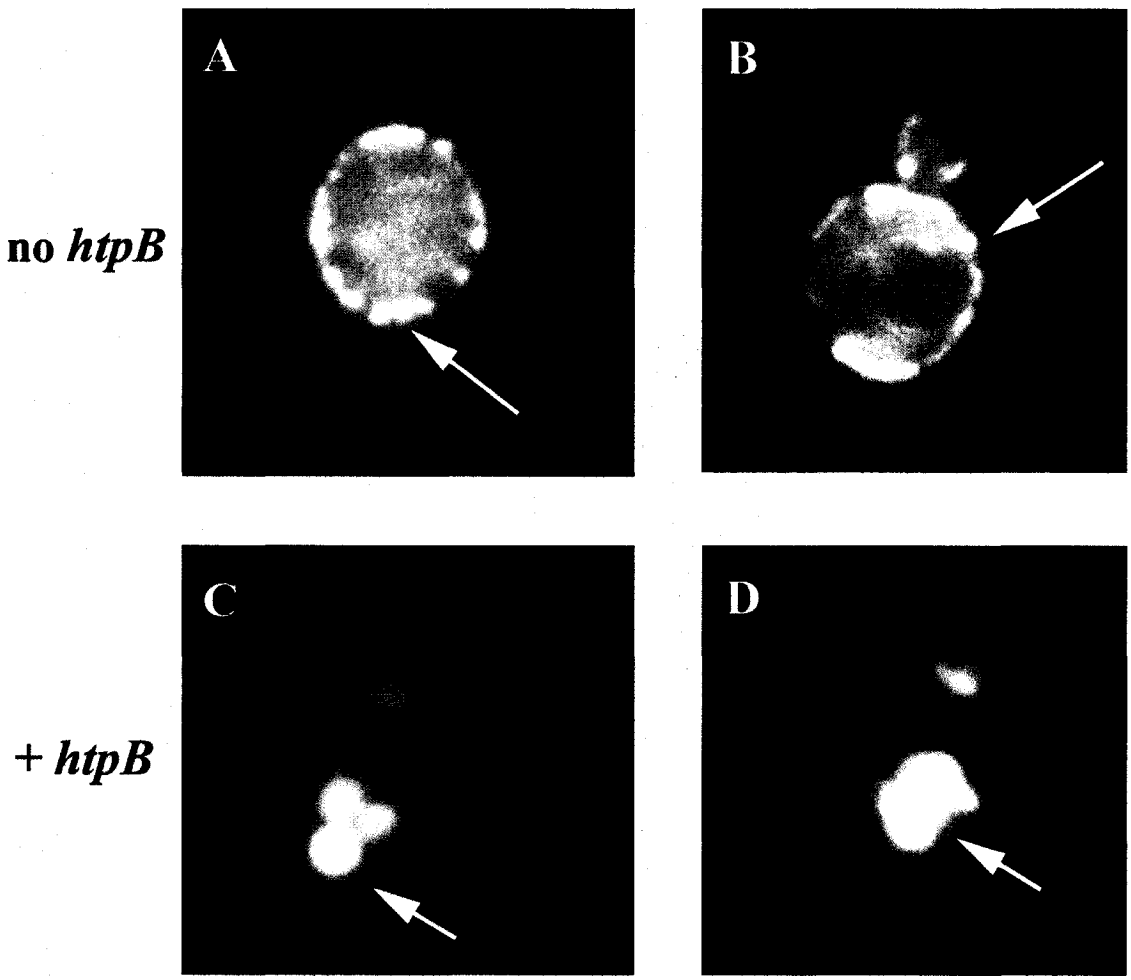


Figure 13. HtpB expression alters lysosome organization in *S. cerevisiae*. Wild-type *S. cerevisiae* cells, strain W303-1b not expressing HtpB (Panels A and B), or expressing HtpB from pEMBLyex4::*htpB* (Panels C and D) were stained with the lysosome specific fluorescent dye, LysoTracker™ and were visualized with fluorescent light. Panel D depicts a visible light image superimposed on the fluorescence-generated image depicted in panel C. Yeast cells were visualized with a 63X objective lens. Arrows depict regions with high level fluorescence.

LysoTracker™ was concentrated near the center of yeast cells (Fig. 13C and 13D). At least 100 cells each for the W303-1b wild-type strain and W303-1b cells expressing HtpB were observed for staining patterns with LysoTracker™. For the W303-1b wild-type strain greater than 90% of a total of 50 cells displayed a punctuated dot distribution as seen in Figure 13A and 13B, and for cells expressing HtpB approximately 20% of a total of 36 cells demonstrated concentrated staining near the center of cells as demonstrated in Figure 13C and 13D. The yeast cells represented in Figure 13 were harvested from cultures in early exponential phase (1×10^7 cells/ml). In cells harvested from cultures in later exponential phase (9×10^7 cells/ml) it was observed that HtpB expression in addition to causing an alteration in the distribution of yeast lysosomes, also induced a morphological change in yeast cells so that they became elongated (data not shown). The latter phenotype was further investigated, and is described in sections below.

1.3. HtpB expression triggers pseudohyphal growth in *S. cerevisiae*, W303-1b.

S. cerevisiae cells become elongated as part of a developmental process termed pseudohyphal growth. This growth process is triggered in diploid yeast cells in response to nitrogen starvation perhaps to facilitate foraging for nutrients (Gancedo, 2001). Other physical manifestations of pseudohyphal growth that often accompany the cell elongation phenotype include the emergence of unipolar buds, persistent cell-cell adherence and capacity for yeast cells to invade solid media (Gancedo, 2001). *S. cerevisiae* haploid strains are reported to undergo a process that is similar to pseudohyphal growth, termed invasive growth, since haploid cells can also invade solid media, and since intracellular

signaling components such as the Ste/MAP kinases play a role in both haploid invasive growth and in pseudohyphal growth in diploids (Gancedo, 2001). However haploid invasive growth is unique in that it is triggered in response to presence of fusel oils such as byproducts of amino acid catabolism, instead of in response to nitrogen depletion (Dickinson, 1996). For the purpose of this work two physical phenotypes, the capacity for yeast cells to form pseudohyphae defined as elongated cells that are budded in a unipolar fashion and to invade solid medium were used as indicators of HtpB-induced pseudohyphal growth. In addition activation of the pseudohyphal growth reporter construct FG(*TyA*)*::lacZ* inside yeast cells (see section 1.9 for a more detailed description of this reporter construct) was used to test whether haploid yeast cells expressing HtpB had a similar transcription profile as compared to diploid cells that undergo pseudohyphal growth in response to nitrogen starvation. Pseudohyphal growth was selected as the term to describe HtpB-induced changes in *S. cerevisiae* haploid strains W303-1b and BY4741for this study, since the term invasive growth that is often used to describe the invasion of solid medium by haploid cells does not allow discrimination between the formation of pseudohyphae (morphological changes) and the active process of agar invasion. In current literature haploid invasive growth is often scored as the ability of haploids to adhere to solid medium after washing with a stream of water, so that the morphological appearance of cells is not always reported. While for this work the phenotypes of agar invasion and the formation of pseudohyphae were treated as independent processes.

The *S. cerevisiae*, haploid strain W303-1b (a standard laboratory yeast strain) formed pseudohyphae when expressing HtpB from pEMBLyex4::*htpB* with overnight incubation at 30 °C on solid inducing medium. That is, HtpB-expressing cells were elongated and were budded in a unipolar fashion (Fig. 14A). Over 50 microscope fields were viewed at 400X magnification, and this phenotype was clearly apparent in greater than 80 % of the cell population. With extended incubation, at 30 °C for five days, HtpB-induced pseudohyphae invaded the agar surface, and remained attached to the solid medium even after washing with ddH_20 (Fig. 14B). Control cells carrying pEMBLyex4 did not display these pseudohyphal growth phenotypes, that is, cells did not elongate, were not budded in a unipolar fashion and did not form invasive pseudohyphae when grown on solid inducing medium (Fig. 14C and 14D, respectively). HtpB-induced pseudohyphal growth closely resembles nitrogen starvation-induced pseudohyphal growth in that the cell were elongated, budded in a unipolar fashion and invaded solid medium, but HtpB-induce pseudohyphal growth is unique in that this process occurred independent of the cues normally required. More specifically HtpB-induced pseudohyphal growth in strain W303-1b was evaluated on medium that had an adequate supply of nitrogen, such that control cells carrying the vector did not form invasive pseudohyphae (Fig. 14C and 14D). Furthermore it is reported that yeast strains W303 and S288C do not form invasive pseudohyphae in response to nitrogen starvation (Liu, Styles, and Fink, 1996), yet the haploid strain W303-1b did form invasive pseudohyphae in response to HtpB expression. Hence it was of interest to investigate whether HtpB

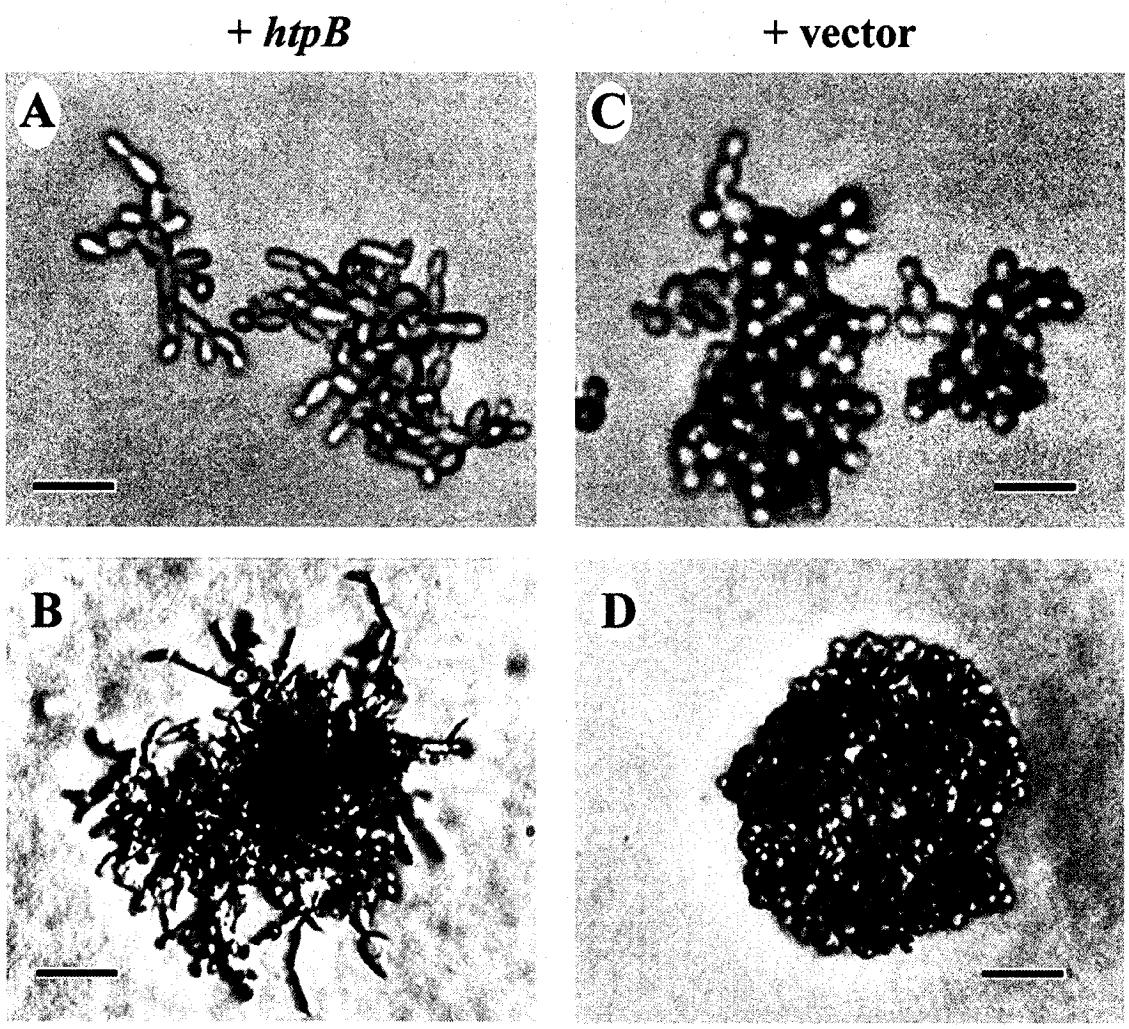


Figure 14. *S. cerevisiae* cells expressing HtpB form invasive pseudohyphae. Haploid *S. cerevisiae* W303-1b cells carrying pEMBLyex4::*htpB* (+ *htpB*: Panels A and B) or the vector, pEMBLyex4 (Panels C and D). Yeast cells shown in Panels A and C were grown overnight in a droplet of liquid inducing medium (Gal+) that was placed on the surface of solid Gal+ medium. Yeast cells shown in Panels B and D were inoculated onto solid Gal+ medium, grown for 5 days and washed from the surface with ddH_2O; cells depicted are those that penetrated the agar surface. Bars indicate 9 μm (Panels A and C) or 25 μm (Panels B and D).

could induce pseudohyphal growth in a strain that has been previously documented to form invasive pseudohyphae (Murray et al., 1998).

1.4. HtpB expression does not trigger pseudohyphal growth in the diploid *S. cerevisiae* strain MLD158.

A diploid strain of the 21R background called MLD158 is reported to form invasive pseudohyphae (Murray *et al.*, 1998). In addition yeast strains derived from MLD158 that contain single deletions in genes that are essential to the process of pseudohyphal growth were available (Murray *et al.*, 1998). It was envisioned that these strains could be used to evaluate whether HtpB relies on these components to induce pseudohyphal growth, by expressing HtpB in these deletion strains followed by an assay for pseudohyphae formation and invasive growth. Hence the capacity for HtpB to induce pseudohyphal growth in the parent strain MLD158 was investigated. When HtpB was expressed from plasmid pEMBLyex4::*htpB* in MLD158 as demonstrated in a Western blot using the *L. pneumophila* MAb that is specific for HtpB (Fig. 15A, panel insert), these cells did not form pseudohyphae (Fig. 15A), and displayed a morphology indistinguishable from MLD158 cells carrying the vector control, pEMBLyex4 (Fig. 15B). As a positive control for pseudohyphal growth on SD-inducing medium (the standard medium used in this study to evaluate HtpB-induced pseudohyphal growth), a derivative of the MLD158 strain called MLD66 that forms pseudohyphae when grown in rich medium (YEPG) was evaluated. MLD66 is homozygous for the *sup70-65* allele,

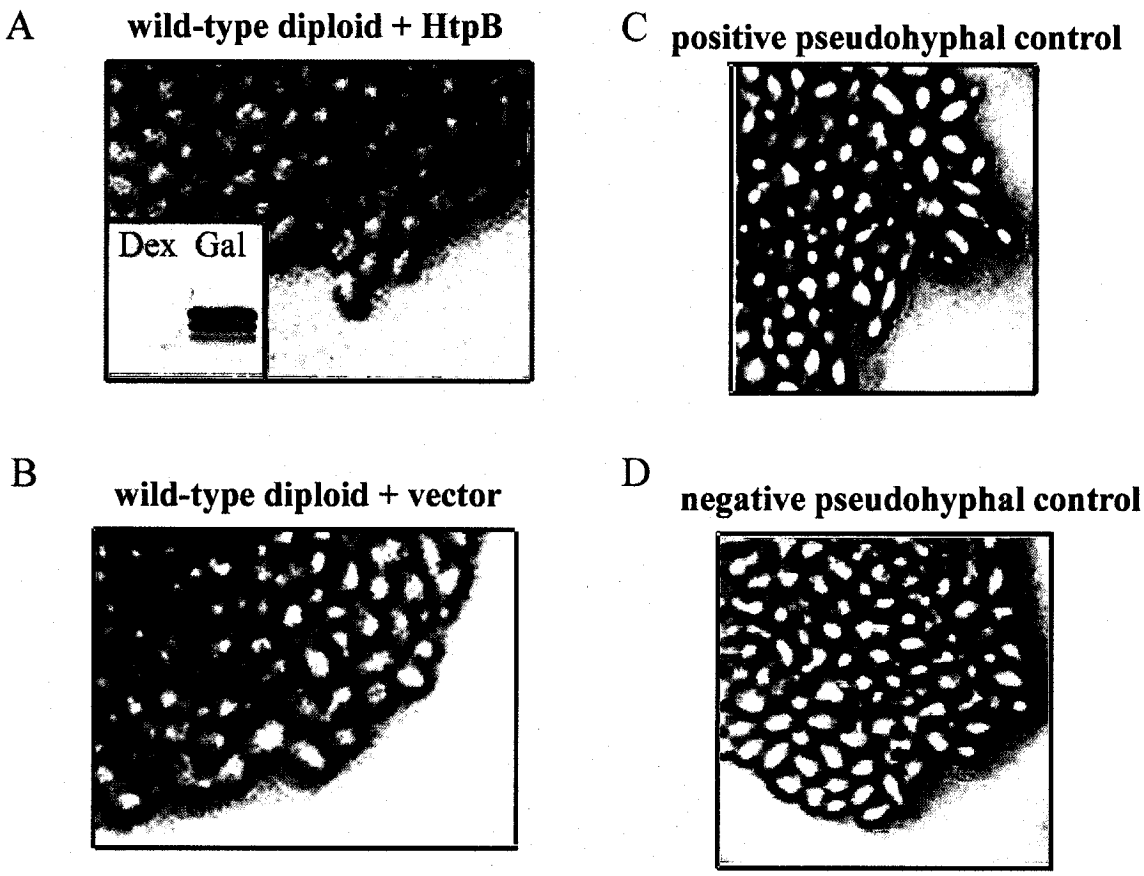


Figure 15. HtpB expression in diploid yeast strains of the *S. cerevisiae* 21R background does not result in the formation of pseudohyphae. The diploid *S. cerevisiae* strain, MLD158 of the 21R strain background expresses HtpB from plasmid pEMBLyex4::*htpB* (Panel A) or carries the vector, pEMBlyex4 (Panel B). As a positive control for pseudohyphal growth, a derivative of strain MLD158 (called MLD66) that bears homozygous mutations for the *sup70-65/sup70-65* alleles that induce constitutive pseudohyphal growth in yeast cells on rich medium was included (Panel C). As a negative control for pseudohyphal growth, strain MLD67, which does not form pseudohyphae in response to nitrogen starvation due to the mutant alleles *ste12Δ/ste12Δ*, was included carrying pEMBLyex4 (Panel D). HtpB expression was confirmed by Western blot with the *L. pneumophila* Mab (Panel A insert). Cells depicted were grown overnight on solid inducing medium, and viewed with a 40X objective lens.

which encodes a mutant version of the yeast glutamine t-RNA that induces yeast cells to undergo pseudohyphal growth. In this study MLD66 did form pseudohyphae on SD-inducing medium, indicating that this strain performed as previously reported (Murray *et al.*, 1998), and that SD-inducing medium is suitable for evaluating pseudohyphal growth in strains of the 21R background. As a negative control for pseudohyphal growth, a derivative of MLD158 called MLD67, containing a homozygous deletion of the *STE12* allele, a gene that is essential for pseudohyphal growth in diploid yeast cells in response to nitrogen starvation was evaluated. MLD67 did not form pseudohyphae on the SD-inducing medium prepared (Fig. 15D), and provided a negative standard for evaluating yeast cell morphology for MLD158. At least 20 microscope fields were viewed for each strain, and greater than 80 % of cells demonstrated the phenotypes illustrated in Figure 15. Taken together these data indicate that the diploid *S. cerevisiae* strain MLD158 of the 21R background is not suitable for assessing HtpB-induced pseudohyphal growth. Hence the W303-1b strain was utilized for further studies.

1.5. Yeast mitochondrial Hsp60p expression does not trigger pseudohyphal growth in the *S. cerevisiae* haploid strain W303-1b.

The *S. cerevisiae* Hsp60 homologue, which is a mitochondrial protein (Hsp60p, Systematic name: YLR259C, SGD™; Reading, Hallberg and Myers, 1989), shares 54.5 % protein sequence identity with the *L. pneumophila* HtpB protein as determined by the Lalign program (Pearson *et al.*, 1997). The yeast Hsp60p protein was over-expressed in *S. cerevisiae* to test whether yeast cells producing large quantities of any chaperonin

would form pseudohyphae, perhaps through non-specific activation of a general stress response and/or the general sequestering of essential yeast proteins. Unlike HtpB, the yeast Hsp60p has a positively charged region at its N-terminus that mediates import into yeast mitochondria (Fig. 16). Since it is possible that sequestration in the mitochondria could prevent the yeast Hsp60p from causing pseudohyphae formation, two versions of the yeast Hsp60p were expressed in *S. cerevisiae*. In addition to the wild-type yeast Hsp60p, a truncated version of the protein, lacking the mitochondrial targeting sequence, Hsp60Δ1-24p, was expressed in *S. cerevisiae*, from plasmids pPP389::*HSP60* and pPP389::*HSP60Δ1-72*, respectively. Sequencing of the *HSP60* and *HSP60Δ1-72* alleles from these constructs revealed that four and five nucleic acid substitutions encoding non-synonymous amino acid changes were introduced into the gene sequences, respectively by Taq polymerase, indicating that these alleles generated by PCR are not identical to the wild-type gene sequence (see Materials and Methods for details), and may display uncharacteristic functions. Expression of the two alleles in *S. cerevisiae* was confirmed by Western blot using a mixture of two non-cross-reacting MAbs, the Yeast MAb, an anti-Hsp60p monoclonal antibody, and the *L. pneumophila* MAb, an anti-HtpB monoclonal antibody (Fig. 17A). *S. cerevisiae* cells expressing the full-length *HSP60* or *HSP60Δ1-72* did not form pseudohyphae (Fig 17B and 17C), demonstrating that yeast cells do not form pseudohyphae as a general response to the hyper-expression of proteins similar to HtpB.

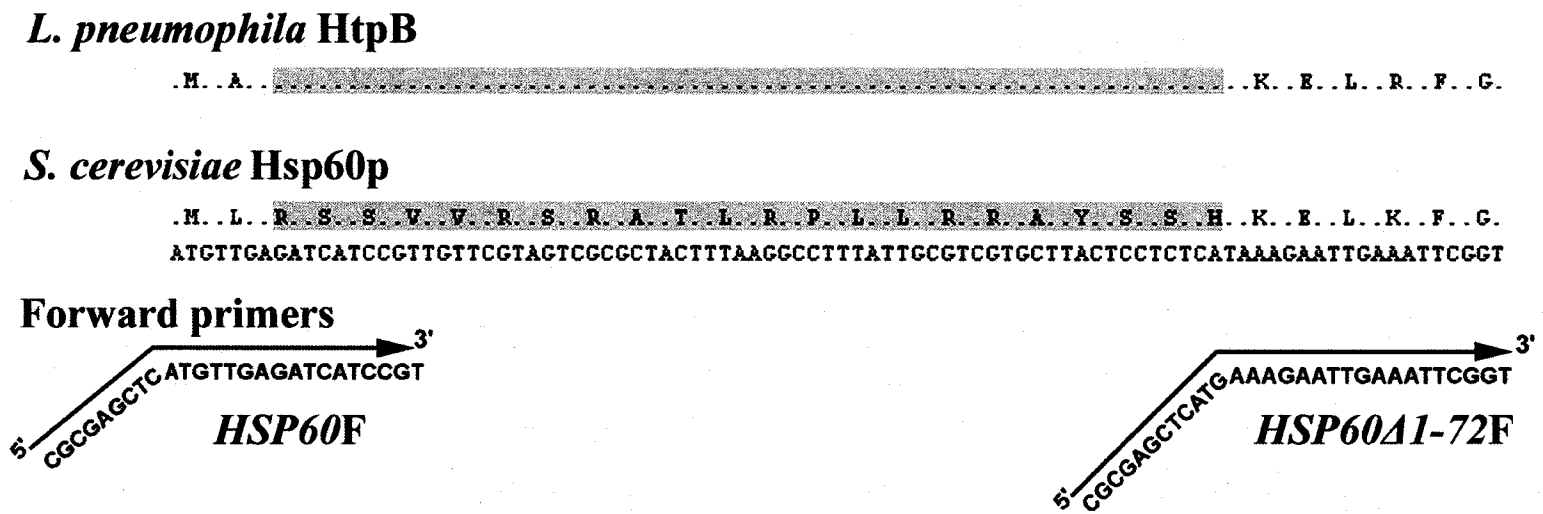


Figure 16. The *S. cerevisiae* Hsp60 homologue has an N-terminal signal sequence, which was either included or deleted during PCR amplification and cloning. This mitochondrial targeting signal sequence is not found in the *L. pneumophila* Hsp60 homologue, HtpB (see highlighted regions). The forward primer, *HSP60*F, was designed to PCR amplify a version of the yeast *HSP60* gene including the mitochondrial targeting sequence. Another forward primer, *HSP60Δ1-72*F, was designed to PCR amplify a second version of the yeast *HSP60* gene that lacks the mitochondrial targeting sequence. Both forward primers were paired with the same reverse primer, *HSP60*R (Table 5).

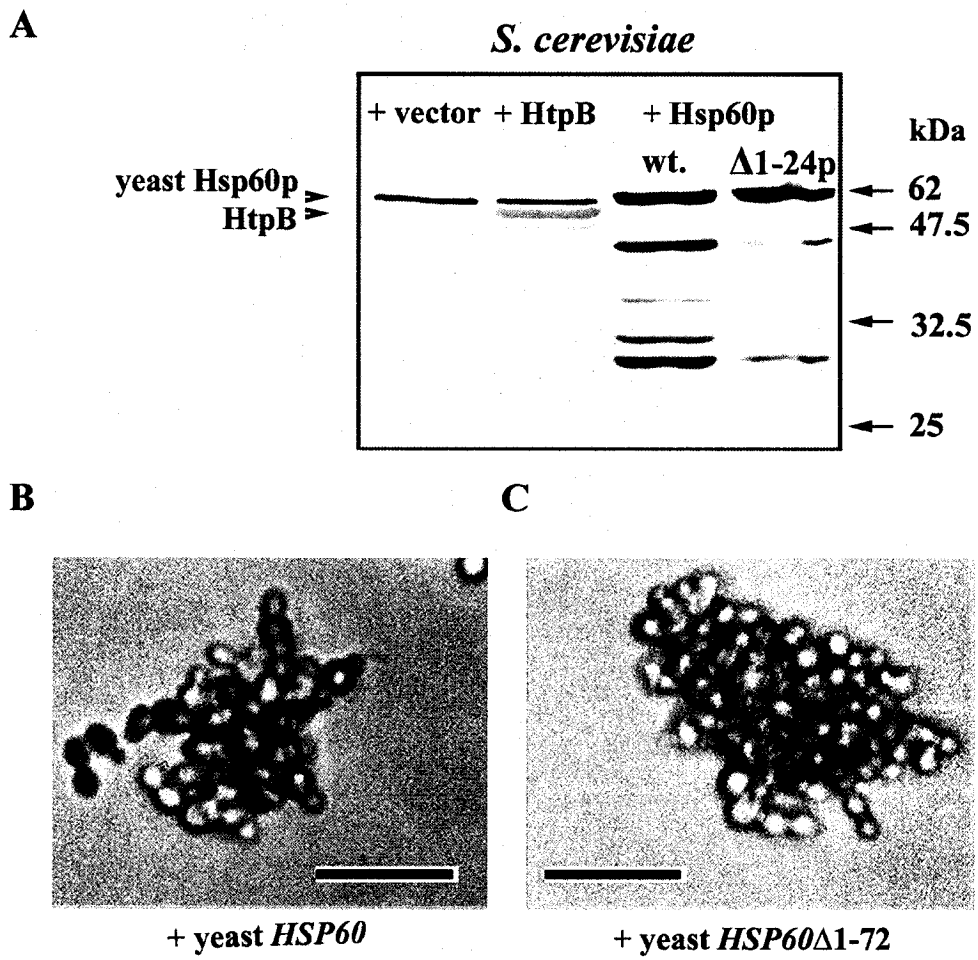


Figure 17. Hyper-expression of the yeast Hsp60p in *S. cerevisiae* does not cause pseudohyphae formation. Proteins were extracted from *S. cerevisiae* cells containing the vector pPP389 (+ vector), pPP389::*htpB* (+ HtpB), pPP389:*HSP60* (+ Hsp60p, wild-type [wt.]) or pPP389::*HSP60Δ1-72* (+ Hsp60p, Δ1-24p), subjected to SDS-PAGE, transferred to nitrocellulose, and probed with a non-crossreacting mixture of the yeast MAb and *L. pneumophila* MAb that are specific for the yeast Hsp60p and the *L. pneumophila* HtpB respectively in a Western blot (Panel A). HtpB was only expressed in the indicated lane (+ HtpB), while the yeast Hsp60p was detected in all lanes (Panel A). *S. cerevisiae* cells expressing Hsp60p from pPP389::*HSP60* (+ yeast *HSP60*) or Hsp60Δ24p from pPP389::*HSP60Δ1-72* (+ yeast *HSP60Δ1-72*) were grown overnight at 30 °C on solid inducing medium. These cells did not form pseudohyphae (Panels B and C). Bars indicate 12.5 μm.

1.6. *E. coli* GroEL expression in *S. cerevisiae* does not cause pseudohyphal growth.

The *E. coli* Hsp60 homologue, GroEL, (Accession No.: AAC77103, NCBI Protein Database) shares 75.5% protein sequence identity with HtpB (Accession No.: AAA25299, NCBI Protein Database) as determined by the Lalign program (Pearson *et al.*, 1997). Many of the inferred characteristics associated with Hsp60 homologues are based on studies with GroEL, which is a cytoplasmic protein that functions to fold denatured and newly synthesized proteins (Zeilstra-Ryalls, Fayet, and Georgopoulos, 1991), and is expressed at high levels in response to heat shock (Arsene, Tomoyasu, and Bukau, 2000). Since HtpB displays contrasting characteristics in that it is surface-associated, expressed constitutively at high levels, upregulated only two-fold in response to heat-shock, and upregulated in response to bacterial contact with host cells (Garduno *et al.*, 1998; Fernandez *et al.*, 1996), it was tempting to express GroEL in *S. cerevisiae* to see whether it could also alter *S. cerevisiae* physiology. The *groEL* gene was amplified by PCR with the high fidelity PFU polymerase from plasmid pTRC99A::*groELS* using primers *GroELpEMBL*-F and *pTRC99A*-R, and was cloned into pEMBLyex4 to generate pEMBLyex4::*groEL*. The *groEL* gene in this plasmid was sequenced and no nucleic acid substitutions were detected in the sequences obtained (see Materials and Methods for details). The expression of recombinant GroEL in yeast from pEMBLyex4::*groEL* was confirmed by Western blot probed with the *L. pneumophila* PAb, a polyclonal anti-sera generated in response to HtpB (Fig. 18A and 18B). Unlike HtpB, GroEL did not induce pseudohyphal growth in *S. cerevisiae* (Fig. 18C-18E). At least 25 fields were viewed for each of three independent transformants, and no cells expressing GroEL demonstrated

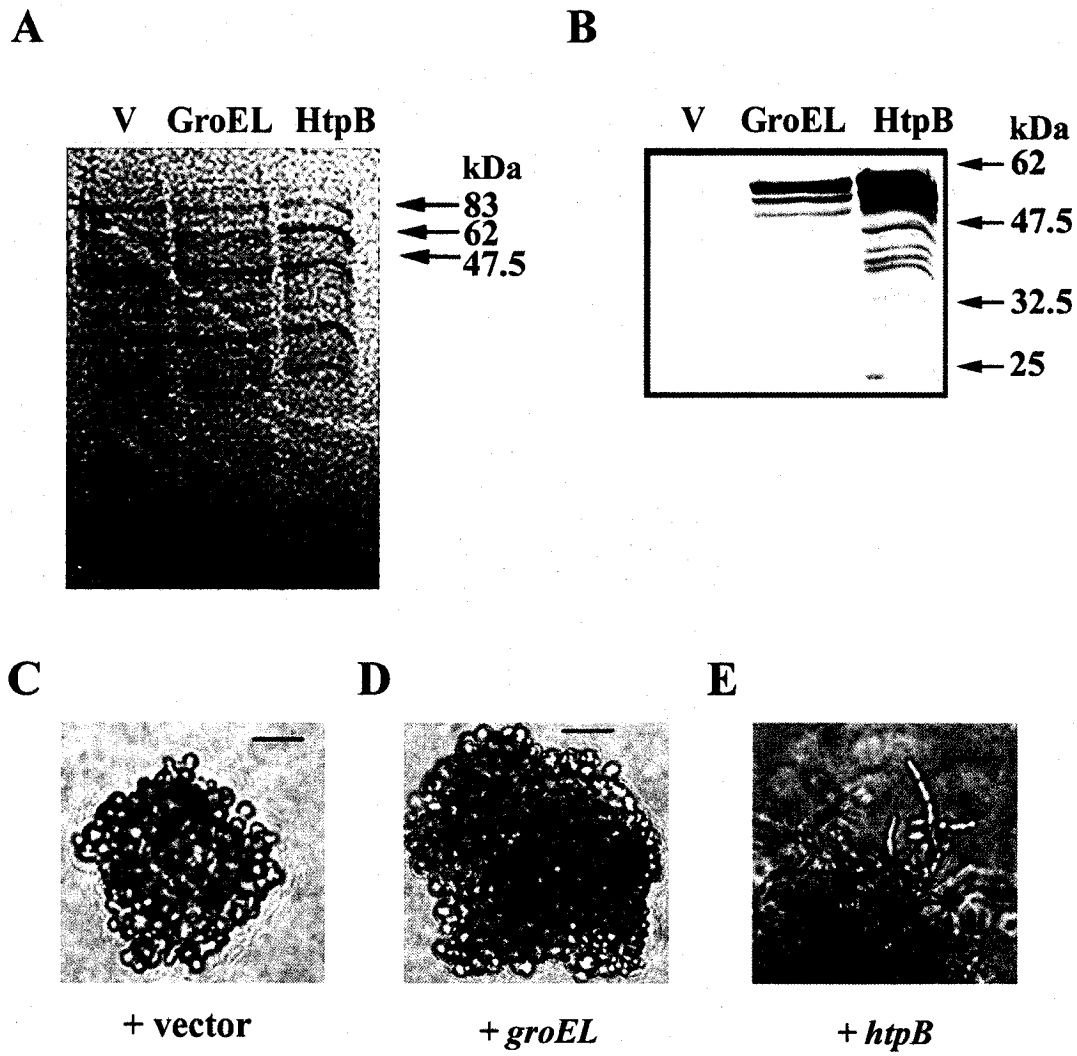


Figure 18. Expression of the *E. coli* Hsp60 homologue, GroEL, in *S. cerevisiae* does not cause pseudohyphal growth. Cell lysates from *S. cerevisiae* cells containing the vector pEMBLyex4 (+V), pEMBLyex4::*groEL* (+GroEL), or pEMBLyex4::*htpB* (+HtpB) were separated by SDS-PAGE, transferred to nitrocellulose and stained with Ponceau S (Panel A), then destained with 1X PBS and probed with the *L. pneumophila* PAb that crossreacts with HtpB and GroEL (Panel B). The recombinant GroEL protein migrated to the expected molecular weight of ~60-kDa. Yeast cells carrying the vector pEMBLyex4 (Panel C) or expressing GroEL from pEMBLyex4::*groEL* (Panel D) did not form invasive pseudohyphae, while cells expressing HtpB from pEMBLyex4::*htpB* (Panel E) did form invasive pseudohyphae. Bars indicate 27.95 μm (Panel C) or 21.5 μm (Panels D and E).

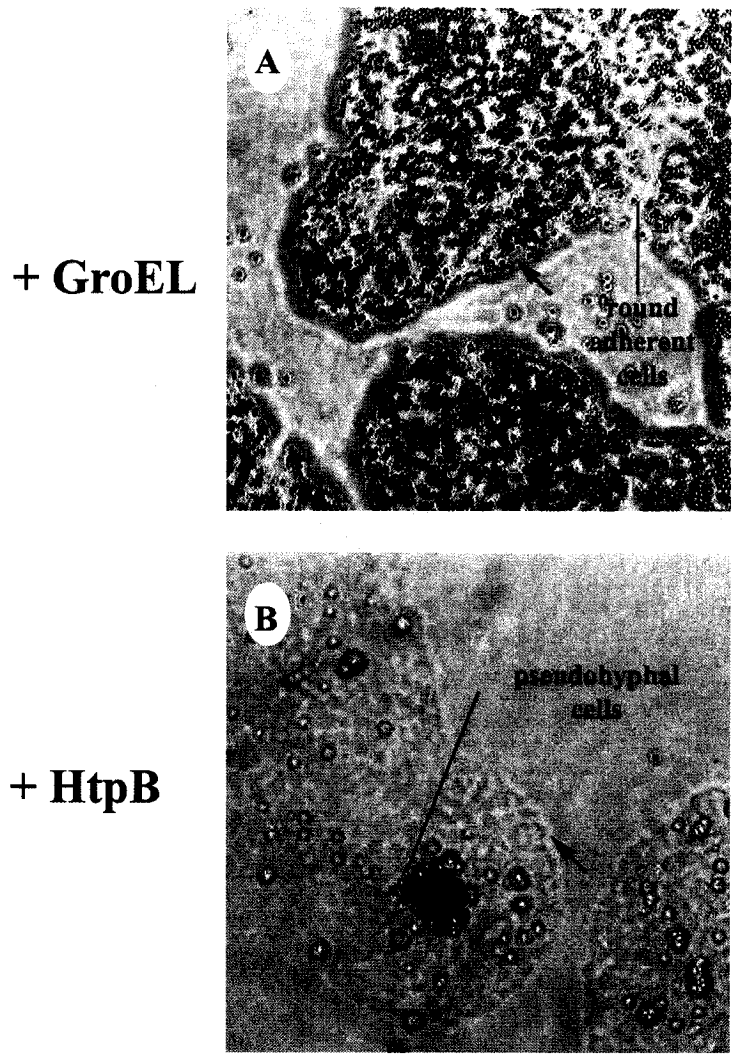


Figure 19. GroEL expression causes yeast cells to adhere to solid medium. Haploid *S. cerevisiae* cells, strain W303-1b expressing GroEL from the galactose-inducible plasmid pEMBLyex4::*groEL* adhere tightly to the surface of solid inducing medium (Panel A). Cells expressing HtpB from pEMBLyex4::*htpB* (Panel B) instead form invasive pseudohyphae. Lines indicate yeast cells that remained adherent to solid inducing medium after growth for five days followed by the washing away of cells with ddH_20. Arrows indicate impressions in the agar medium made by yeast colonies that were washed away with ddH_2O. Light micrographs of the agar surface taken with a 10X objective lens are presented.

pseudohyphal growth phenotypes, while in the positive control culture expressing HtpB greater than 80 % of cells were elongated, budded in a unipolar fashion and/or could invade solid medium. However, GroEL expression did cause yeast cells to become more adherent to the surface of solid inducing medium as compared to HtpB-expressing cells (Fig. 19).

1.7. Expression of the HtpB-H400G mutant allele in *S. cerevisiae*.

HtpB (Accession No.: M31918, NCBI Protein Database) shares 70 % amino acid sequence identity with the Hsp60 homologue called symbionin, from the endosymbiont, *Buchnera aphidicola* (Accession No.: CAA43460, NCBI Protein Database) as determined by the Lalign program (Pearson *et al.*, 1997). An interesting feature of symbionin is that it is reported to have histidine kinase activity (Morioka *et al.*, 1994). Based on the premise that the chaperonins are proteins that display conserved function it was hypothesized that HtpB could also have histidine kinase activity, which might signal pseudohyphal growth in *S. cerevisiae*.

The amino acid sequences encoding HtpB (the *L. pneumophila* Hsp60 homologue), symbionin (the *B. aphidicola* Hsp60 homologue), GroEL (the *E. coli* Hsp60 homologue), Cpn60 (the *B. pertussis* Hsp60 homologue Accession No.: I40331, Entrez Protein Database) and Hsp60p (the *S. cerevisiae* Hsp60 homologue) were aligned using the web-based program Clustal W (Chenna *et al.*, 2003). Histidine (H) residues were then highlighted using the web-based program GeneDoc (Karl Nicholas, http://www.psc.edu/biomed/genedoc), so that the histidine residues that are conserved in

the bacterial Hsp60 homologues, but absent in the *S. cerevisiae* Hsp60p could be identified (Fig. 20). Since Hsp60p lacks the ability to induce pseudohyphal growth in *S. cerevisiae* (Fig 17) it was hypothesized that if the Histidine residue was required for pseudohyphal development it would not be present in the yeast Hsp60p. Histidine residue 400 (H400) in HtpB is the only Histidine residue in HtpB that meets these criteria (Fig. 20). Furthermore H400 is situated near to the ATP-binding pocket in Hsp60 homologues (Yoshida *et al.*, 2001; Brocchieri and Karlin, 2000), which is necessary for histidine kinases as they require a source of ATP nucleotides (Alex and Simon, 1994) to be active. To determine whether H400 plays an essential role in the HtpB-induced pseudohyphal growth in *S. cerevisiae* using site directed mutagenesis, it was replaced with glycine (G). The H400 change to glycine encoded by nucleotides 5'-CAT-3' and 5'-GGT-3' respectively was confirmed by restriction digest of 686 bp PCR amplicons that contained the nucleotides residues that code for residue 400. At the equivalent nucleotide position, 1197, there exists a *Nla*III restriction enzyme site (5'-CATG-3') in the wild-type *htpB* gene that is lost when H400 is replaced with glycine, hence plasmids containing the mutant *htpB* allele, *htpB-1197* (Fig. 21, Lanes1, 2, 5 and 10) could be differentiated from those containing wild-type copies of the *htpB* gene (Fig. 21, Lanes 3, 4, 6, 7, 8 and 9), since the *htpB-1197* mutant allele lacks the *Nla*III site that is present in wild-type *htpB*. Expression of HtpB-H400G from pEMBLyex4::*htpB-1197* was confirmed by Western blot (Fig. 22A) using the *L. pneumophila* MAb. The mutant protein HtpB-H400G induced pseudohyphal growth in *S. cerevisiae* (Fig. 22B - 22D), as well as the wild-type HtpB protein. In at least 50 microscope fields that were assessed at 200X magnification

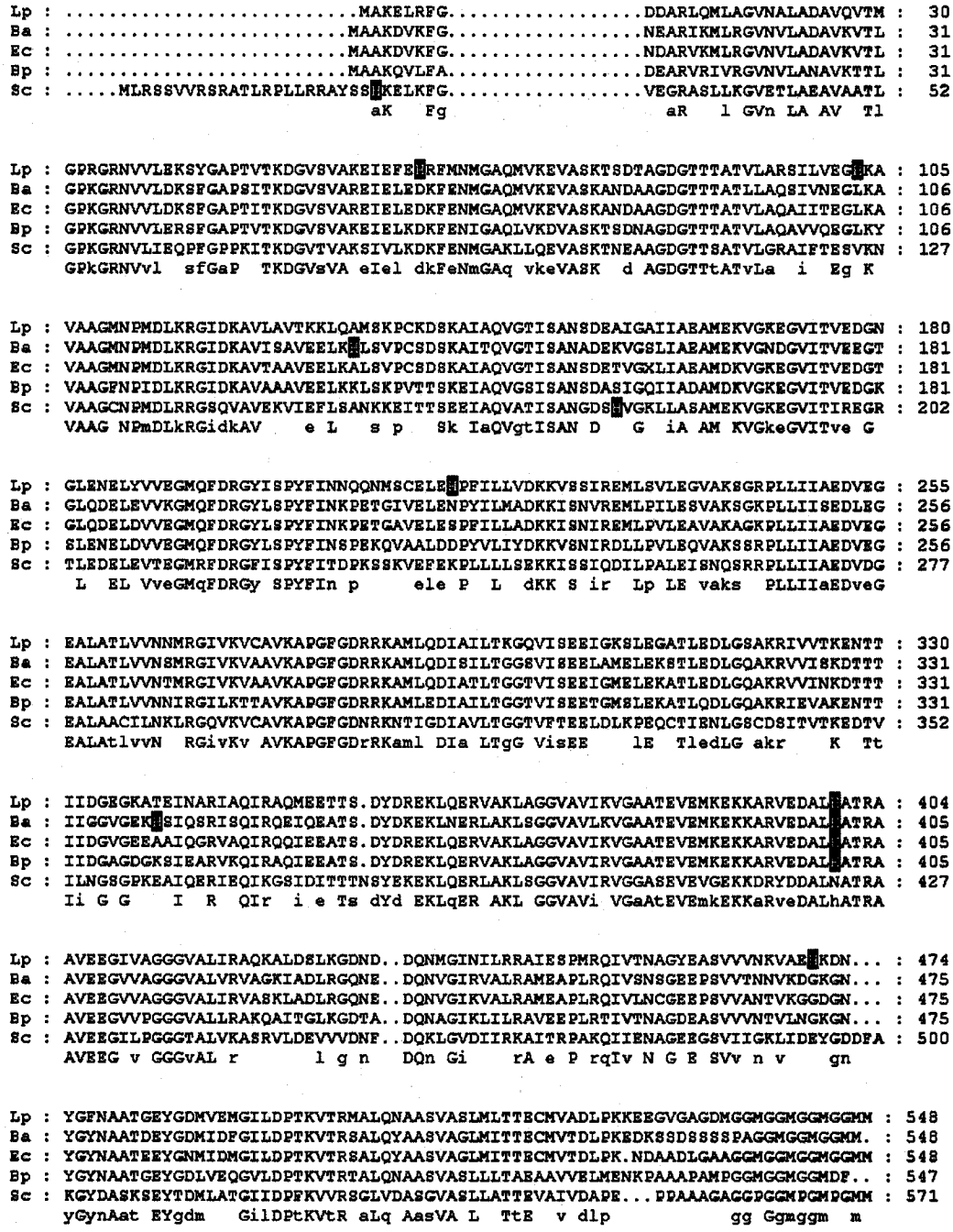


Figure 20. Alignment of Hsp60 homologues. The amino acid sequences for the Hsp60 homologues from *L. pneumophila* (Lp), *B. aphidicola* (Ba), *E. coli* (Ec), *B. pertussis* (Bp) and *S. cerevisiae* (Sc) were aligned using Clustal W and edited using GeneDoc. Histidine residues are highlighted. Identical and similar residues are indicated in the bottom row. Numbers indicate amino acid residue positions for each of the Hsp60 homologues.

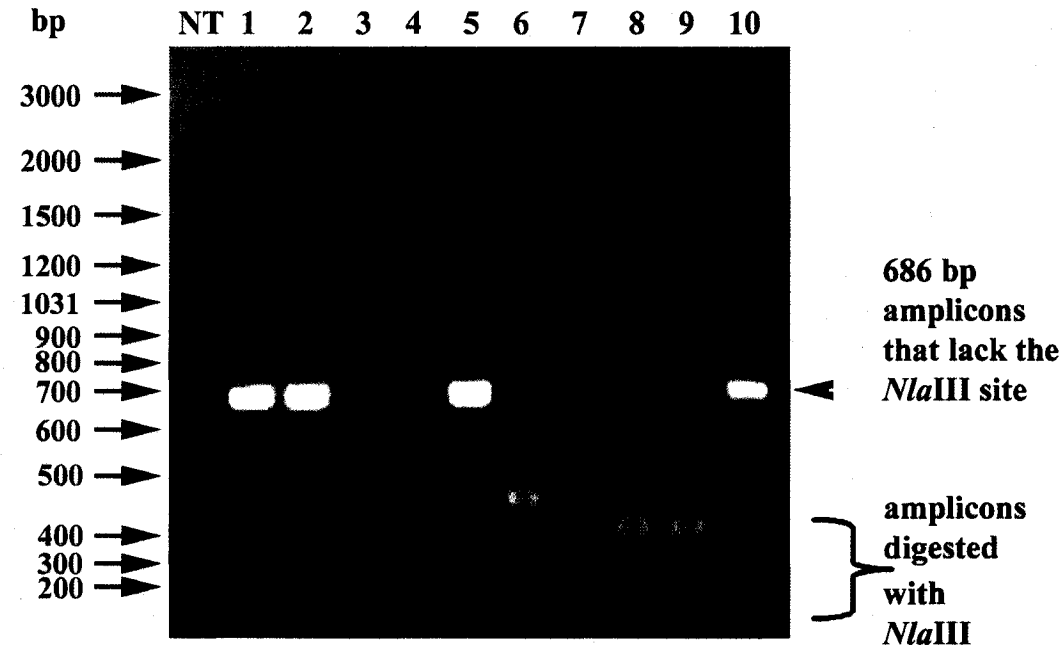


Figure 21. The *Nla*III restriction enzyme site that is present in the wild-type *htpB* gene is lost when the nucleotides encoding histidine residue 400 in the HtpB protein are changed to encode glycine by site-directed mutagenesis. To test whether Histidine (H) residue 400 in HtpB is critical for the process of HtpB-induced pseudohyphal growth, sense strand and anti-sense strand primers for *htpB* (Table5) were designed to encode a change in the nucleotides encoding H400 located at position 1197 in the wild-type *htpB* gene, and were used for site-directed mutagenesis, so that these nucleotides could be changed from 5'-CAT-3' to 5'-GGT-3'. This mutant allele was to be expressed in *S. cerevisiae* from plasmid pEMBLyex4 to test for the presence or absence of pseudohyphal growth. Located at position 1197 in the wild-type *htpB* gene is the *Nla*III restriction enzyme site (5'-catg-3'), hence replacement of the 5'-cat-3' nucleotides with 5'-ggt-3' in the wild-type *htpB* gene resulted in a loss of the *Nla*III restriction enzyme site (Lanes 1, 2, 5, and 10), while Lanes 3, 4, 6, 7, 8, and 9 represent negative plasmid clones that still contained the *NlaIII* site. The 686 bp PCR products that were used for restriction enzyme analysis contain nucleotides encoding amino acid residue 400 in HtpB and were generated using primers, (*htpB*686Fand *htpB*686R, in Table 5). NT = no template PCR negative control. The GeneRuler™ 100 base pair (bp) DNA Ladder Plus from MBI Fermentas (Catalogue No: SM0321) was used.

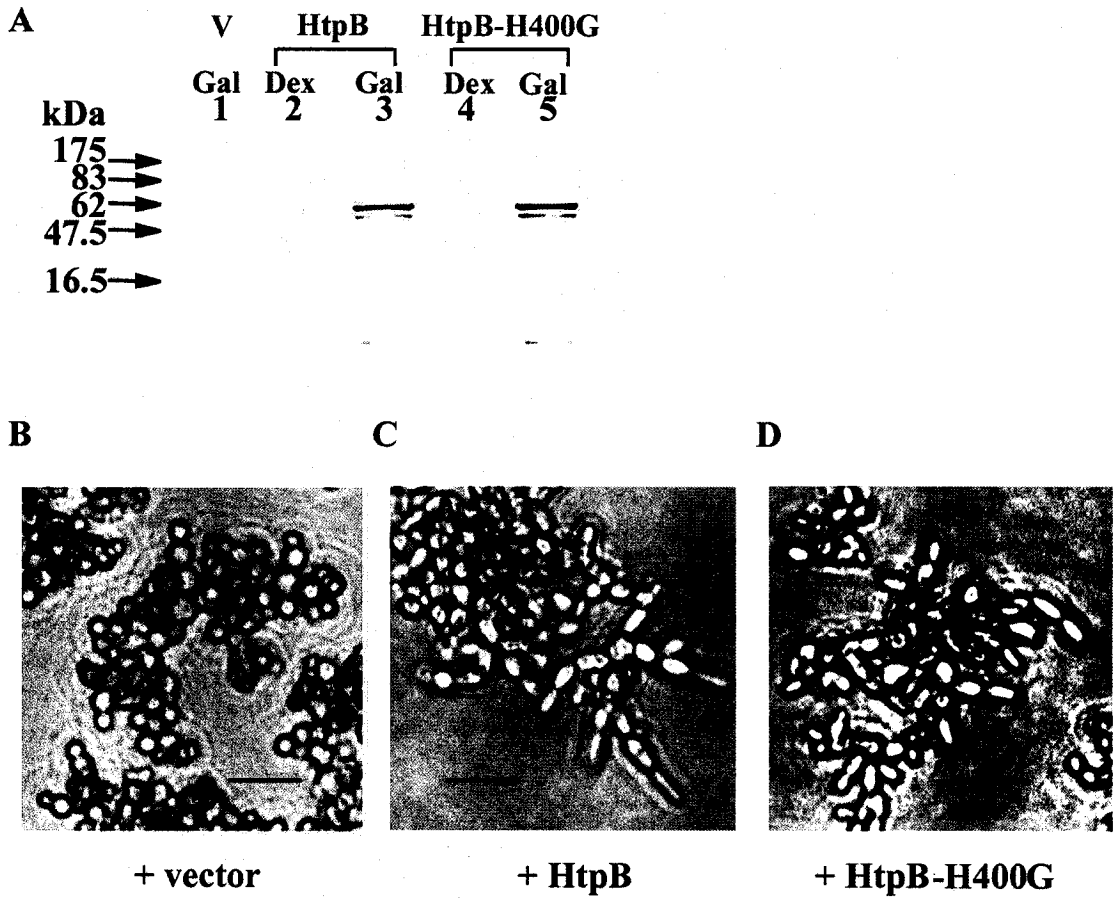


Figure 22. Expression of HtpB-H400G in *S. cerevisiae* mediates pseudohyphal growth. Panel A: Protein extracts from *S. cerevisiae* cells carrying pEMBLyex4 (vector [V], Lane 1), pEMBLyex4::*htpB* (HtpB, Lanes 2 and 3) or pEMBLyex4::*htpB-1197* (HtpBH400G, Lanes 4 and 5) were probed with the *L. pneumophila* MAb in a Western Blot. S. *cerevisiae* cells were grown in medium that induced (Gal) or did not induce (Dex) HtpB or HtpB-H400G expression from a galactose-inducible promoter. *S. cerevisiae* cells carrying the control vector, pEMBLyex4 (+ vector, Panel B), expressing wild-type HtpB from the yeast expression plasmid, pEMBLyex4::*htpB* (+ HtpB, Panel C), and expressing HtpB-H400G from the yeast expression plasmid, pEMBLyex4::*htpB-1197* (+ HtpB-H400G, Panel D) are shown. Cells depicted were grown overnight at 30 °C in a liquid droplet of induding medium for HtpB expression that was placed on the equivalent solid medium. Bars indicate 11.25 μm.

for two independent transformants, greater than 80 % of cells expressed the phenotypes depicted in panels B, C and D. This result indicates that H400 in HtpB is not essential for HtpB-induced pseudohyphal growth.

1.8. HtpB utilizes known regulators of pseudohyphal growth in *S. cerevisiae*.

Pseudohyphal growth can be triggered through the activation of the G-protein Ras2p, which stimulates at least two signaling pathways, the cAMP-dependent Tpk pathway and the Ste-kinase pathway (a highly conserved eukaryotic MAP-kinase pathway), both extensively reviewed in Gancedo, 2001 and Heitman, 2000 and depicted in (Fig. 46). The two Ras2p-activated pathways merge at the *FLO11* promoter via their respective transcription factors, Flo8 and Ste12 (Rupp *et al.*, 1999; Lengeler *et al.*, 2000). Flo11p is a lectin-like surface protein in yeast that facilitates cell-cell adherence during pseudohyphal growth (Lo and Dranginis, 1996).

Since Ras2p activates the signaling hierarchy that regulates pseudohyphal growth in *S. cerevisiae* (Mosch, Roberts, and Fink, 1996), a haploid *S. cerevisiae* strain of the W303-1b background bearing a *ras2Δ* mutation was constructed to test its effect on HtpB-driven pseudohyphal growth. The strain was constructed by gene replacement with a *ras2*-containing fragment from plasmid *pras2::URA3* (see Materials and Methods for details) in strain W303-1b. HtpB expression in the W303-1b strain bearing the *ras2Δ* mutation, from the plasmid pPP389*::htpB*, was confirmed by Western blot using the *L. pneumophila* MAb (Fig. 23B). The mutant strain was transformed with plasmids encoding HtpB or Ras2p or both HtpB and Ras2p, and tested for pseudohyphal growth.

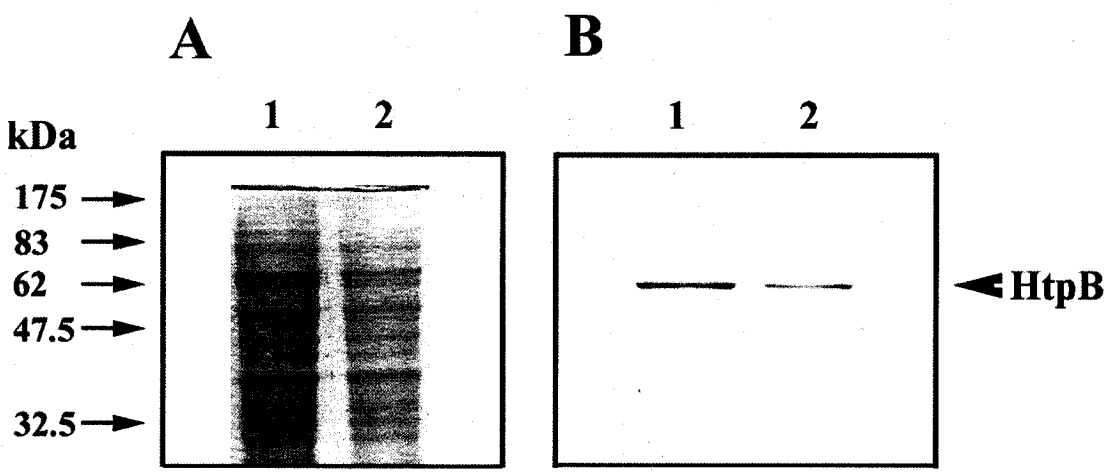


Figure 23. HtpB is expressed in *S. cerevisiae* strain W303-1b bearing a *ras2*Δ mutation. *S. cerevisiae* protein extracts were separated by SDS-PAGE, transferred to a nitrocellulose membrane, stained with Ponceau S (Panel A), destained with 1X PBS and probed with a mixture of the *L. pneumophila* MAb and the pan-ras MAb (Panel B). The W303-1b strain bearing the *ras2*Δ mutation carried pPP389::*htpB* and pRS313 (Lane 1) or pPP389::*htpB* and pRS313::*RAS2* (Lane 2).

The *S. cerevisiae* strain bearing the *ras2Δ* mutation was completely defective in its ability to grow pseudohyphally in response to HtpB expression (Fig. 24A). Genetic complementation of the *ras2Δ* mutation with *RAS2* expressed from the low copy number plasmid pRS313::*RAS2* completely restored the mutant's ability to elongate, bud in a unipolar fashion and invade the agar surface in response to HtpB expression from pPP389::*htpB* (Fig. 24B). These findings indicate that HtpB requires Ras2p to induce cell elongation and invasive growth in *S. cerevisiae*, and confirms that functional Ras2p was expressed form pRS313::*RAS2*.

Since the Ste/MAPK signaling cascade is required for nitrogen starvation-induced pseudohyphal growth, and is activated by Ras2p, a role for the signaling cascade in mediating HtpB-driven pseudohyphal growth was investigated (Gancedo, 2001). W303-1b yeast mutants with single deletions in individual members of the Ste-kinase pathway (*STE20*, *STE7*, *STE11* and *STE12*) were generated by gene replacement with restriction fragment from plasmids pEL45, pSL1311, pSl1077, and pSl1094 respectively (see Materials and Methods for details), and HtpB was expressed in each deletion mutant. Since the deletion of any of the *STE20*, *STE7*, *STE11* and *STE12* genes results in sterility in haploid yeast strains, the inability to mate was used as the criterion for selecting *steΔ* mutants. HtpB expression from pPP389::*htpB* in each of the *steΔ* mutants was confirmed by Western blot using the *L. pneumophila* MAb (Fig. 25). For each of the *steΔ* mutant cells expressing HtpB in two separate experiments including two independent transformants between 20 – 60 % of cells expressing HtpB were elongated, budded in a unipolar fashion (Fig. 26). The remaining cells demonstrated a less intense elongated

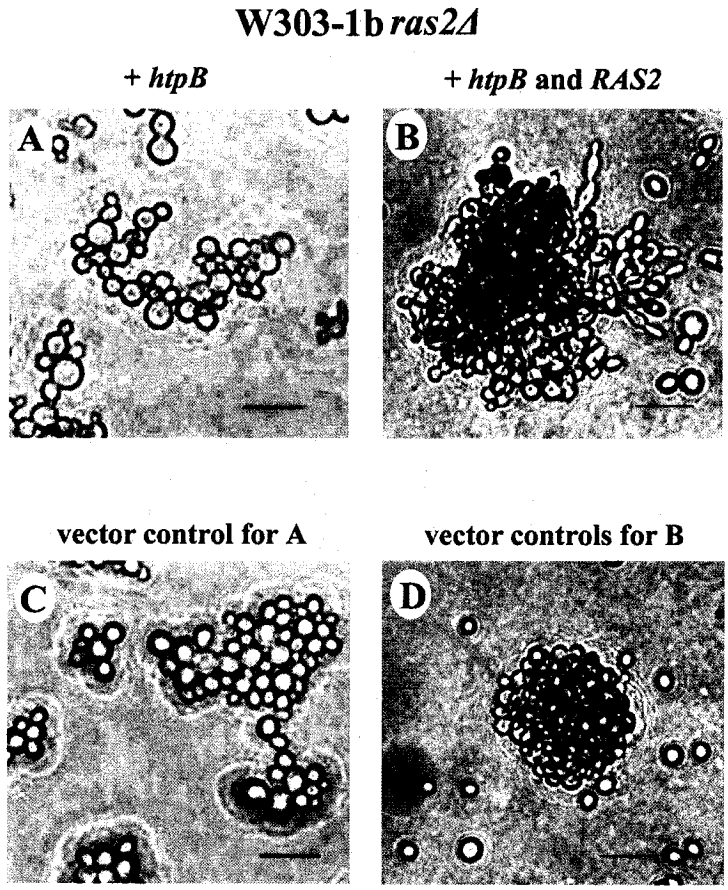


Figure 24. HtpB-induced pseudohyphal growth is dependent on Ras2p in *S. cerevisiae*. *S. cerevisiae* cells of the W303-1b strain background bearing a *ras2Δ* mutation were transformed with plasmids pPP389::*htpB* (Panel A), pPP389::*htpB* and pRS313:*RAS2* (Panel B), pPP389 (Panel C) or pPP389 and pRS313 (Panel D), and grown under conditions that induced HtpB expression from the galactose-inducible construct, pPP389::*htpB*. Yeast cells were photographed either after overnight incubation in liquid droplets of medium resting on the surface of solid medium (Panels A and C) or after growth for 5 days on solid medium followed by the washing away of non-adherent cells (Panels B and D), such that the cells depicted in panel B have invaded the agar surface. Bars indicate 12.5μm.

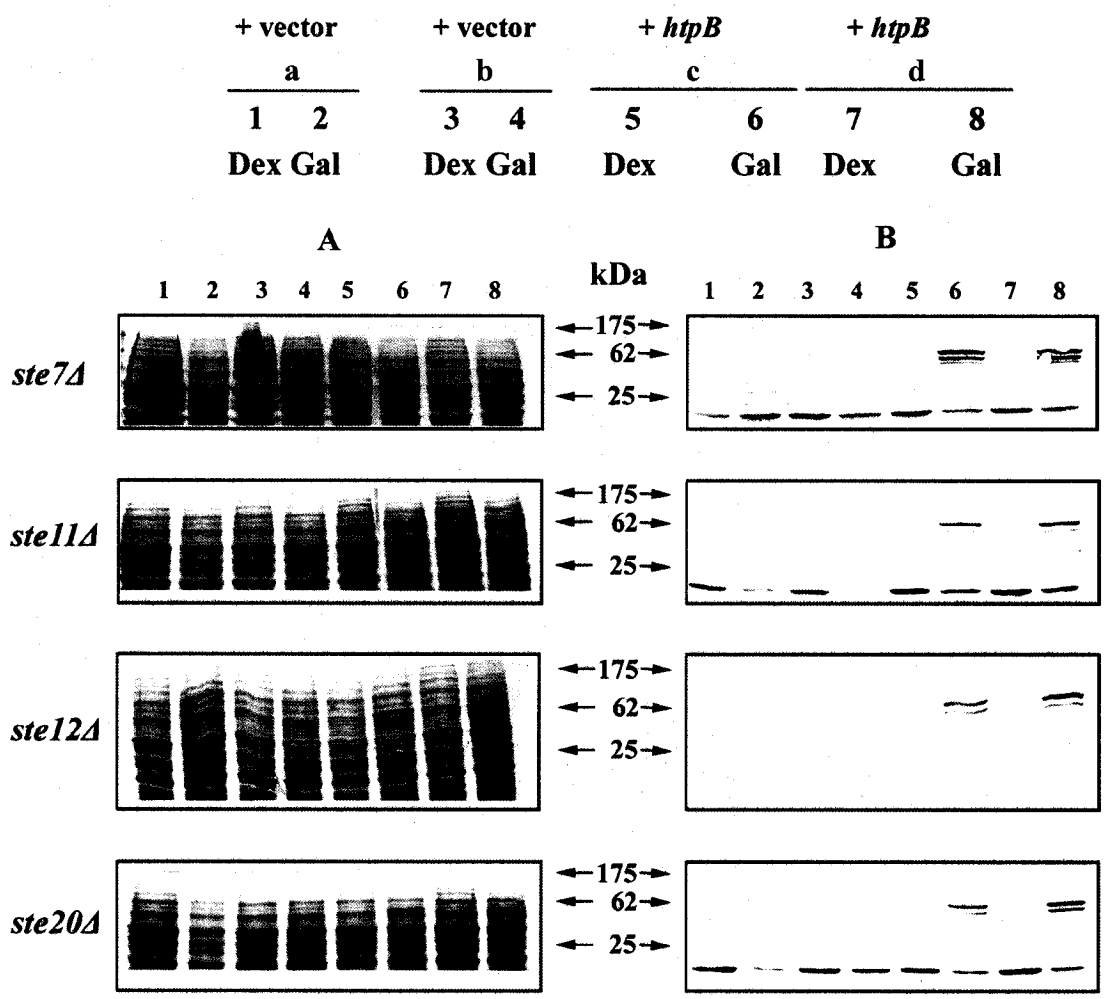


Figure 25. HtpB is expressed in the *S. cerevisiae* mutant deletion (Δ) strains *ste7*Δ, *ste11*Δ, *ste12*Δ and *ste20*Δ. Protein extracts from the *steΔ* mutants that carry the vector pPP389 (+ vector) or the expression plasmid pPP389::*htpB* (+ *htpB*) were separated by SDS-PAGE (Panel A) and were probed with the *L. pneumophila* MAb in Western blots (Panel B). *S. cerevisiae* transformants (a, b, c and d) were grown in medium that induced (Gal) or did not induce (Dex) HtpB expression from a galactose-inducible promoter. The loading key applies to all SDS-polyacrylamide gels and blots shown here. The molecular weights (kDa) of proteins are indicated.

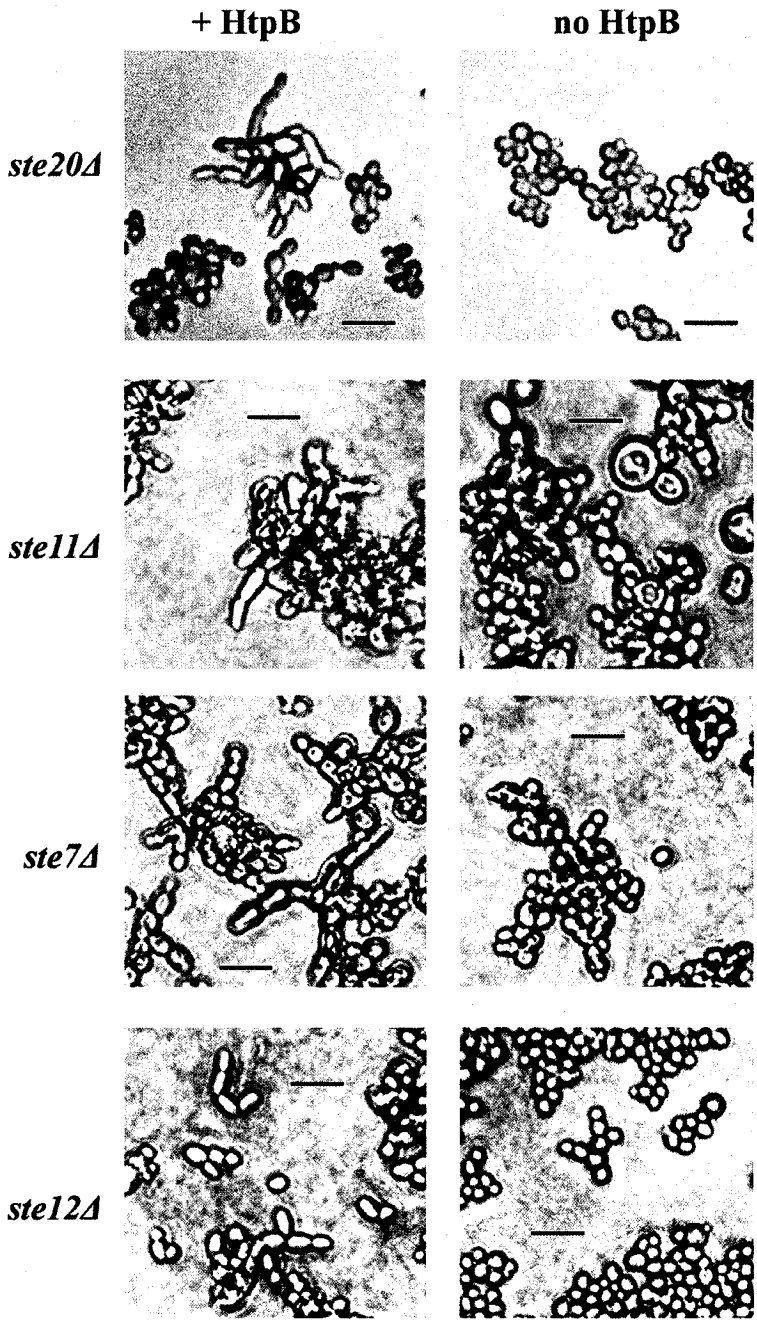


Figure 26. The Ste-kinase pathway is not essential for HtpB-induced elongation and unipolar bud formation in *S. cerevisiae* cells. Haploid yeast strains with the indicated gene deletion and containing either pPP389::*htpB* (+ HtpB) or pPP389 (no HtpB) were incubated overnight on inducing medium. Cells were elongated and budded ina unipolar fashion while still immersed in the liquid inocula that were placed on solid medium. Bars indicate 12.5μm.

phenotype as compared to wild-type W303-1b cells expressing HtpB (data not shown). In addition the *steΔ* mutant strains did not invade solid inducing medium in response to HtpB expression as indicated by the absence of adherent cells on solid-inducing medium after washing with ddH_2O. These observations indicate that the Ste/MAPK pathway is not essential for HtpB-induced yeast cell elongation and the formation of unipolar buds, but contributes to the process. However, the Ste/MAPK pathway is required for the agar invasive phenotype associated with HtpB expression. Since the Ste/MAPK pathway was found not to be essential for all phenotypes associated with HtpB-induced pseuodohyphal development, the role of the cAMP/PKA signaling cascade, which is also a Ras2p activated pathway that plays a role in nitrogen starvation-induced pseudohyphal growth, for HtpB-induced pseudohyphal growth was investigated.

Since Flo8p is the transcription factor that is activated by the cAMP/PKA pathway, it was of interest to create a deletion of the *FLO8* gene in W303-1b, to test for its role in HtpB-induced pseudohyphal development in *S. cerevisiae*. Despite numerous attempts to construct a *flo8*Δ W303-1b strain using a restriction fragment from plasmid pHL129 for gene replacement, no *flo8*Δ mutants were obtained (see Discussion for possible explanations). A second method of generating a construct for gene replacement was attempted. The *FLO8* gene was PCR amplified from *S. cerevisiae* strains W303-1b and MLD158 using primers *FLO8*up-S and *FLO8*dwn-AS, which consist of nucleotide sequences identical to DNA flanking *FLO8* gene sequences from *S. cerevisiae* strain S288C (Systematic name: YER109C, SGD™), for which the entire genome sequence is available in the Saccharomyces Genome Database™. Amplicons obtained with primers

*FLO8*up-S and *FLO8*dwn-AS from W303-1b whole cells was less intense in comparison to amplicons obtained by PCR amplification from MLD58 whole cells, which served as the positive control in three separate PCR runs as depicted in Figure. 27, which could indicate a mismatch between the *FLO8* primer sequences and template DNA from the haploid strain W303-1b or could result since MLD158 is a diploid strain and has a genome content of 2N as opposed to 1N as is the case for W303-1b. It is also possible that non-equivalent numbers of yeast cells for W303-1b and MLD158 were transferred to the PCR reactions, so that more MLD158 cells were available to supply template for PCR amplification. Several attempts were made to clone either the putative *FLO8*-containing fragment amplified from either W303-1b or MLD158 into pBluescript KS using DH5α as a host, but these attempts were unsuccessful, the reason for which is unclear. Since a *FLO8* deletion construct could not be generated, an alternative method was employed to obtain an *S. cerevisiae* strain bearing a *flo8Δ* deletion. It is reported that in particular in commonly used laboratory strains such as S288C, genes such as *FLO8* involved in the process of flocculation, that is cell-cell adherence related to pseudohyphal growth are defective (Lui, Styles and Fink, 1996). The reason for this is that the ability to isolate individual colonies is positive attribute for scientific experimentation such that strains, such as S288C, which are less "sticky" would prove better specimens for scientific investigation. In the case of W303-1b another standard laboratory strain, while it is reported that this strain does not undergo pseudohyphal growth in response to nitrogen starvation the genetic basis for this phenotype is still unclear (Lui, Styles and Fink, 1996).

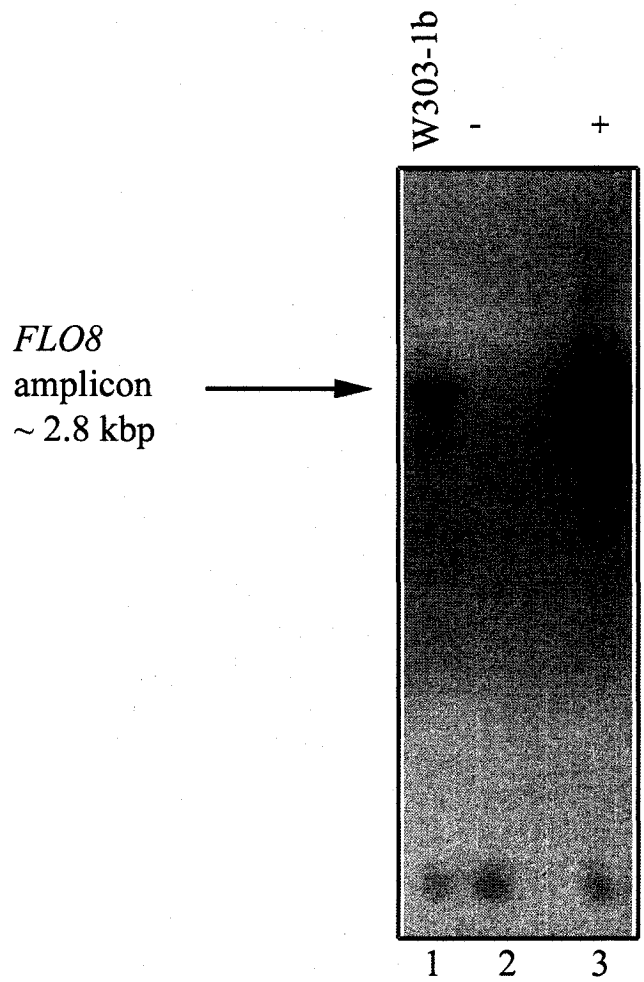


Figure 27. PCR amplification of *FLO8* from *S. cerevisiae* strains W303-1b and MLD158. The *FLO8* gene was amplified by colony PCR using *S. cerevisiae* whole cells from strains W303-1b (Lane 1) and MLD158 (+ control, Lane 3) as template for the reaction and primers *FLO8*up-S and *FLO8*dwn-AS. Lane 2: No template control (-).

As an alternative strategy to investigate a role for Flo8p in HtpB-induced pseudohyphal growth, a *flo8*Δ mutant strain of the S288C strain background called BY4741 from a gene-deletion strain collection (Brachmann *et al.*, 1998) was utilized for HtpB-expression studies. HtpB was expressed in this strain from plasmid pLM86. The pLM86 transformant was also transformed with plasmid pHL135 (pRS202::*FLO8*) to complement the *flo8*Δ mutant allele or with the vector pRS426 (a pRS202 equivalent to serve as a control for pHL135) (Fig. 28). Greater than 90 % of the BY4714 cells expressing HtpB were elongated and budded in a unipolar direction as determined for two independent transformants, for which at least 25 microscope fields were viewed at 400X magnification as depicted in (Fig.29), but were not invasive on solid inducing medium (data not shown). These data indicate that *FLO8* is necessary for agar invasion, but not for yeast cell elongation and for the formation of unipolar buds. BY4714 cells complemented with a wild-type *FLO8* gene on plasmid pHL135 formed clumps when grown in selective medium (data not shown), as is expected since *FLO8* codes for a transcription factor that promotes the transcription of numerous genes encoding flocculins that promote cell-cell adherence (Kobayashi, Yoshimoto and Sone, 1999). In addition these cells were hyper-adherent to the surface of solid medium, that is, cells remained tightly bound to the agar surface after washing with ddH_20 and rubbing of the agar surface (Fig. 29). BY4741 complemented with *FLO8*, however, did not elongate or bud in a unipolar fashion (Fig. 29). In contrast BY4714 cells complemented with *FLO8* and expressing HtpB demonstrated a hyper-adherent phenotype on solid inducing medium, and in addition were elongated, and budded in a unipolar direction (Fig.29).

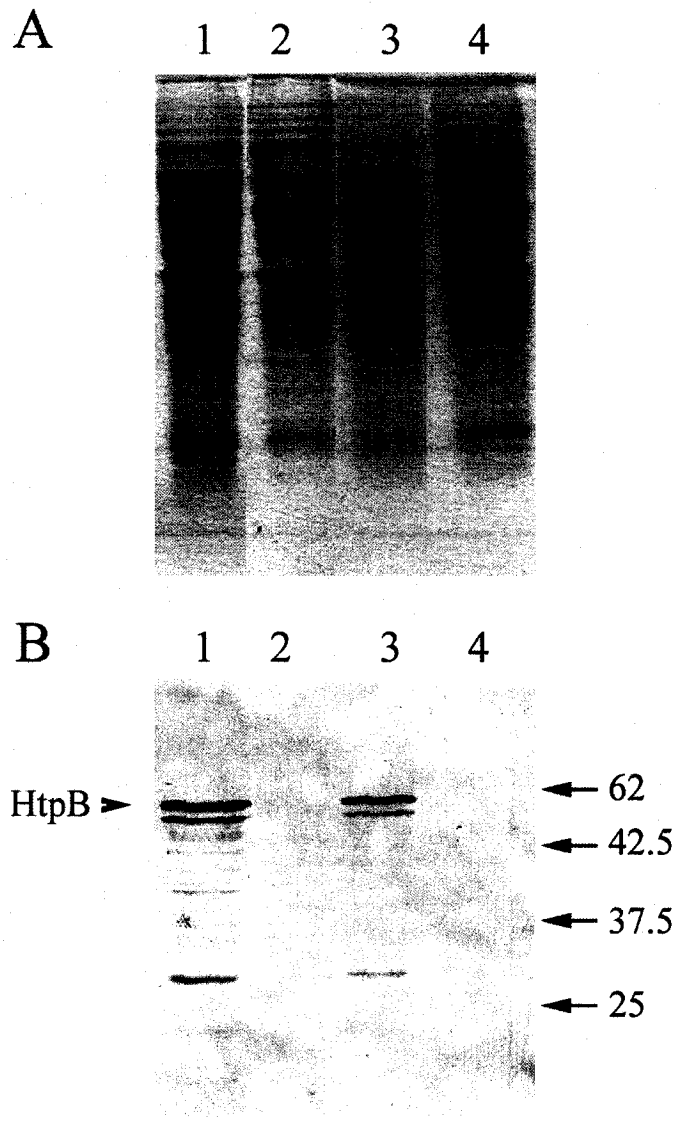


Figure 28. HtpB is expressed in the *S. cerevisiae* haploid strain BY4741 that bears a *flo8Δ* mutation. BY4741 protein extracts were separated by SDS-PAGE (Panel A), transferred to nitrocellulose and probed with the *L. pneumophila* MAb in a Western blot (Panel B). BY4741 cells harbor plasmids pHL135 (pRS202::*FLO8*) and pLM86 from which *FLO8* and *htpB* are expressed, respectively (Lane 1), vector pRS426 (a pRS202 equivalent) (Lane 2), plasmid pLM86 and vector pRS426 (Lane 3), or plasmid pHL135 alone (Lane 4). Yeast cells were grown in galactose medium that induced HtpB expression from a galactose-inducible promoter.

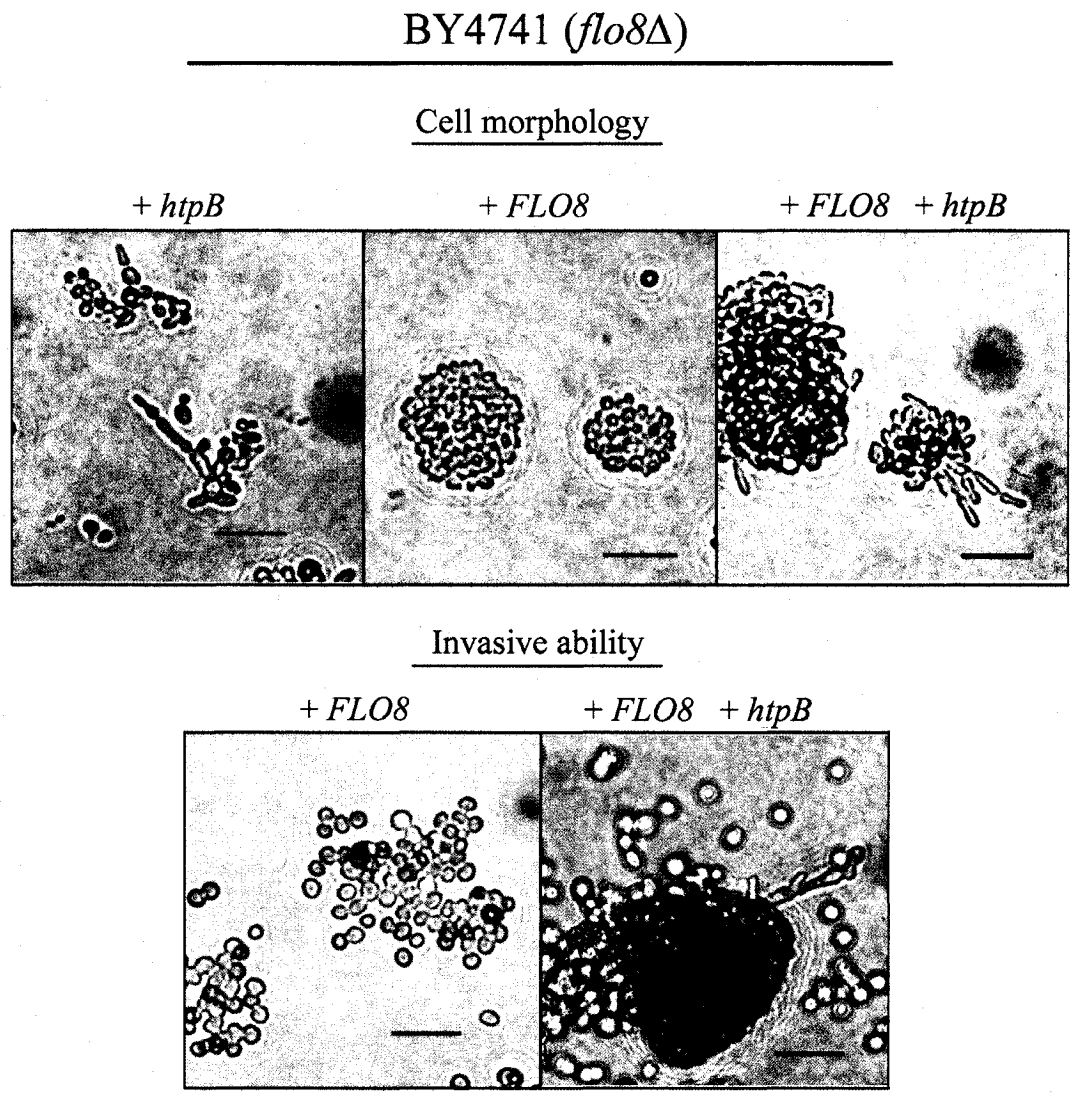


Figure 29. HtpB-induces the pseudohyphae formation in the BY4741 yeast strain that bears a *flo8Δ* mutation. HtpB was expressed from plasmid pLM86 (+ *htpB*) in the *S. cerevisiae* strain background BY4741 that lacks *FLO8*. Plasmid pHL135 (+ *FLO8*) was used for *flo8*Δ complementation. Yeast cells were grown under conditions that induced HtpB expression from a galactose-inducible promoter, and were assessed for cell morphology (Panels A, B and C) and invasive ability (Panels D and E). Cells in the bottom panels represent those that remain attached to solid inducing medium after washing with ddH_2O. A panel indicating invasive ability for the BY4741 strain expressing HtpB from plasmid pLM86 is not demonstrated, since these cells were neither invasive not adherent to solid medium. Bars indicate 10μm.

These data indicate that *FLO8* is required for HtpB-induced agar invasion, but is not essential for yeast cell elongation and the formation of unipolar buds. It is not surprising that *FLO8* and *STE12* were found to be essential for HtpB-induced agar invasion, since these transcription factors are essential for the expression of Flo11p, the surface lectin that is required for persistent cell-cell adherence, an essential criteria for agar invasion. A *FLO8-STE12* double deletion was recently generated by crossing the W303-1b *ste12*Δ strain made in this study with BY4741, and selecting for their haploid progeny in which the two mutant alleles, *ste12Δ* and *flo8Δ* were segregated. When HtpB was expressed in the latter haploid strain, yeast cells did not elongate or bud in a unipolar fashion (pers. commun. Dr. L. Murray). These data indicate that either a functional *FLO8* gene or *STE12* gene is essential for HtpB-induced cell elongation and the formation of unipolar buds, and that the Ste/MAPK and cAMP/PKA signaling pathways pseudohyphal growth.

1.9. Expression of the dominant negative *CDC42*^{Ala118} allele and *htpB* in *S. cerevisiae*.

Cdc42p acts downstream of the small GTP-binding protein, Ras2p, and upstream of the Ste/MAPK pathway to trigger pseudohyphal growth in *S. cerevisiae* (Mosch, Roberts, and Fink, 1996; Peter *et al.*, 1996). Since it was demonstrated that Ras2p is required for both HtpB-induced pseudohyphae formation and agar invasion, while the Ste/MAPK pathway was essential only for agar invasion it was of particular interest to determine whether Cdc42p was required for pseudohyphae formation, that is the morphological changes that are associated with HtpB expression. Cdc42p also coordinates other cellular

functions including the delivery of secretory vesicles to sites of bud growth (Adamo *et al.*, 2001), the directing cell polarity (Kozminski *et al.*, 2003), regulating G2/M cell-cycle transition (Richman, Sawyer, and Johnson, 1999) and controlling bud-site selection (Nelson, 2003) in *S. cerevisiae* . For example yeast cells expressing, one particular mutant protein that is defective in a Cdc42p effector domain required for Cla4p association, called Cdc42(V44A)p, are elongated, indicative of Cdc42p's involvement in the apical to isotropic switch for bud growth (Richman, Sawyer, and Johnson, 1999). Since Cdc42p is a protein that is essential for *S. cerevisiae* viability, a deletion mutant could not be generated; instead, the dominant negative allele, $CDC42^{Ala118}$, which confers a mutation in the Cdc42p GTP-binding domain that causes yeast cells to arrest as large unbudded cells (Ziman *et al.*, 1991) was chosen for this work. It was envisioned that if wild-type Cdc42p plays a key role in HtpB-induced pseudohyphae formation in yeast cells, then the $CDC42^{Ala118}$ allele would block HtpB-induced yeast cell elongation and the formation of unipolar buds, since it has dominant negative effect on wild-type Cdc42p function (Ziman *et al.*, 1991). HtpB alone or HtpB and Cdc42(A118)p were expressed in *S. cerevisiae* W303-1b from galactose-inducible promoters in pLM87 for HtpB expression and P_{GAL}::$CDC42^{Ala118}$, named B3081, for CDC42(A118)p expression. Cells were grown in Dex medium (SD medium + 2 % Dextrose), Raff medium (SD medium + 3 % raffinose and 0.1 % Galactose) or Gal medium (SD medium + 2 % galactose). Raff medium has been utilized to reduce the expression of $CDC42^{Ala118}$ from P_{GAL}(Griggs and Johnston, 1991), since $CDC42^{Ala118}$ inhibits growth in *S. cerevisiae* when expressed at high levels (Mosch, Roberts, and Fink, 1996). In this study HtpB

protein levels in *S. cerevisiae* cells grown in Raff medium was variable (Fig.30, lanes 1, 3, 5, 7, 9 and 11), while HtpB protein levels in *S. cerevisiae* cells grown in Gal medium was consistent (Fig.30, lanes 2, 4, 6, 8, 10 and 12) as detected by Western blot with the *L. pneumophila* MAb. In one case HtpB expression from pLM87 was not detected in *S. cerevisiae* cells grown in Raff medium even by Western blot with the *L. pneumophila* MAb (Fig. 30, lane 7). This finding indicates that the use of raffinose to reduce gene expression from a galactose inducible promoter such as in pLM87 is unreliable, and that Gal media yielded more consistent HtpB protein levels; therefore, Gal media were used to promote the expression of HtpB and Cdc42(A118)p expression from pLM86 and B3081 in *S. cerevisiae* W303-1b. *S. cerevisiae* cells expressing *CDC42*^{Ala118} from plasmid B3081 in Gal medium became large and round, and did not bear daughter buds (Fig.31A), while cells expressing HtpB from plasmid pLM87 formed pseudohyphae (Fig.31B). The morphologies of the yeast cells expressing Cdc42(A118)p and HtpB from plasmids B3081 and pLM87 consisted of a mixture of large round cells, large elongated cells, and smaller elongated cells (Fig. 31C). Of interest was that some large cells expressing both Cdc42(A118)p and HtpB could still form pseudohyphae when grown in Gal medium (Fig. 31C, arrowheads), which indicates that the *CDC42*^{A118} allele did not completely block HtpB-induced pseudohyphae formation.

To further analyze the effects of Cdc42(A118)p expression on HtpB-induced pseudohyphal growth, it was envisioned that a transcriptional reporter called FG(*TyA*)::*lacZ* that is used to measure pseudohyphal growth activation could be used to measure the effects of *CDC42*^{Ala118} expression on putative HtpB-induced activation of

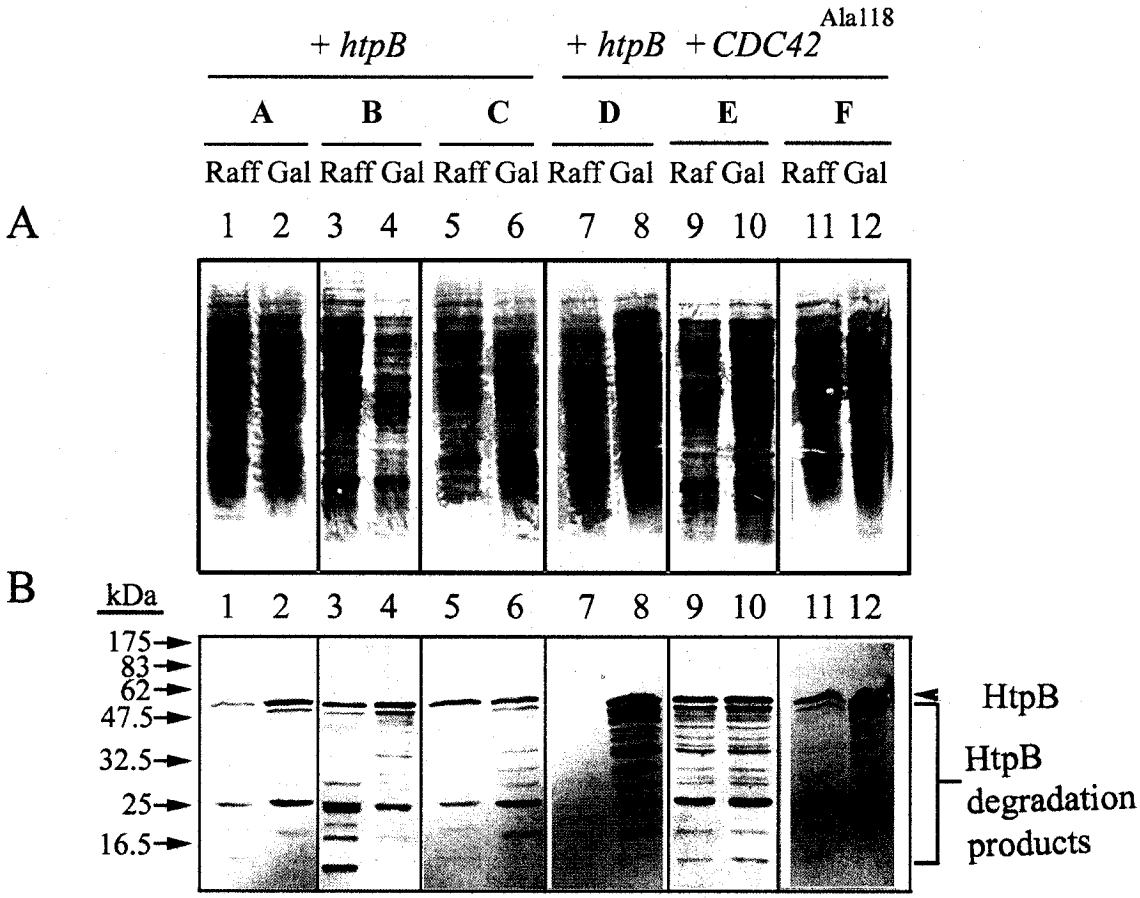


Figure 30. HtpB protein levels in the *S. cerevisiae* strain W303-1B bearing the galactose-inducible plasmid pLM87 are consistent when yeast cells are grown in galactose medium, but variable when yeast cells are grown in raffinose medium. *S. cerevisiae* W303-1b protein extracts were separated by SDS-PAGE, transferred to nitrocellulose, stained with Ponceau S (Panel A), destained with 1X PBS and probed with the *L. pneumophila* MAb in Western blots (Panel B). Yeast cells tranformants carrying plasmid pLM87 (A, B and C) to express *htpB* alone (Lanes 1-6) or plasmids pLM87 and B3081(D, E and F) to express *htpB* and $CDC42^{Ala118}$, respectively (Lanes 8-12) were grown in either galactose medium (Gal, SD medium containing 2 % galactose) or raffinose medium (Raff, SD medium containing 3 % raffinose and 0.1 % galactose). Yeast cell transformant D bearing plasmids pLM87 and B3081did not express *htpB* from plasmid pLM87 when grown in raffinose medium (Lane 7).

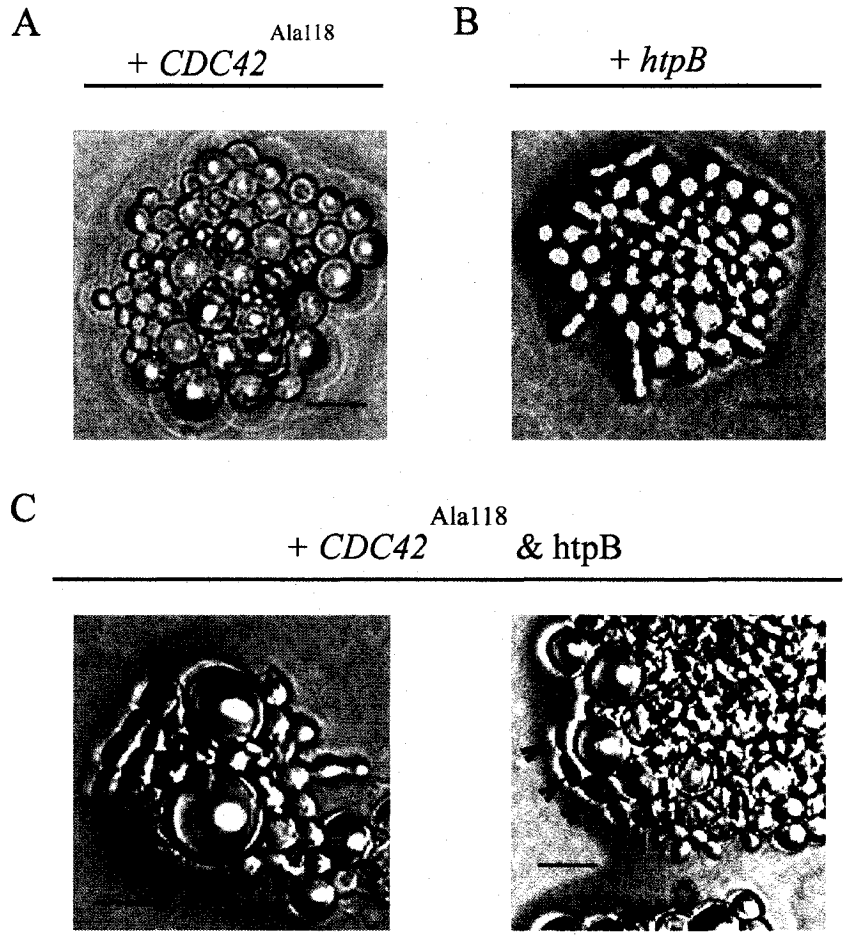


Figure 31. *S. cerevisiae* W303-1b expressing HtpB and Cdc42(A118)p form pseudohyphae. *S. cerevisiae* cells were grown for 48 hours at 30 °C in inducing-medium (SD medium containing 2% galactose) so that *CDC42*^{Ala118} (Panel A), *htpB* (Panel B) or *CDC42*^{Ala118} and *htpB* (Panel C) expression were induced from galactose-inducible promoters in plasmids B3081 (P_{GAL}::*CDC42*^{Ala118}) and pLM87 (pEMBLyex4::*HIS3*::*htpB*). Yeast cells expressing Cdc42(A118)p alone arrested as large unbudded cells (Panel A); yeast cells expressing HtpB alone formed pseudohyphae (Panel B), and the yeast cell population expressing HtpB and CDC42(A118)p was comprised of a mixture of large cells and pseudohyphal cells (Panels C and D). In some cases large yeast cells that appeared as arrested cells still formed pseudohyphae (arrowheads, Panel D). Bars indicate 10 μm.

of this repoter construct. FG(*TyA*)::*lacZ* expression is regulated from a Ty1-derived promoter sequence that consists of the phermone response element (PRE, 5'-A/TGAACA - 3') that facilitates Ste12p binding (Dolan, Kirkman, and Fields, 1989), and the TEA/ATTS consensus sequence (TCS, 5' - CATTCC - 3') that is necessary for Tec1p binding (Madhani and Fink, 1997; Laloux, Jacobs, and Dubois, 1994). Ste12p and Tec1p are transcription factors for pseudohyphal development (Gancedo, 2001), so that expression of the FG(*TyA*)::*lacZ* construct is indicative of pseudohyphal growth activation. Liquid β-galactosidase enzyme assays were used to monitor *TyA* mediated expression of the *lacZ* fusion in three independent transformants each for *S. cerevisiae* cells that expressed *htpB* only (A, B and C) or *htpB* and $CDC42^{Ala118}$ (E, F and G) as depicted in Figure 33 and Tables 6 and 7. This experiment was performed twice and the experiments are referred to as replicate 1 and replicate 2 in Figure 33 and in Tables 6 and 7. For these experiments, the transformants expressing HtpB alone (A, B and C) or HtpB and Cdc42(A118)p (D, E and F) were cultured in Dex, Raff, or Gal medium (formulated from a base mixture consisting of SD medium supplemented with the appropriate amino acids to maintain plasmid selection) simultaneously, and cells were collected from each culture once it had arrived at a sufficient density to allow for the harvesting of between 1 $x10^6$ – 1 x 10^7 cells for measuring β-galactosidase activity and 1 x 10^8 cells for the testing HtpB protein levels by Western blot. Samples of these cells from replicate 2 were harvested for testing HtpB protein levels (Fig. 29). Activation of the FG(*TyA*)::*lacZ* reporter construct from plasmid pFG(TyA)::lacZ-HIS3 was

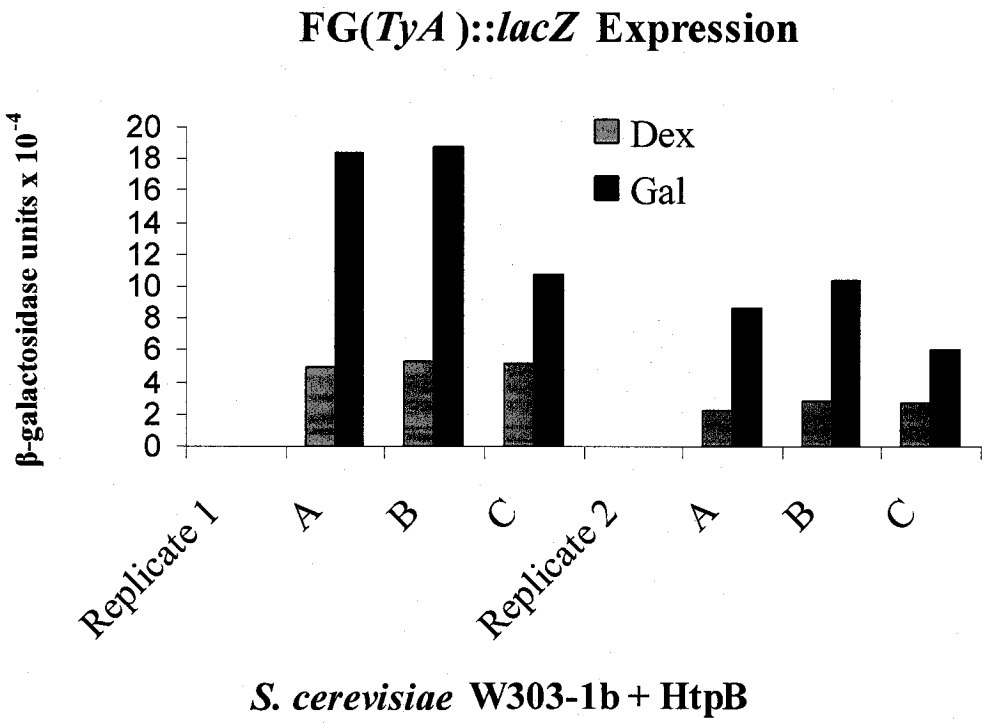


Figure 32. HtpB activates expression from the reporter construct FG*(TyA)::lacZ* for pseudohyphal growth. *S. cerevisiae* cells were grown in non-inducing medium (Dex), or inducing-medium (Gal) for *htpB* expression from the galactose-inducible plasmid pLM87. The reporter construct for pseudohyphal growth, FG*(TyA)::lacZ*, was expressed from plasmid, pFG(TyA)::lacZ-HIS3. Three transformants (A, B and C) were tested for FG(*TyA)::lacZ* activation in two separate experiments (Replicates 1 and 2). Reporter gene activity is expressed as β-galactosidase units, which were calculated as described in the Appendix. By the two-tailed t-test as calculated using GraphPad Prism software it was determined with 99% confidence ($p \leq 0.01$) that in each of the two experiments (Replicates 1 and 2) the mean β-galactosidase units obtained for transformants A, B, and C, when grown in inducing medium were significantly different from the means obtained for β-galactosidase units for these transforrmants when grown in non-inducing medium. See Table 6 for the means and standard deviations.

Replicate Number	Growth Condition (protein expressed or not)	Mean β-galactosidase units x 10^{-4} +/- standard deviation for transformants (A, B and C, Fig. 32)	Are the means for the different growth conditions significantly different at p=.01 (99% confidence)
1	Dextrose (no HtpB)	5.1 +/- 0.1	Yes
	Galactose (+ HtpB)	16.0 +/- 4.6	
2	Dextrose (no HtpB)	2.6 +/- 0.2	Yes
	Galactose (+ HtpB)	8.4 +/- 1.25	

Table 6: HtpB-induced activation of the pseudohyphal growth indicator construct, FG(*TyA*)::*lacZ* is statistically significant. W303-1b cells either expressed or did not express HtpB from plasmid pEMBLyex4::*htpB* when grown in medium containing galactose or dextrose, respectively. FG(*TyA*)::*lacZ* activation was measured by way of a liquid β-galactosidase assay (see Materials and Methods). Significance for the difference in means for the two growth conditions were calculated by the two-tailed t-test using GraphPad Prism software. See Figure 32 for raw data.

Replicate Number	Protein(s) Expressed (Transformants)	Mean β-galactosidase units x 10^{-4} +/- standard deviation	Are the means for the different growth conditions significantly different to p</=.05 (95% confidence level)
1	HtpB (A, B and C) HtpB and Cdc42^{Ala118}p (D, E and F))	16.0 +/- 4.6* 11.5 +/- 3.8	No
2	HtpB (A, B and C) HtpB and Cdc42^{Ala118}p (D, E and F)	8.4 +/- 2.2* 10.2 +/- 4.0	No

*See Figure 33 for raw data graphically presented.

Table 7. Expression of the *CDC42*^{Ala118} allele does not significantly affect HtpB-induced FG(*TyA*)*::lacZ* activation. *S. cerevisiae* cells were grown in non-inducing or inducing-medium for HtpB and Cdc42(A118)p expression from the galactose-inducible promoters in plasmids pLM87 and B3081, respectively. Data for inducing medium are presented. The reporter construct for pseudohyphal growth, FG*(TyA)::lacZ*, was expressed from plasmid, pFG(TyA)::lacZ-HIS3. Six transformants, three expressing HtpB alone (A, B, C) and three expressing HtpB and Cdc42(A118)p (D, E, F) were tested for FG*(TyA)::lacZ* activity in two separate experiments (Replicate 1 and Replicate 2). By the two-tailed t-test using GraphPad Prism software it was determined that there was no significant difference between the mean values for β-galactosidase activity for cells expressing HtpB alone or HtpB and Cdc42(A118)p, indicating that Cdc42A118p expression had no significant effect on HtpB-induced FG(*TyA*)::lacZ expression.

significantly different, for cells expressing *htpB* from pLM86 in Gal medium (inducing medium) as compared to cells grown in Dex medium (non-inducing medium), as is apparent by comparing the mean value for β-galactosidase units obtained for transformants A, B, and C grown in Dex as compared to Gal medium in both replicates (Fig. 31 and Table 8). When *CDC42*^{Ala118} was expressed in three yeast cell transformants that also expressed *htpB* (C, D and E, Table 9), there was no significant difference in the mean values for β-galactosidase units obtained for these three transformant as compared to the mean β-galactosidase units obtained for the three transformants (A, B and C) that expressed htpB alone (Table 9). These data indicate that *CDC42*^{Ala118} expression does not have an effect on HtpB-induced activation of the FG(*TyA)::lacZ* reporter, and perhaps pseudohyphal growth. This finding is supported by the observation that large yeast cells initially arrested from cell cycle due to Cdc42(A118)p expression responded to HtpB expression, and form elongated cells that were budded in a unipolar fashion. Based on these data; however, it is still not possible to determine whether the function of wild-type Cdc42p is essential for HtpB-induced pseudohyphae formation. If the Cdc42(A118)p mutant protein did block HtpB activity then it would be clear that Cdc42p is necessary for HtpB-induced pseudohyphal growth activation, since Cdc42(A118)p blocks wild-type Cdc42p function. However, since this was not the case, and the Cdc42(A118)p mutant protein did not completely block HtpB-induced pseudohyphal growth, then two possibilities remain (i) HtpB may interact with wild-type Cdc42p present in the W303-1b transformants that were co-expressing HtpB and Cdc42(A118)p, to produce an effect that is dominant to Cdc42(A118)p-mediated cell cycle arrest to induce elongated bud

formation or (ii) HtpB-induces the formation of elongated cells that bud in a unipolar pattern independent of Cdc42p. Hence the role of Cdc42p for in HtpB-induced pseudohyphae formation remains unclear.

Although a role for Cdc42p in HtpB-induced pseudohyphae formation could not be determined based on these experiments, other known regulators of pseudohyphal growth including Ras2p and members of the Ste/MAPK and cAMP/TPK pathways were shown to be involved in mediating the phenotypes HtpB-induced pseudohyphal growth.. Perhaps it would be useful to screen a yeast two-hybrid library for yeast proteins that directly interact with HtpB as this may helps to identify core regulators of pseudohyphal growth in *S. cerevisiae*. This is of particular interest since HtpB could trigger this developmental change in a yeast strain W303-1b that does not form pseudohyphae in response to nitrogen deprivation.

Part 2. Hsp60 Homologues and HeLa cells.

2.1. Development of a methodology for Cpn60 protein purification.

When virulent *L. pneumophila* cells infect host cells, they delay the fusion of the endosomes, within which they replicate, with host lysosomes for at least eight hours post infection (Sturgill-Koszycki and Swanson, 2000); in contrast the replicative endosomes containing *L. pneumophila* mutant strains can fuse with host lysosomes as quickly as five minutes post infection (Roy, Berger, and Isberg, 1998). While BSA-coated beads are housed within loose endosomes inside HeLa cells (indicative of extensive fusion with

host endosomes), HtpB-coated beads reside within tight endosomes (indicative of limited fusion with host endosomes) (Garduno, Garduno, and Hoffman, 1998). These observations are of interest, since they indicate that the HtpB protein may possess unique properties that could play a role in preventing fusion of host endosome with endosomes that contain the HtpB protein. Since the evasion of intracellular digestion, a process that is mediated by endosome fusion events, is essential of *L. pneumophila* survival inside eukaryotic cells (Swanson and Hammer, 2000), and HtpB is expressed on the surface of *L. pneumophila*, it is proposed that perhaps HtpB contributes to this process. Since chaperonins are conserved with respect to their capacity to assist proteins in assuming a functional conformation, but also possess unique functions it was of interest to determine whether other bacterial chaperonins including the including the *E. coli* GroEL protein and the *B. pertussis* Cpn60 protein could influence the interaction of latex beads with the eukaryotic endocytic pathway. To undertake this work, purified GroEL and Cpn60 proteins were required, and while GroEL was available for purchase from a commercial source (Stressgen, Victoria BC), a protocol had to be developed for the purification of Cpn60. In this study a methodology was developed for the purification of recombinant Cpn60 and Cpn10 as expressed from plasmid pBs::*cpn10cpn60* in *E. coli* DH5α by. Anion exchange and gel filtration (size exclusion) chromatography were employed.

Since a good starting pH for binding to the insoluble Diethylaminoethyl (DEAE) matrix used for protein binding in anion exchange chromatography is 1 pH unit above the isoelectric point (pI) of the protein to be purified (Pharmacia Biotech, 1982), a starting

buffer with a pH of 6.0 and containing 35 mM KCl was selected for binding Cpn60 to the anion exchange matrix. A theoretical pI for Cpn60 (I40331, NCBI Protein Database) of 5.22 was calculated using the Protein Calculator (The Scripps Research Institute). The test-tube method for determining the optimal pH for Cpn60 binding to DEAE, as outlined in the Materials and Methods, was attempted to determine an actual value for Cpn60 binding; however, the efficiency of Cpn60 binding to the DEAE matrix was not obviously different within a range of pH 7.5 to pH 5.8; although there was slightly less unbound protein found at pH 6.0 and pH 5.8 as compared to the higher pH values (Fig. 33). Perhaps a more clear distinction in protein binding at the different pH values was masked due to saturation of the DEAE matrix with Cpn60, and reducing protein load may have improved results. An increase in KCl concentration to 135 mM was sufficient for high level elution of Cpn60 from the DEAE matrix at pH 6.0 (Fig. 33). When the crude DH5α protein extract containing Cpn10 and Cpn60 was added to the anion exchange column, equilibrated to pH 6.0, Cpn60 did not bind to the DEAE matrix, and was released into the run-off fraction (Fig. 34). The fact that pH is altered by changes in temperature was not taken into consideration; therefore, perhaps the test-tube assay should have been performed at the temperature of protein purification, 4 °C, instead of at room temperature. A theoretical calculation of Cpn60 net charge across a pH range of pH 4.0 to pH 10 was calculated using the Protein Calculator, and pH 7.5 was selected as a good pH for binding, since it resulted in a large theoretical net negative charge for Cpn60 (-11.2 as compared to -8.4 at pH 6.0). In addition since Cpn60 shares 72.2 % amino acid identity with HtpB, and pH 7.6 was recommended as a good buffer pH for HtpB binding

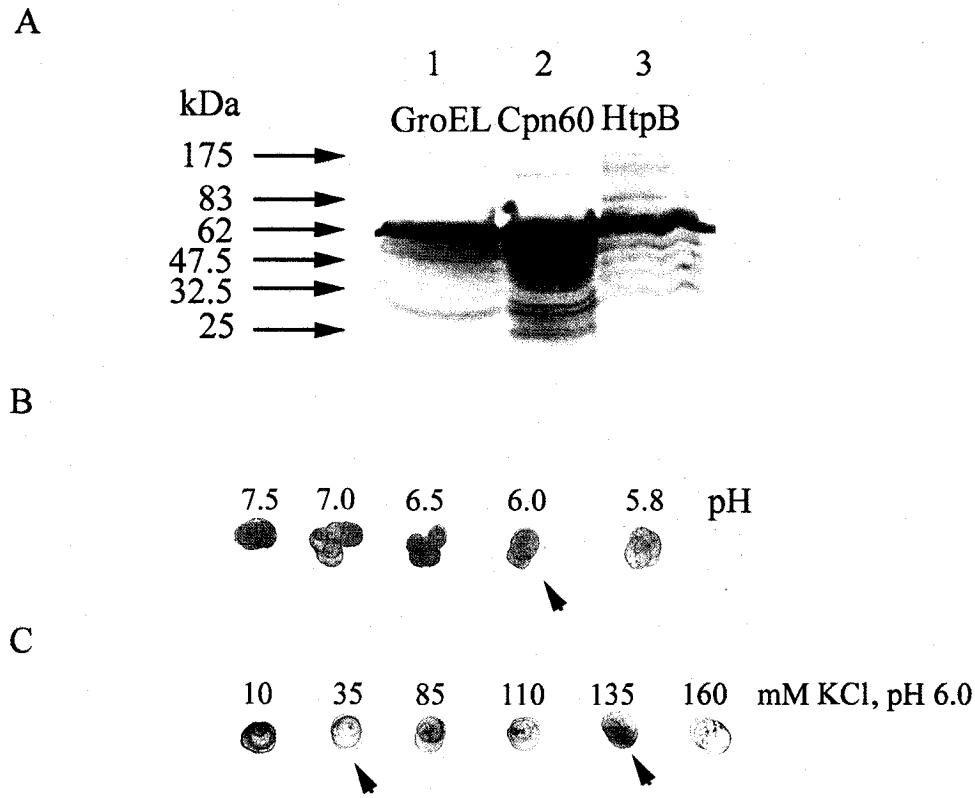


Figure 33. Determination of buffer conditions for the purification of Cpn60 by anion exchange chromatography. Protein extracts from heat-shocked *E. coli* DH5α carrying no plasmid (Panel A, Lane 1) or pBs:*cpn10cpn60* (Panel A, Lane 2) and a protein extract from *L. pneumophila* (Panel A, Lane 3) were separated by SDS-PAGE and subjected to Western blot with the *E. coli* PAb. A sample of the protein extracts were also added to 0.1 ml DEAE test-tube columns that were previously equilibrated in column buffer ranging from pH 7.5 - 5.8. After mixing and incubation at room temperature for 10 minutes, 10 µl of each sample was spotted onto nitrocellulose and subjected to Western blotting with the *E. coli* PAb (Panel B). The latter procedure was repeated with column buffer pH 6.0, but with [KCl] ranging from 10 mM - 160 mM (Panel C). The starting buffer conditions of pH 6.0 and 35 mM [KCl] was selected for binding to DEAE matrix, and 135 mM KCl was considered sufficient for protein elution from the columns (Panels B and C, arrows).

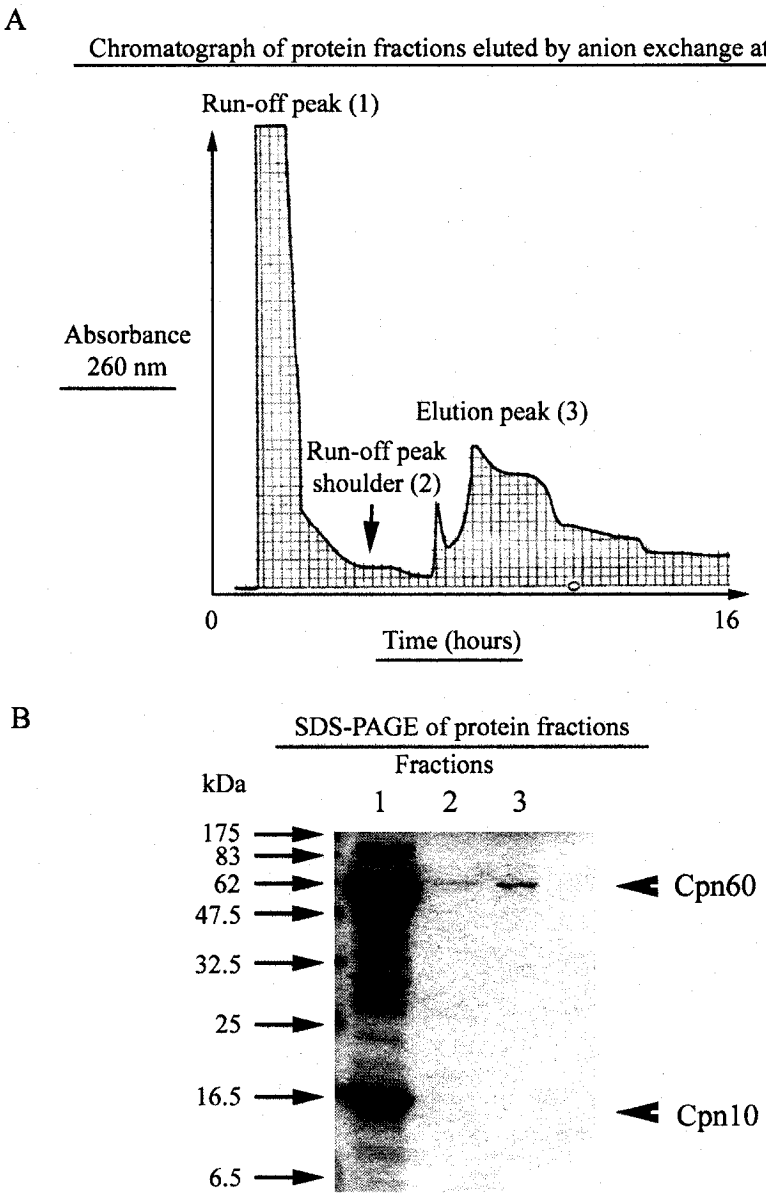


Figure 34. Buffer pH 6.0 did not promote Cpn60 binding to an anion exchange column. A cleared protein lysate of DH5α expressing Cpn10 and Cpn60 from plasmid pBs::*cpn10cpn60* was subjected to anion exchange in a DEAE matrix equilibrated to pH 6.0. Protein fractionation was recorded (panel A) and protein fractions were visualized by SDS-PAGE (Panel B). Run-off refers to the fractions collected prior to addition of the KCl concentration gradient (Panel A, peaks 1 and 2; Panel B, lanes 1 and 2), and elution refers to fractions collected after addition of the gradient (Panel A, peak 3; Panel B, lane 3).

to an anion exchange column (pers. comm. Dr. P. S. Hoffman); therefore, I attempted to use this pH to purify Cpn60. At a buffer pH of 7.5 with no KCl or NH_4Cl added to the column buffer, Cpn60 and Cpn10 did bind to the anion exchange column. The proteins were eluted using a KCl concentration gradient ranging from 200 mM to 400 mM, and fractionated in 10 ml fractions (Fig. 35). Cpn60 identity was confirmed by Western blot with the *L. pneumophila* MAb (Fig. 35).

Cpn60 and Cpn10 isolated in the run-off fraction were stored at -70 °C, which unexpectedly resulted in protein precipitation. The addition of Triton X detergent did not improve solubility; perhaps sonication could have also been attempted. Cpn60, Cpn10, and other proteins that remained in solution were then stored at 4 °C, and the sample was fractionated after passage through a Sephadex G200 gel-filtration column (Fig.36), as described in the Materials and Methods, which resulted in protein separation based on size differences. Two Cpn60-containing pools and two Cpn10-containing pools were silver-stained to assess purity (Fig. 37A), and were quantified using the Bradford assay (see Materials and Methods). Despite the addition of PMSF and DTT, storage at 4 °C resulted in the truncation of Cpn60 (Fig.37). Cpn60 pools had concentrations of 240 ug/ml and 140 ug/ml protein, and the two Cpn10-containing pools had concentrations of 140 ug/ml and 400 ug/ml protein (Fig. 37B). The Lowry method (see Materials and Methods) was also used to measure protein concentration, but this method proved unreliable. It is reported that DTT, which was used as a preserving agent, is incompatible with the Lowry Reagent; and that the drying of protein samples in a stream of nitrogen is necessary prior to quantification by the Lowry method (Lowry *et al.*, 1951).

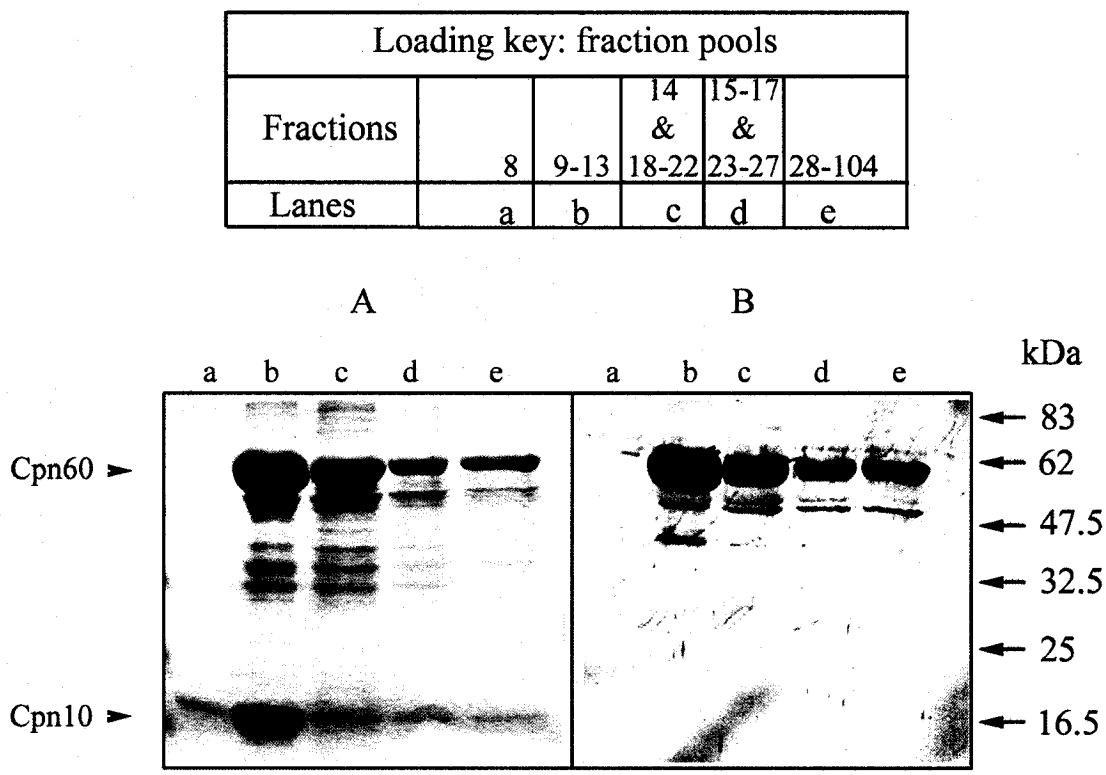
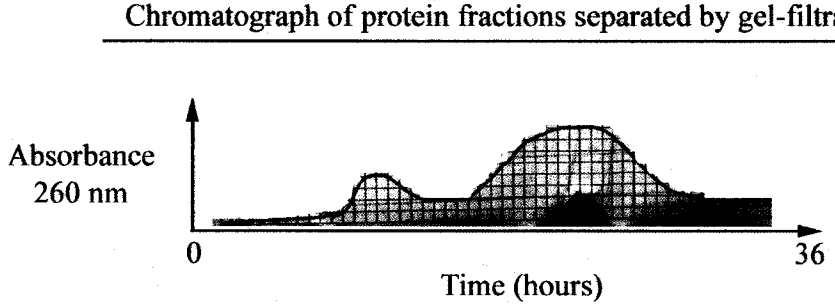


Figure 35. Cpn60 was partially purified by anion exchange chromatography at pH 7.5. A cleared protein lysate from DH5α expressing Cpn60 and Cpn10 from pBs::*cpn10cpn60* was subjected to anion exchange chromatography in a DEAE matrix equilibrated to pH 7.5. Fractions eluted, after the addition of a KCl concentration gradient ranging from 200 mM to 400 mM, were visualized by SDS-PAGE (not shown) , and stained with Coomasie stain (Panel A), or transferred to nitrocellulose, and probed in a Western blot (Panel B), using the *L. pneumophila* MAb, which is raised against HtpB, and cross-reacts with Cpn60.

A



B

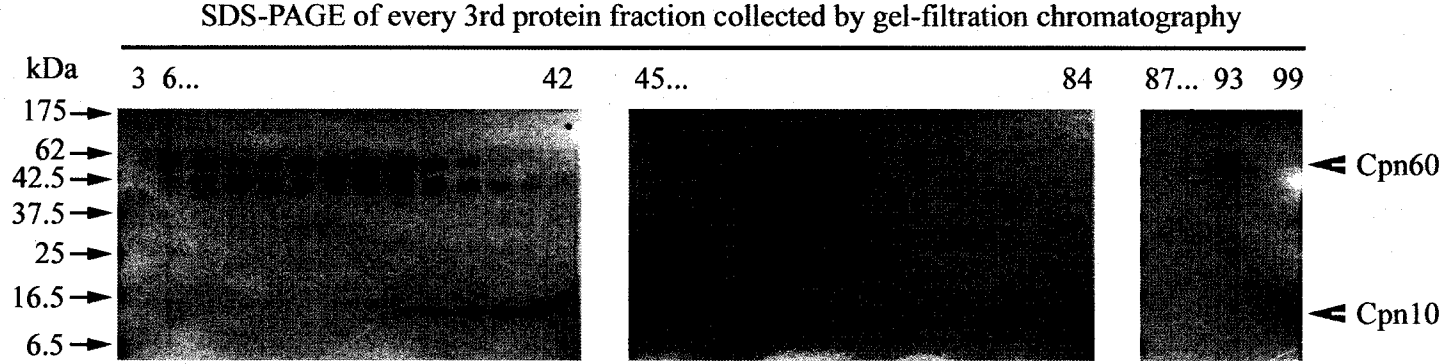
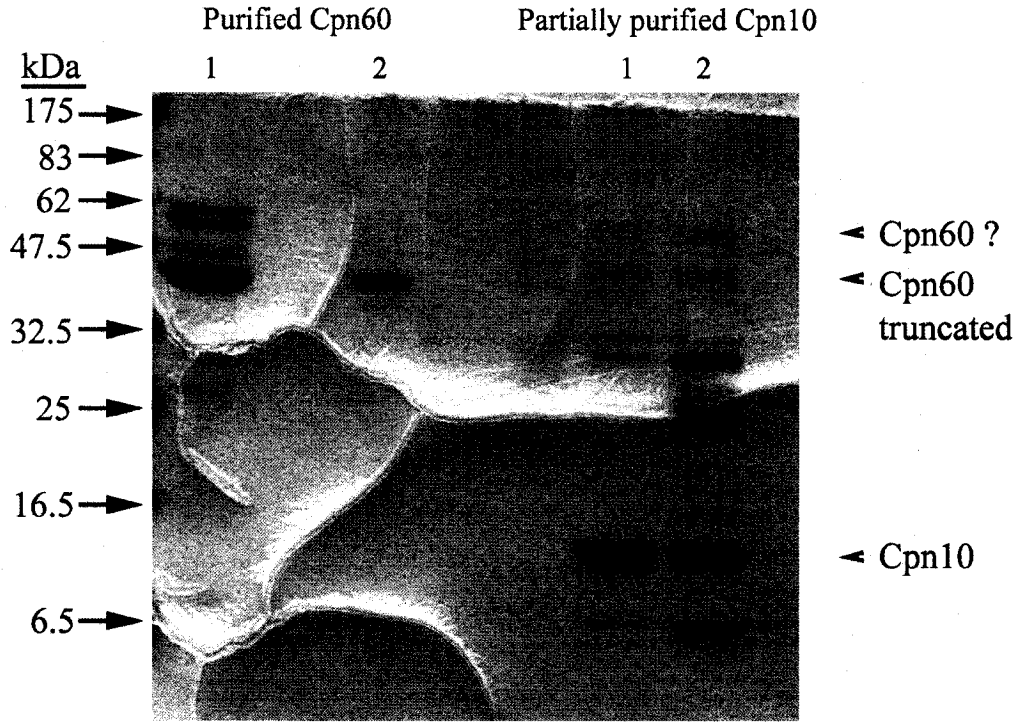


Figure 36. Cpn60 and Cpn10 protein purificaiton by gel-filtration chromatography. A protein fraction containing Cpn60 and Cpn10, partially purified from a DH5α cell lysate by anion exchange chromatography, was further purified by gel-filtration chromatography in a Sephadex G200 superfine matrix. Protein fractionation was recorded (Panel A) and every third protein fraction collected was visualized by SDS-PAGE (Panel B).

A

Silver-stained polyacrylamide gel of purified Cpn60 and Cpn10 proteins



B

Cpn60 and Cpn10 concentrations as determined by the Bradford Assay

	Cpn60 µg/ml	Cpn10 µg/ml
1	240	260
2	140	400

Figure 37. SDS-PAGE confirms that Cpn60 and Cpn10 were partially purified by anion exchange and gel-filtration chromatography. Protein fractions containing purified Cpn60 and partially purified Cpn10 were separated by SDS-PAGE and silver-stained (Panel A). A sample of the protein fractions were quantified using the Bradford protein assay with comparison to a BSA standard curve, see materials and methods (Panel B).

Purified protein samples were then stored at -70 °C. These protein samples were to be used for coating latex beads for use in experiments to investigate whether Cpn60 could perturb the eukaryotic endocytic process as is the case for HtpB (Garduno, Garduno and Hoffman, 1998). The anion exchange and gel filtration matrices were sanitized and checked for contamination by monitoring the absorbance readings of the effluent using the ISCO UA-5 absorbance/fluorescence detector, and these columns were then utilized by another graduate student, Vanessa Landry, using the method developed in this study, for the purification of the *L. pneumophila* HtpB protein from DH5α. The purified HtpB protein was used to produce the *L. pneumophila* polyclonal antibody used in this study.

2.2. HeLa cell staining.

L. pneumophila has the capacity to delay the maturation of its replicative endosome into an acidic organelle inside eukaryotic cells (Sturgill-Koszycki and Swanson, 2000). The maturation of organelles is dependent on the transient fusion of early endosomes with later endosomes, such as lysosomes that contain acid hydrolases (Tjelle, Lovdal, and Berg, 2000). Since endosome maturation is dependent on movement along cytoskeletal tracts inside eukaryotic cells (Maples, Ruiz, and Apodaca, 1997), and it has been demonstrated that microtubule polymerization is required for the entry of *L. pneumophila* into Vero cells, it seemed plausible that *L. pneumophila* could delay phagosome maturation by perturbing the host cytoskeleton. Also since eukaryotic and bacterial chaperonins have been reported to associate with tubulin subunits that make up

microtubule filaments (Dunn, Melville, and Frydman, 2001; Linder, Schliwa, and Kube-Granderath, 1998); it was of interest to determine whether the *L. pneumophila* chaperonin, HtpB, which is a surface-exposed protein could perturb the architecture of microtubules inside the eukaryotic cells. In this study a methodology was developed to stain HeLa cell microtubules. It was envisioned that this method could be used to assess the effect on microtubules, when HtpB was expressed in the cytoplasm of a HeLa cells. An immuno-fluorescence protocol developed by Dr. Thomas MacRae for staining the microtubule cytoskeleton in mouse fibroblasts (Walling, Criel, and MacRae, 1998) was adapted for staining HeLa cells as described in the Materials and Methods. The HeLa cell microtubule cytoskeleton was detected with an anti-α-tubulin MAb raised against *Trypanosoma brucei* (Woods *et al.*, 1989), and an FITC-conjugated secondary antibody. The microtubule cytoskeleton appeared as a cross-linked network that extended throughout the cell structure (Fig. 38A). The HeLa cell nucleic acid was stained with DAPI as described in the materials and methods and the resulting image was superimposed onto an image of the microtubule network (Fig. 38B). This methodology will be utilized by others in future experiments to investigate the effects on HtpB expression on the HeLa cell microtubule arrangement. For this study as a method to investigate the function of HtpB inside a eukaryotic cell, a HeLa cell cDNA library was screened by the two-hybrid method to identify HeLa cell proteins that may interact with HtpB inside mammalian cells. This is described below.

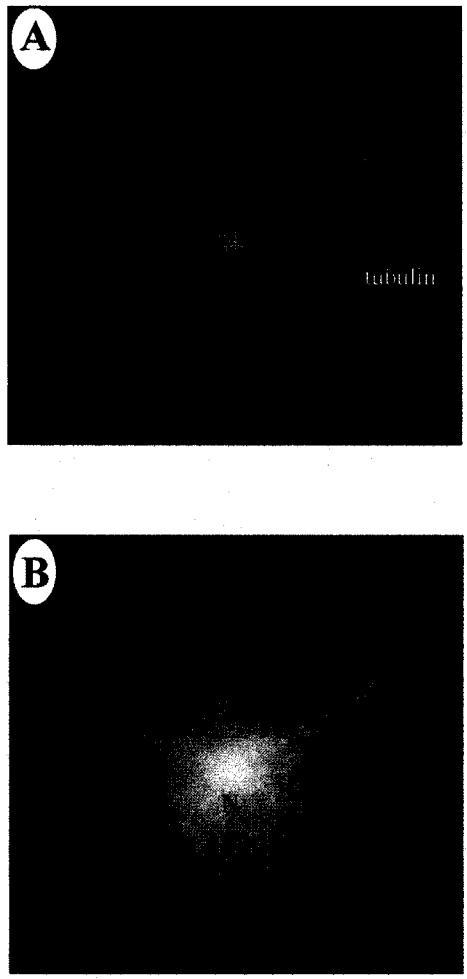


Figure 38. HeLa cell with cytoskeleton and nuclear material stained. HeLa cells were grown on coverslips, fixed in methanol and stained with the anti-α-tubulin MAb called TAT, and a secondary FITC-conjugated anti-IgG antibody (Panel A) and the nuclear stain DAPI (Panel B). FITC and DAPI were visualized on a Leitz Aristoplan microscope with a 63X objective lens with filter cubes I3 (513683) and A (513678) respectively. In Panel B the DAPI-generated image is superimposed on the FITC-generated image. N = nucleus

2.3. Identification of human HeLa cell proteins that interact with HtpB.

The two-hybrid method was used to isolate, from a commercially available human HeLa cell MATCHMAKER cDNA library (BD Biosciences, Palo Alto, CA), open reading frames that encode proteins, which physically interact with HtpB *in vivo*. This HeLa cDNA library was constructed in the Gal4 activation domain (AD) vector, pGADT7-Rec, in which cDNA inserts are fused to DNA sequences that encode the Gal4 AD. The AD library was maintained in the yeast strain Y187. The "bait" plasmid encoding a fusion of the Gal4 DNA-binding domain (DNA-BD) and HtpB, called pGBD- C1::*htpB*, was constructed for this study as described in the Materials and Methods. Expression of the Gal4 DNA-BD-HtpB fusion in yeast strain AH109 was confirmed by Western blot using the *L. pneumophila* MAb (Fig. 39). The Gal4 DNA-BD-HtpB fusion ran at the expected molecular weight of approximately 80 kDa, near the sum of the weights for the Gal4 DNA-BD (22 kDa) and HtpB (60 kDa). Lower molecular weight products were also visualized indicating that a fraction of the hybrid protein was degraded in the yeast protein extracts.

Approximately 4×10^{11} HeLa cell cDNA clones were screened for potential two-hybrid interactions with the Gal4 DNA-BD-HtpB fusion protein. The Y187 strain carrying the AD library was mated with AH109 strain carrying pGBD-C1::*htpB*. The AH109 strain is engineered to express four reporter genes including *HIS3*, *ADE2*, *lacZ*, and *MEL1* when it is activated by a functional Gal4 transcription factor. Y187/AH109

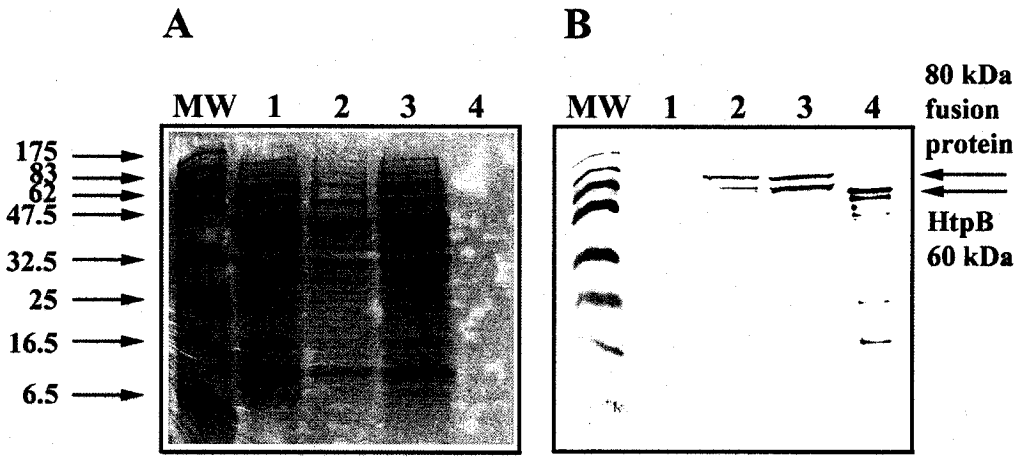


Figure 39. The HtpB-Gal4 DNA Binding Domain fusion protein is expressed in *S. cerevisiae* strain AH109. The HtpB-Gal4-DNA Binding Domain fusion protein was expressed from plasmid pGBD-C1::*htpB*. Protein extracts from strain AH109 carrying pGBD-C1 alone (Lane 1) or pGBD-C1::*htpB* (Lanes 2 & Lane 3) and a protein extract from strain W303-1b carrying pPP389::*htpB* (Lane 4) were separated by SDS-PAGE, transferred to nitrocellulose, stained with Ponceau S (Panel A), destained with 1X PBS, and probed with the *L. pneumophila* MAb in a Western blot (Panel B).

diploid progeny that produced a blue pigment, and that could form colonies at least 2 mm in diameter on either triple dropout (TDO)for less stringent selection or quadruple dropout (QDO) medium for more stringent selection were studied further (see Materials and Methods for a more detailed explanation of the selection media).

Plasmid DNA from five transformants named p7, p22, p24, p53, and p301 were transferred directly from the yeast cells to the *E. coli* KC8 strain by electro-transformation to facilitate AD library plasmid isolation. The KC8 strain is auxotrophic for leucine due to a mutation in its *leuB* gene. Since the yeast *LEU2* gene complements the *E. coli leuB* mutation it was useful for isolating the *LEU2*-based AD library plasmids when plated on selective M9 minimal medium lacking leucine. Six different HeLa cell cDNA plasmids were isolated (clone 301 yielded two different plasmids). To eliminate false positives, the AH109 strain was transformed with each of six AD library plasmids and then co-transformed with pGBD-C1::*htpB*, the vector control pGBD-C1, or plasmid pGBKT7-53, which encodes a Gal4 DNA-BD-p53 fusion to serve as the negative control, and these transformants were spotted onto selective medium containing X-α-gal. Four of the six co-transformants, bearing AD library plasmids p22, p24, p53 or p301-1 and pGBD-C1::*htpB*, produced more blue pigment than AH109 cells bearing the same library plasmids co-transformed with pGBD-C1 (Fig. 40) or pGBKT7-53 (data not shown). The other two AD library plasmids, p7 and p301-2, did not produce more blue pigment when co-transformed with pGBD-C1::*htpB* as compared to pGBC-C1 in AH109, so that they were considered false positives. Plating of the AH109 strain, devoid of plasmids, on

YPDA medium containing X-α-galactosidase revealed that weak transcription may occur from the *MEL1* reporter gene in the absence of an active Gal4 transcription factor (data not shown). This may explain the weak blue signal detected for the vector control.

To determine the relative sizes cDNA inserts present in plasmids p22, p24, p53 and p301-1, these plasmids were digested with *Hin*dIII to release the cDNA inserts from the pGADT7-Rec plasmid backbone (~7.2 kbp). In addition the cDNA inserts in these plasmids were PCR amplified with primers pGADT7-F and pGADT7-R to yield amplicons of the approximate sizes: 1.4-kbp (p22), 3.5-kbp(p24), 1.200-kbp (p53) and 2.2-kbp (p301-1), respectively. The cDNA inserts were then sequenced from both 5' and 3' directions using the primers, T7-F and AD-R, respectively (Table 5), and the sequences were analyzed using the Blastn alignment program (NCBI Entrez Database) to identify the genes of interest. The four cDNA inserts encoded the ribosomal protein L4 (RPL4), merlin-associated protein (Map), the cyclin-dependent protein kinase regulatory subunit 1b (CksHs1), and phosphoribosylaminoimidazole succinocarboxamide synthetase (PAICS) (Fig. 41). By analyzing the sequence data and noting the PCR amplicon sizes, it was clear that only one open reading frame was present in each of the four AD library plasmids, so that there was no question as to which protein was responsible for the putative interaction with HtpB (Fig. 41). Of the four putative proteins identified as interacting partners for HtpB only the cDNA fragment encoding RPL4 was found to be in frame with the gene sequence encoding the Gal4p-AD. However, it is recommended to

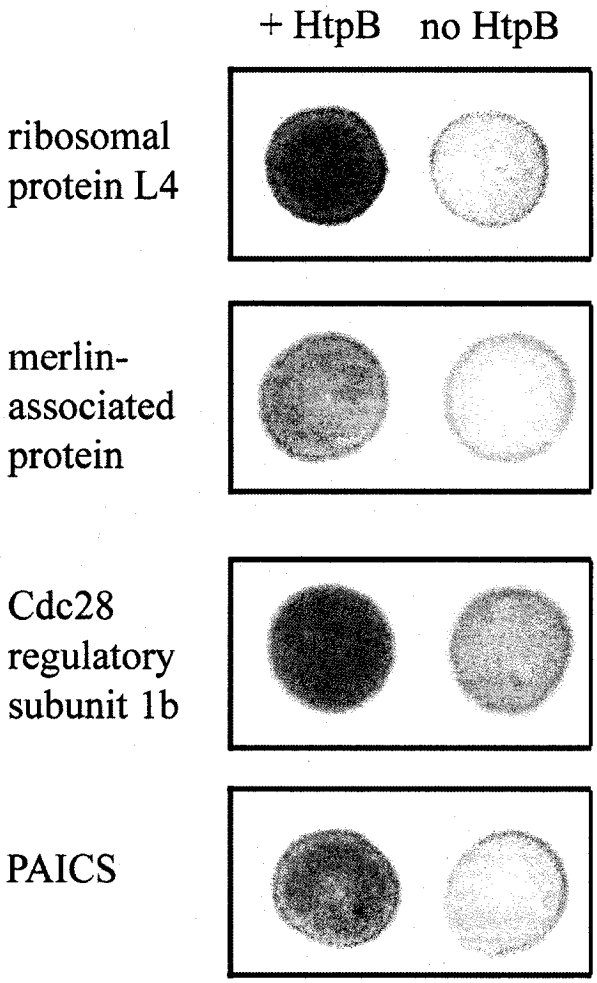


Figure 40. Yeast cell transformants bearing plasmids containing HeLa cDNA inserts that were isolated in a 2-hybrid screen using HtpB as "bait".
S. cerevisiae strain AH109 was co-transformed with either pGBD::*htpB* (+ HtpB) or pGDB-C1 (no HtpB) and purified plasmids containing HeLa cDNA inserts. The cDNA inserts were identified by DNA sequencing to encode ribosomal protein L4, merlin associated protein, the Cdc28 regularoty subunit 1b, and phosphoribosylaminoimidazple succinocarboxamide synthetase (PAICS). Yeast cells were plated on double dropout medium containing the chromogenic substrate, X-α-galactosidase.

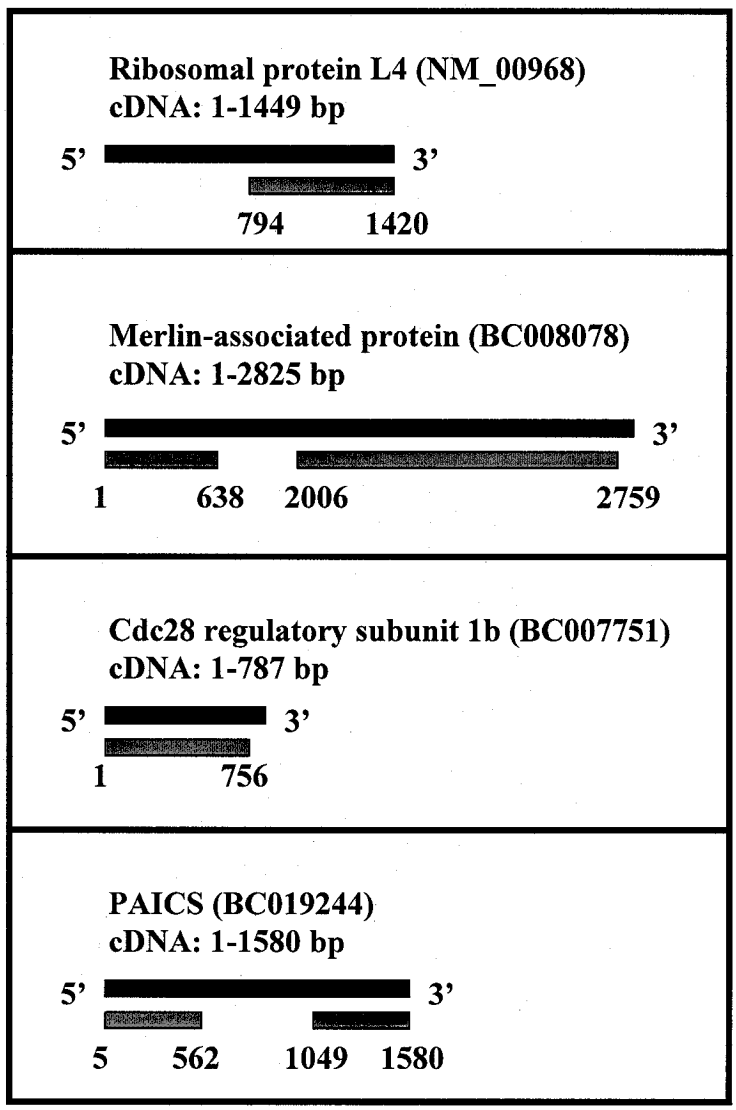


Figure 41. Homology matches for HeLa cell cDNA inserts, which were isolated in a two-hybrid screen using HtpB as "bait". Black boxes represent full-length cDNA sequences that share homology with HeLa cDNA partial sequences, indicated by the grey boxes. Partial sequences for the HeLa cell cDNA inserts in plasmid pGADT7-Rec were obtained using primers T7 (5' sequencing primer) and AD (3' sequencing primer); bp: nucleotide base pair; PAICS: phosphoribosyl succinocarboxamide synthetase. NCBI database accession numbers are indicated in brackets. Actual DNA sequences are available upon request from Dr. Rafael A, Garduno, Dalhousie University, Halifax, NS.

test AD library plasmids found to contain cDNA fragments that encode proteins that are not in-frame with the gene encoding the Gal4pAD in other assays such as immuno-precipitation to confirm fusion protein expression (BD biosciences, 2001), since translational frameshifts that allow for expression of a fusion protein are known to occur in *S. cerevisiae* (Staht *et al.*, 2001 and Bekaert et. al. 2006). These translational frameshifts reported were most often +/-1 frameshifts as was the case for the other three AD library plasmids that encoded Cdc28, MAP and PAICS. Hence the interactions between HtpB and HeLA cell proteins identified by the two-hybrid method indicate putative protein interactions that will be tested by co-precipitation studies and coimmunolocalization studies as indicated in the Discussion section of this work.

Part 3. A Secretion Mechanism for Hsp60 Homologues.

While in the pathogenic organisms such as, *L. pneumophila*, *Brucella abortus, Bordetella pertussis*, and *Bordetella bronchiseptica* (Garduno et al., 1998; Watarai *et al.*, 2003 and this study), Hsp60 homologues are found in association with the bacterial outer membrane and cell surface, in non-pathogenic *E. coli*, Hsp60 homologues are cytoplasmic proteins (Garduno *et al.*, 1998 and Zeilstra-Ryalls, Fayet, and Georgopoulos, 1991). It is still unknown by what mechanism(s) many of these pathogenic organisms transport Hsp60 homologues to their outer membranes and cell surfaces; however, it was recently reported that Hsp60 surface mobilization in *B. abortus* is dependent on a Type IV secretion-associated process (Watarai *et al.*, 2003).

3.1. Hsp60 secretion in *E. coli.*

To elucidate the mechanism for Hsp60 surface localization, studies were undertaken to investigate whether the inability of *E. coli* to localize GroEL to its cell surface is due to properties in the GroEL protein, or due to the absence of a secretion system in *E. coli* that is present in *L. pneumophila*, *B. pertussis* and/or *B. bronchiseptica*. *E. coli* DH5α lacks a type III and a type IV secretion system, while *L. pneumophila* and *B. pertussis* have functional type IV secretion systems, but no functional type III secretion system, and *B. Bronchiseptica* has a functional type III secretion system, but not a functional type IV secretion system (Mattoo *et al.*, 2004; Mattoo *et al.*, 2001), leading us to hypothesize that GroEL could be confined to the DH5α cytoplasm due to the absence of a type III or type IV secretion mechanism.

Type III secretion is a one-step process that occurs in response to bacterial contact with its host cell, which triggers the direct injection of virulence factors from inside the bacterial cell into the eukaryotic host cell (Cornelis, 2000). Type IV secretion systems are known for sharing homology with DNA conjugation systems (Ding, Atmakuri, and Christie, 2003). In the case of *L. pneumophila*, its Dot/Icm type IV secretion system is essential for intracellular survival (Brand, Sadosky, and Shuman, 1994; Berger and Isberg, 1993), while *B. pertussis* uses its Type IV secretion system for liberating pertussis toxin (Weiss, Johnson, and Burns, 1993).

Enteropathogenic *E. coli* (EPEC), a pathogenic *E. coli* strain that causes infantile diarrhea, has a functional type III secretion system (Zaharik *et al.*, 2002) unlike nonpathogenic *E. coli* strains that lack this secretion system, hence it was of interest to

localize GroEL and HtpB, expressed from plasmid pSH16, in EPEC to assess whether the type III secretion system in this strain could facilitate the surface mobilization of HtpB or GroEL to the bacterial cell surface. HtpB expression in EPEC was confirmed by Western blot with the *L. pneumophila* MAb (Fig. 42) that does not cross-react with GroEL. Since GroEL expression is upregulated after heat-shock, and type III secretion is activated by host-cell contact, EPEC cells expressing the *L. pneumophila* HtpB protein, from plasmid pSH16, were heat-shocked at 42 °C, and/or placed in contact with HeLa cells, and were visualized by immunogold-electron microscopy, using the *E. coli* PAb or the *L. pneumophila* PAb. In both cases greater than 90% of the HtpB and GroEL proteins remained in the EPEC cytoplasm, indicating that a type III secretion system in EPEC did not mobilize GroEL or HtpB to the cell-surface (Fig. 43, Table 8 and Allan D.S., 2002). These data indicated that type III secretion is EPEC is not sufficient to mobilize either GroEL or HtpB to the EPEC cell surface.

3.2. Cpn60 secretion in *Bordetella spp.*

Bordetella bronchiseptica has a functional type III secretion system, and its involvement in mobilizing the endogenous *B. bronchiseptica* Hsp60 homologue, called Cpn60, to the bacterial cell envelope was assessed. In two *B. bronchiseptica* strains, the wild-type strain RB50, and the type III secretion defective mutant WD3, Cpn60 was localized by immunogold electron microscopy. In these experiments, Cpn60 was predominantly found in association with the cell envelope of strains RB50 and WD3 (Fig. 44, Table 9),

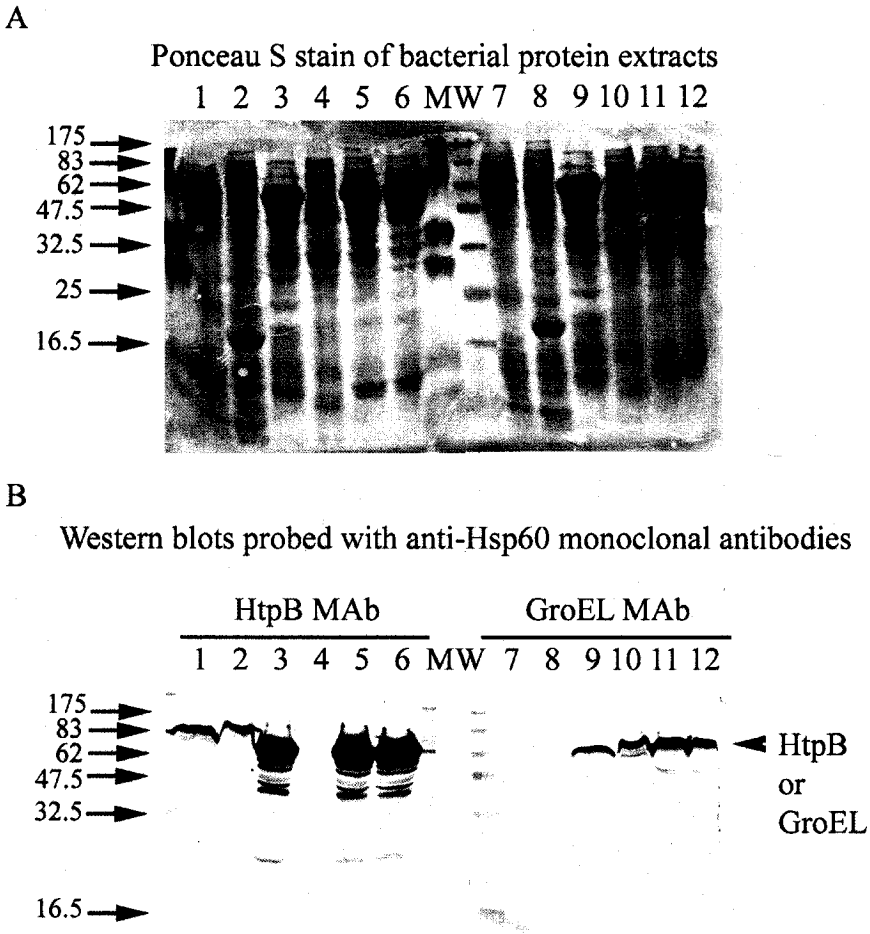


Figure 42. HtpB is expressed in enteropathogenic *E. coli*. HtpB was expressed from plasmid pSH16. Protein extracts from *L. pneumophila* (Lanes 1, 2, 7 and 8), *E. coli* DH5α carrying pSH16 (Lanes 3 and 9), and Enteropathogenic *E. coli* (EPEC) cells carrying either no plasmid (Lanes 4 and 10) or pSH16 (Lanes 5, 6, 11 and 12) were separated by SDS-PAGE, transferred to nitrocellulose, stained with Ponceau S, destained with 1 PBS, and then labeled with either of two non-crossreacting antibodies, the *L. pneumophila* MAb (a HtpB specific MAb) or the *E. coli* MAb (a GroEL specific MAb). EPEC cells were grown at 30 °C and then transferred to 42 °C for 30 minutes prior to lysis to increase GroEL expression by heat-shock. MW: molecular weight markers. Arrowhead indicates expected size for GroEL and HtpB.

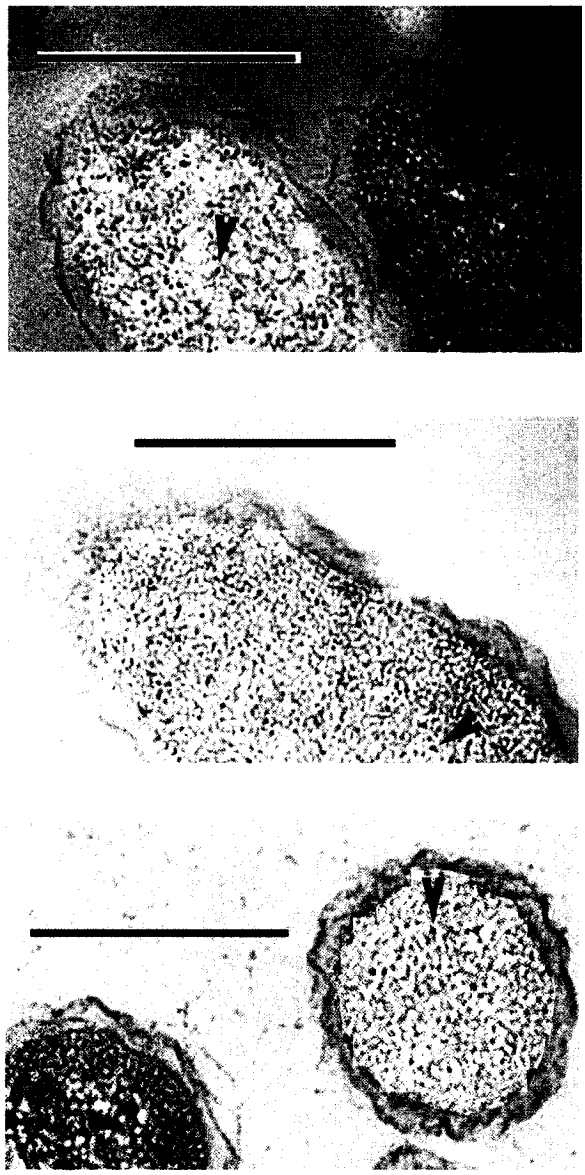
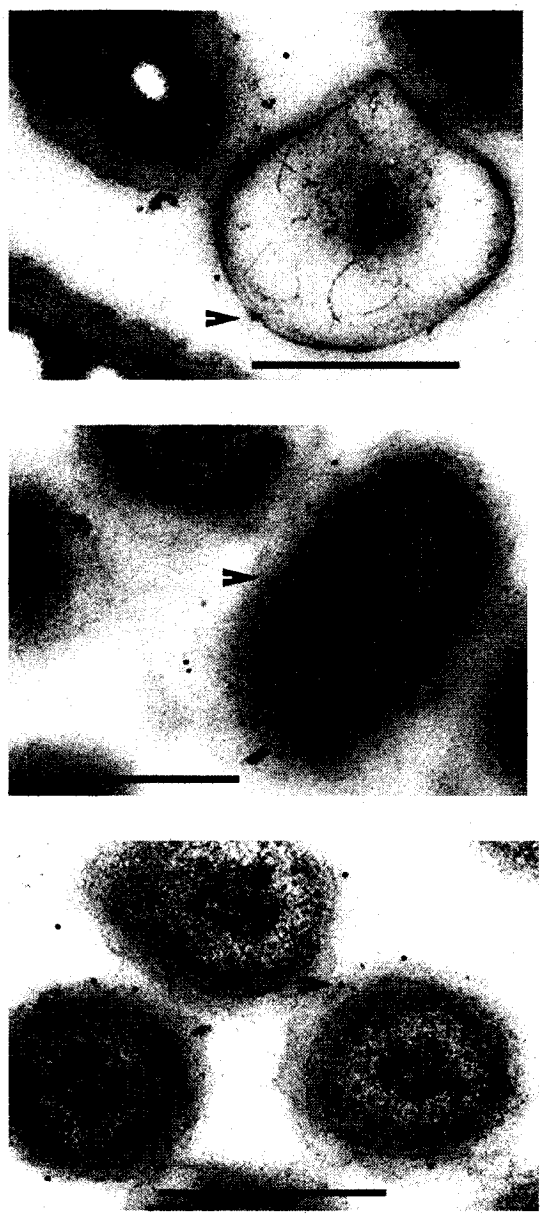


Figure 43. GroEL and HtpB are localized in the cytoplasm in enteropathogenic *E. coli*. EPEC expressing HtpB from pSH16 or carrying no plasmid were grown at 30 °C, shifted to 42 °C for 30 minutes, fixed in paraformaldehyde and prepared for thin sectioning for electron microscopy. Thin sections were labeled with the *L. pneumophila* PAb (HtpB PAb) or the *E. coli* (GroEL PAb) PAb and an anti-rabbit immunoglobulin gold conjugate (10 nm). Bars indicate 0.5 μm. Arrows indicate immunogold particles present in the EPEC cytoplasm.

Bacterial strain/antibody used for immunogold labelling	**Percentage gold particles present in the cytoplasm**	**Percentage gold particles associated with the cell envelope**	**Number of cells counted/ number of gold particles counted**
EPEC wild-type/ *E. coli* PAb	96.4	3.6	9/472
EPEC expressing HtpB/ *E. coli* PAb	92	8	12/435
EPECexpressing HtpB / *L. pneumophila* PAb	94.7	5.3	12/150

Table 8. The Hsp60 homologues, GroEL and HtpB, were predominantly localized to the cytoplasm in an enteropathogenic *E. coli* wild-type strain, as indicated by the percentage distribution of immunogold particles. GroEL (wild-type) and HtpB (expressed as a recombinant protein from plasmid pSH16) were localized in a wild-type EPEC strain. The *E.coli* PAb and the *L. pneumophila* PAb that can bind to shared epitopes in both GroEL and HtpB were used independently to localize the the Hsp60 homologues inside EPEC. These primary antibodies were labelled with an anti-rabbit gold-conjugated secondary antibody (Table 4).



B. pertussis wild-type
probed with HtpB PAb

B. bronchiseptica wild-type
probed with HtpB PAb

B. bronchiseptica
Type III secretion mutant
probed with HtpB PAb

Figure 44. Cpn60 is localized to the cell envelope of *B. pertussis* and *B. bronchiseptica* wild-type strains, and in a *B. bronchiseptica* type III secretion mutant. Wild-type *B. pertussis* BP388, wild-type *B. bronchiseptica* RB50 and a *B. bronchiseptica* type III secretion defective mutant WD3 were labeled in thin sections prepared for electron microscopy with the *L. pneumophila* PAb (HtpB PAb) that cross-reacts with Cpn60. A secondary gold-conjugated antibody was used for primary antibody detection as described in the materials and methods. Bars represent 0.5 μm.

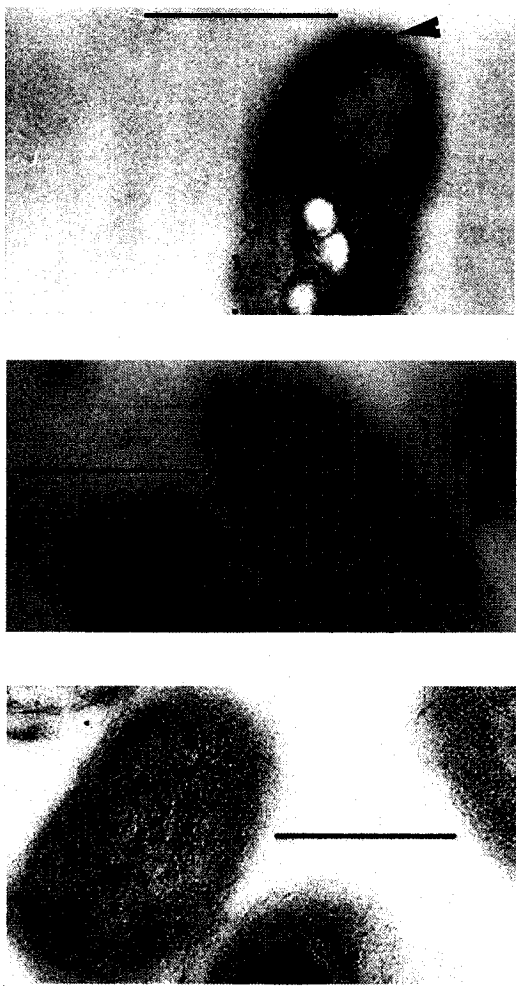


Figure 45. Cpn60 is localized to the cell envelope of a *B. pertussis* wild-type strain, and a type IV secretion mutant, and is localized to the cytoplasm of a sensor kinase mutant. Wild-type strain *B. pertussis* BP388 (wild-type), a type IV secretion mutant BP3171 (Ptl-), and a sensor kinase mutant BP347 (BvgS-) were labeled with the *E. coli* PAb (a GroEL PAb that cross-reacts with Cpn60) in thin sections prepared for electron microscopy. A secondary gold-conjugated antibody was used for primary antibody detection as described in the Materials and Methods. Bars indicate 0.5 μm.

Bacterial strain/antibody used for immunogold labeling	**Percentage gold particles present in the cytoplasm**	**Percentage gold particles associated with the cell envelope**	**Number of cells counted/ number of gold particles counted**
Bordetella pertussis wild-type strain BP388/ E. *coli* PAb	4.90	95.1	1/41
Bordetella pertussis type IV secretion mutant BP3171/ *L. pneumophila* PAb	8.5	91.5	4/188
Bordetella pertussis bvgS mutant strain BP347/ *L. pneumophila* PAb	94	6	12/100
Bordetella pertussis wild-type strain BP388/ *L. pneumophila* PAb	23.4	76.6	40/586
Bordetella bronchiseptica wild-type strain RB50/*L. pneumophila* PAb	8.1	91.9	41/123
Bordetella bronchiseptica type III secretion mutant WD3/ *L. pneumophila* PAb	20.4	79.6	56/255

Table 9. The Hsp60 homologue, Cpn60, was predominantly associated with the bacterial cell envelope in *Bordetella spp.* wild-type strains and in type III and type IV secretion mutants, as indicated by the percentage distribution of immunogold particles. In contrast, in a *bvgS* mutant, Cpn60 was predominantly localized in the bacterial cytoplasm.The *E. coli* PAb and the *L. pneumophila* PAb (Table 4) cross-react with Cpn60, and these were used independently as the primary antibodies to detect Cpn60 (wild-type protein) in the indicated *Bordetella spp.* These primary antibodies were labelled with an anti-rabbit gold-conjugated secondary antibody

which indicates that type III secretion is not essential for Cpn60 association with the bacterial cell envelope in *B. bronchiseptica*. Since *B. bronchiseptica* has a functional type III secretion system, but not a functional type IV secretion system, although it contains the complement of genes required for type IV secretion, the endogenous *B. pertussis* Cpn60 was localized in two *B. pertussis* strains, the wild-type strain BP388, which has a functional type IV secretion system, and in the Ptl$^-$ strain BP3171, defective in type IV secretion. In addition localization of the endogenous *B. pertussis* Cpn60 was measured in the BvgS-negative mutant BP347, since BvgS is a sensor kinase that regulates virulence factor gene expression including the expression of type III secretion genes in *B. bronchiseptica* (Yuk, Harvill, and Miller, 1998). In both the wild-type *B. pertussis* strain BP388 and in the Ptl-negative mutant Bp3171, Cpn60 was localized predominantly to the *B. pertussis* cell envelope (Fig.45, Table 9), which indicates that type IV secretion is not required for Cpn60 association with the bacterial cell envelope. In contrast, greater than 90 % of the Cpn60 protein was localized in the cytoplasm of the BvgS-negative mutant, Bp347, (Table 9) indicating that the association of Cpn60 with the *B. pertussis* cell-envelope is dependent on a BvgS-activated system. For each of these experiments cells were also labeled without a primary antibody to control for non-specific binding. Taken together these studies using *Bordetella spp.* indicate that Cpn60 surface translocation is independent of known type III and type IV (Ptl) secretion systems in *Bordetella spp.*, and demonstrates that the surface expression of Cpn60 is regulated with virulence gene expression in *B. pertussis*.

CHAPTER 4: DISCUSSION.

Part 1. Rationale.

The opportunistic human pathogen, *L. pneumophila*, can replicate inside mammalian cells (derived from monocytes, fibroblasts and epithelial cells) and protozoan cells (amoeba and ciliated protozoa)(Fields, 1996), which highlights the versatility of *L. pneumophila*'s strategy for intracellular replication. The process of infection can be separated into different phases (i) attachment and invasion, (ii) intracellular replication, and (iii) egress from the host cell for transmission. It is reported that a type IVB secretion system called the Dot/Icm secretion system is essential for the process of intracellular infection (Berger and Isberg, 1993; Brand, Sadosky, and Shuman, 1994). Indeed it was demonstrated that the replicative endosomes of *L. pneumophila dotA* mutants are labeled with the lysosomal glycoprotein, LAMP-1, an indication of exposure to acid hydrolases housed within lysosomes (intracellular digestion) (Roy, Berger, and Isberg, 1998). Using an inducible expression system for DotA, it was demonstrated that the DotA protein was required very early in the infection process (within five minutes), but was not required during later stages of intracellular replication (Roy, Berger, and Isberg, 1998). Therefore it is evident that the mechanism(s) involved in promoting *L. pneumophila* intracellular replication takes control of the host endocytic process very early in infection, perhaps at the point of attachment and invasion. It has been demonstrated that *L. pneumophila* utilizes its surface-exposed HtpB protein to mediate the attachment to and invasion of non-phagocytic HeLa cells (Garduno, Garduno, and Hoffman, 1998), and that *L. pneumophila* releases HtpB into its replicative endosomes

(Fernandez *et al.*, 1996; Garduno *et al.*, 1998). Avirulent strains of *L. pneumophila* express less HtpB on the cell surface as compared to virulent strains as determined by immunofluorescence (Hoffman, Houston, and Butler, 1990; Garduno, Garduno, and Hoffman, 1998), and HtpB expression is upregulated upon contact with eukaryotic host cells. These observations highlight that HtpB is available to make contact with host cells during the early infection process, which makes HtpB a very interesting candidate to investigate further.

Part 2. An Investigation of HtpB Biological Function in a Yeast Model.

HtpB is an essential protein for *L. pneumophila*; therefore, it was not possible to create a gene deletion mutant to assess the protein's role in virulence. Instead, a strategy to investigate the biological function of the protein in the context of the eukaryotic cell was undertaken. Since *S. cerevisiae* is a genetically tractable eukaryotic model for which many genetic tools are available, it was chosen as a model to explore HtpB biological function. It was fascinating to observe that when HtpB was expressed in the cytoplasm of *S. cerevisiae* (from plasmid pEMBLyex4::*htpB*), *S. cerevisiae* cells underwent a developmental change called pseudohyphal growth (Fig. 14). Pseudohyphal growth can otherwise be triggered in *S. cerevisiae* by various cues. External cues include nitrogen depletion (Gimeno *et al.*, 1992), the exposure to fusel alcohols such as 0.5% isoamyl alcohol (Dickinson, 1996), and exposure to the plant hormone, indoleacetic acid (Prusty, Grisafi, and Fink, 2004). Internal cues include the suppression of a stress response (Stanhill, Schick, and Engelberg, 1999), slowed DNA synthesis (Jiang and Kang, 2003),

and perturbations in the process of mitotic exit (Zhu *et al.*, 2000). The phenotypes of pseudohyphal growth include cell-elongation, the formation of unipolar buds, persistent cell-cell adhesion, and invasion of the growth substrate (Lengeler *et al.*, 2000). Various physiological processes must be coordinated to achieve the physical attributes of pseudohyphal growth including: specific patterns for gene expression and signal transduction, a delay in cell-cycle, the selection of a unipolar and distal bud-site, the polarization of actin to bud tips, and the maintenance of physical attachment between cells (Rua, Tobe, and Kron, 2001). The molecular mediators that are relevant to the process of HtpB-induced pseudohyphal growth in *S. cerevisiae* are discussed below.

2.1 Gene expression.

Gene expression during pseudohyphal growth is controlled by a large group of transcription factors including the transcriptional activators Flo8p, Ste12p Tec1p and Mss1p, which bind to the complex promoter of the *FLO11* gene (Rupp *et al.*, 1999; Kohler *et al.*, 2002; Gagiano *et al.*, 1999), and Sfl1p, a Flo8p antagonist that suppresses the expression of *FLO11* (Conlan and Tzamarias, 2001). Flo11p is a lectin-like protein also known as a flocculin that mediates prolonged cell-cell adherence that is essential for the process of invasive growth by *S. cerevisiae* (Lo and Dranginis, 1998; Lo and Dranginis, 1996).

In this study when HtpB was expressed in haploid yeast cells whose chromosomal copies of either *STE12* or *FLO8* were deleted, the cells became elongated, but did not invade the growth substrate (Fig. 26 and Fig. 29, respectively). In contrast when both

FLO8 and *STE12* were deleted simultaneously, the yeast cells did not elongate and did not invade the surface of their growth media (pers. commun. Dr. L. Murray). These data indicate that both Flo8p and Ste12p are necessary for HtpB-induced agar invasion by *S. cerevisiae* cells, but that either is sufficient for the process of cell elongation. Since Flo8p and Ste12p are both necessary for *FLO11* activation it is probable that in the absence of Flo11p, cell-cell adherence, a requirement for pseudohyphal growth, does not occur, thus HtpB is unable to induce agar invasion in the yeast strains containing deletions in *FLO8* and/or *STE12*.

Hence it would be informative to test directly whether the *FLO11* gene is required for HtpB-induced pseudohyphal growth, by expressing HtpB in a *flo11Δ* strain, and then testing for the formation of elongated cells, cells budded in a unipolar fashion and the capacity for cells to invade the agar surface. This experiment could also more clearly define which of these pseudohyphal growth phenotypes are Flo11p-dependent. The capacity for HtpB to induce pseudohyphal growth in a haploid strain with a *flo11Δ* mutation that was derived from strain S288C (a strain background that has a natural *flo8Δ* mutation, but that is responsive to HtpB-induced pseuodohyphal growth once complemented with the wild-type *FLO8* gene) is currently under investigation. Since the S288C strain bears a natural nonsense mutation in its *flo8* allele, both the wild-type *FLO8* gene and *htpB* must be introduced into this *flo11Δ* mutant strain to test directly the role of *FLO11* gene in HtpB-induced pseudohyphal growth. This work is currently under investigation (pers. commun. Dr. L. Murray).

FLO11 gene transcription is also activated by Tec1p, which interacts with the

Ste12p transcription factor to promote gene transcription (Gavrias *et al.*, 1996; Rupp *et al.*, 1999). Tec1p (for transposon enhancement control) binds to the promoter sequences of the retrotransposon Ty1, and is required for its full expression (Laloux *et al.*, 1990). In *S. cerevisiae* the insertion of retrotransposons within the genome can result in the activation of adjacent genes (Laloux *et al.*, 1990). Tec1p interacts specifically with Ty1-derived sequences associated with genes involved in pseudohyphal growth to activate their transcription; therefore the FG (*Ty*A)::*lacZ* reporter construct, which contains Ty1-derived sequences in addition to the pheromone response element that facilitates Ste12p binding (Laloux, Jacobs, and Dubois, 1994; Madhani and Fink, 1997).is sensitive to Tec1p and Ste12p activation, and hence serves as a reporter construct for pseudohyphal growth. This reporter construct is used to as an indicator of nitrogen-starvation-induced pseudohyphal growth in diploids (Madhani and Fink, 1997), and was also used in this study to determine whether HtpB-induced pseudohyphal growth is a process similar to nitrogen-starvation-induced pseudohyphal growth in yeast. It was demonstrated, using β-galactosidase liquid assays, that HtpB expression results in the transcription of the FG(*TyA)::lacZ* reporter construct (Fig. 32). These data indicate that HtpB requires and may activate known transcriptional regulators of pseudohyphal growth to induce cell elongation and agar invasion in *S. cerevisiae*.

2.2. Signaling cascades activate pseudohyphal growth in response to nitrogen starvation.

HtpB induces yeast cells to form elongated cells that bud in a unipolar fashion and that can invade the solid medium, which are phenotypes associated with pseudohyphal growth in *S. cerevisiae*. Hence it was of interest to investigate whether components that induce natural pseudohyphal growth in *S. cerevisiae* are utilized by HtpB when expressed in yeast cells. These critical components are discussed.

A number of signaling cascades have been identified that transduce the external and internal signals that regulate pseudohyphal growth in *S. cerevisiae*. One can envision that the existence of multiple regulatory pathways for the stimulation of a single developmental process such as pseudohyphal growth would provide *S. cerevisiae* with the dexterity to survive in a changing environment. In response to nitrogen deprivation, and in the presence of an abundant carbon source, *S. cerevisiae* diploid cells assume a pseudohyphal mode of growth (Gimeno and Fink, 1994). This developmental change can be stimulated by at least two signaling pathways, the cAMP-dependent protein kinase (PKA) pathway, and a conserved MAP kinase pathway, the Ste-kinase cascade (Fig. 46 adapted from Gancedo, 2001 and Lengeler *et al.*, 2000).

Components of this conserved MAPK pathway (Fig. 46) in *S. cerevisiae* are involved in regulating mating and invasive growth in haploid cells, and pseudohyphal growth in diploid cells (Roberts and Fink, 1994). Haploid invasive growth is phenotypically similar to pseudohyphal growth in diploids; here, haploid yeast cells elongate, form filaments and invade solid medium (Roberts and Fink, 1994).

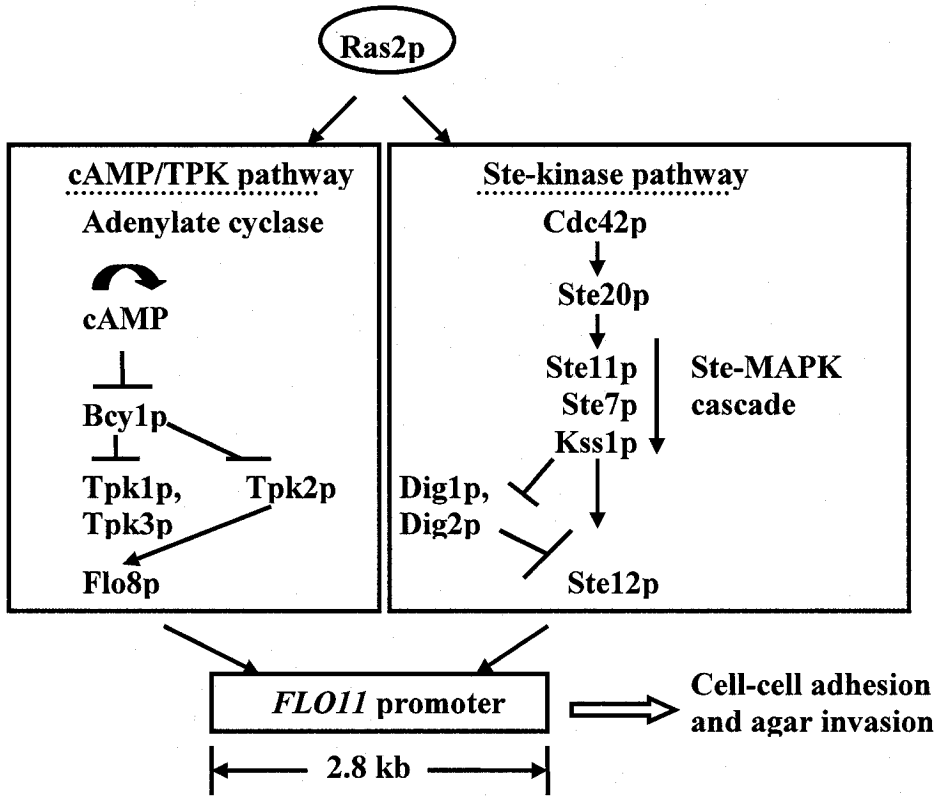


Figure 46. Two Ras2p-controlled pathways that mediate cell-cell adhesion and invasive growth in *S. cerevisiae*. Kinases include Bcy1p, Tpk1p, Tpk2p, Tpk3p, Ste20p, Ste11p, Kss1p, Dig1p and Dig2p; transcription factors include Flo8p and Ste12p GTP-binding proteins include Ras2p and Cdc42p, and the structural gene for the yeast flocculin (lectin) that promotes cell-cell adhesion for invasive growth is encoded by *FLO11*; arrows indicate stimulation and bars denote inhibition.

Pseudohyphal growth and invasive growth depend on Ras2p activation the Ste/MAPK cascade (Gancedo, 2001). In a *ras2/ ras2* mutant diploid cell, starved for nitrogen, pseudohyphae are composed of cells that are round instead of elongated (Kubler *et al.*, 1997). Ras2p activates the guanine exchange factor, Cdc24p, which positively regulates the small GTP-binding protein Cdc42p (Gancedo, 2001; Zheng, Cerione, and Bender, 1994). Activated Cdc42p interacts with Ste20p (a homologue of the mammalian $p65^{PAK}$ kinase) relieving its repression by the negative regulator, Hsl7 (Fujita *et al.*, 1999). In addition. Cdc42p is required for mobilization of Ste20p to the site of polarized growth, which is necessary for bud emergence especially in the absence or Cla4p, a kinase that is essential for bud emergence and that shares homology with Ste20p (Peter *et al.*, 1996). Activated Ste20p then signals through the remaining members of the Ste/MAPK pathway in the following order: Ste11p (a MAPK Kinase Kinase), Ste7p (a MAPK Kinase), and then Kss1p (a MAPK) (Roberts and Fink, 1994). Phosphorylated Kss1p then phosphorylates and activates Ste12p, and inactivates negative regulators of Ste12p, namely Dig1,2, also by phosphorylation (Cook *et al.*, 1996). Another downstream target of the Ste/MAPK cascade is the stimulatory transcription factor Tec1p, which is not phosphorylated by Kss1p, but has a Ste12p-recognition sequence in its promoter that could allow for positive regulation by Ste12p (Gancedo, 2001; Madhani and Fink, 1997).

In this study the involvement of some of these pseudohyphal growth regulators in mediating HtpB-induced pseudohyphal growth was investigated. It was demonstrated that in a *ste12Δ* mutant HtpB could induce the formation of elongated cells that budded in a unipolar direction, but could not induce agar invasion (Fig. 26), since Ste12p is the

transcriptional factor that is activated downstream of the Ste/MAPK pathway it was inferred that HtpB must act upstream of Ste12p to activate pseudohyphal growth in *S. cerevisiae*. As such yeast strains were constructed bearing chromosomal deletions of *STE20*, *STE11* or *STE7*. When HtpB was expressed in the deletion strains bearing *ste20*Δ, *ste11*Δ or *ste7*Δ, the cells elongated and budded in a unipolar direction, but did not penetrate solid medium (Fig. 26), indicating that HtpB must act upstream of the MAPK pathway to activate agar invasion, but may act through a different pathway to induce cell elongation and the formation of unipolar buds. Yeast cell elongation can result due to a delay in the exit from mitosis (Zhu *et al.*, 2000), and from this study there exists preliminary evidence, which indicates that HtpB may utilize this mechanism to induce cell elongation in yeast cells. A model describing a possible mechanism for HtpB-induced regulation of cell-cycle regulators to promote yeast cell elongation is discussed in the following sections of this discussion.

The Protein Kinase A pathway (PKA, Fig. 46) is activated by the G-protein coupled receptor, Gpr1p, for which glucose and other related sugars serve as activating ligands (Lorenz *et al.*, 2000). The PKA pathway can also be activated by the Mep2p ammonium transporter (Lorenz and Heitman, 1998). Gpr1p and Mep2p act upstream of the heterotrimeric G-protein alpha subunit, Gpa2 (Lorenz and Heitman, 1997; Lorenz and Heitman, 1998). Gpa2 activity results in an intracellular increase in cAMP (Gancedo, 2001; Kubler *et al.*, 1997; Dumortier *et al.*, 2000). The small GTPase, Ras2p, also plays a role in regulating pseudohyphal growth by upregulating cAMP concentrations; Ras2p has the capacity to activate adenylate cyclase, encoded by the *CYR1* gene, to catalyze the

synthesis of cAMP (Pan and Heitman, 1999; Mosch, Roberts, and Fink, 1996; Mosch *et al.*, 1999; Uno *et al.*, 1985). Ras2p- and Gpa2-induced cAMP upregulation results in the activation of protein kinase A (PKA) (Pan and Heitman, 1999; Mosch, Roberts, and Fink, 1996; Mosch *et al.*, 1999; Dumortier *et al.*, 2000; Kubler *et al.*, 1997). PKA consists of four subunits: the regulatory subunit, encoded by *BCY1*, and three catalytic subunits, encoded by *TPK1*, *TPK2*, and *TPK3* (Gancedo, 2001). The three Tpk catalytic subunits are redundant for vegetative growth, but have different roles in regulating pseudohyphal growth; the Tpk1 subunit inhibits pseudohyphal growth, while the Tpk2 subunit is stimulatory, and there are contrasting opinions as to whether Tpk3 plays no role or an inhibitory role in the process (Robertson and Fink, 1998; Pan and Heitman, 1999). PKA exists as a tetramer, and the binding of cAMP to the regulatory subunit, Bcy1p, facilitates the release of the catalytic subunits Tpk1-3 (Lengeler *et al.*, 2000). The Tpk2 catalytic subunit triggers pseudohyphal growth by activating the stimulatory transcription factor Flo8p and by repressing the inhibitory transcription factor Sfl1, both of which function at the *FLO11* promoter (Pan and Heitman, 2002; Rupp *et al.*, 1999). The Tpk1 subunit and perhaps the Tpk3 subunit may act to repress cAMP synthesis as part of a negative feedback loop (Lengeler *et al.*, 2000).

The PKA and Ste/MAPK pathways are responsible for activation of the Flo8p and Ste12p transcription factors, respectively. Since both Flo8p and Ste12p activation are required for the appearance of all phenotypes associated with HtpB-induced pseudohyphal growth it seemed reasonable to investigate a role for Ras2p in this process. Indeed Ras2p was shown to be essential for both agar invasion and cell elongation (Fig.

24). Of interest is that *RAS2*, *RAS1*, or the ras-related gene, *RSR1*, must be functional for completing the late mitotic phase of the *S. cerevisiae* cell cycle (Morishita *et al.*, 1995). Triple *RAS* gene deletion mutants are not viable, but can be complemented with the over expression of Cdc5p, Tem1p and other proteins that play a role in the completion of mitosis (Morishita *et al.*, 1995). Although Ras activation results in *FLO11* gene transcription, it seems unlikely that the expression of a surface lectin is all that is needed for the process of pseudohyphal growth. Indeed when I overexpressed the *FLO8* gene in *S. cerevisiae* S288C, yeast cells became flocculent and sticky, an indication of *FLO11* hyper-expression. However, these cells were not elongated, indicating that *FLO11* hyper-expression may not be sufficient for the changes in cell morphology that accompany pseudohyphal growth. In fact others have shown that yeast cells bearing a *flo11Δ* mutation may still have the capacity to form elongated cells (Rua, Tobe, and Kron, 2001). It is reported that the cAMP/PKA and Ste/MAP kinase pathways possibly through the activation of the Flo8p and Ste12p can promote mitosis, a process that is required for cell-elongation in *S. cerevisiae* (Kotani *et al.*, 1998; Ahn, Acurio, and Kron, 1999). This provides an avenue by which the cAMP/PKA and Ste/MAP Kinase can promote cell elongation. However it should be noted that HtpB requires Ras2p, which in addition to activating the cAMP/PKA and Ste/MAPK pathways for inducing pseudohyphal growth, activates other processes, such as bud formation and the progression through mitosis via Cdc42p (Hofken and Schiebel, 2004). Hence there exists multiple paths by which HtpB could be acting to induce pseudohyphal growth in *S. cerevisiae*.

2.3. Cell-cycle delay and pseudohyphal growth.

The cell cycle involves growth (the propagation of DNA and increased cell mass), and mitosis (the segregation of duplicated chromosomes and cell division) (Russell, 1996). The cell cycle is divided into four phases including Gap1 (G1), Synthesis (S), Gap 2 (G2), and the Mitotic (M) phase (Russell, 1996). G1 is called the pre-synthesis stage, and is the point at which cells prepare for DNA synthesis, which occurs in S phase; the G2 phase or post-synthesis stage follows S phase, and is the point at which cells prepare for chromosome segregation and cell division, which occurs in M phase (Russell, 1996).

To ensure that cells do not replicate in an uncontrolled manner the cell contains checkpoints; that is, biochemical pathways that ensure that cell cycle does not progress unless all necessary factors are in place (Elledge, 1996). The DNA damage checkpoint elicits a signal that arrests cells in G1 or G2 or slows down DNA synthesis (depending on what stage of the cell cycle the cell is in at the time of insult), and activates transcription of DNA repair genes or apoptotic genes (in mammalian cells) (Elledge, 1996). The spindle assembly checkpoint coordinates chromosome segregation with spindle assembly, and the morphogenesis checkpoint maintains the coordination of bud formation and nuclear division in *S. cerevisiae* (Elledge, 1996; Sia, Herald, and Lew, 1996).

Cyclin dependent kinases (Cdks) are serine/threonine kinases that regulate cell cycle transitions, and they are effectors of check points, so that the inhibition of Cdks results in cell cycle arrest (Pines, 1994; Elledge, 1996). The major Cdk in yeast is Cdc28p/Cdk1/Cdc2 (for cell division cycle), while mammals utilize several different Cdks, specialized for different cell cycle transitions including Cdk1/Cdc2 (G2-M), Cdk2

(G1-S), Cdk3 (G1 START), Cdk4 (G1 START) , and Cdk6 (G1 START) (Pines, 1994). Cdks are regulated by Cdk inhibitors (CKIs), a poorly characterized regulatory subunit called Cks1 in *S. cerevisiae* (Tang and Reed, 1993) and CksHs1 in mammals (Mongay *et al.*, 2001), as well as by cyclins (Elledge, 1996). The cyclins make up a diverse family that are divided into two groups G1 or START cyclins (Cln) that activate Cdks to initiate DNA replication, and the G2 or mitotic cyclins (also called B-type cyclins, Clb) that are required for mitosis (Pines, 1994).

The induction of developmental processes in yeast requires perturbations in the cell cycle. For example yeast cells can be stalled at the G1 START of the cell cycle, so that a decision can be made to initiate DNA replication, to mate, or to sporulate (Pines, 1994). Similarly, yeast cells stall at the G2 phase, delaying mitosis during pseudohyphal growth (Wittenberg and La Valle, 2003). In the case of pseudohyphal growth, extended activation of the G1 cyclin-Cdk complex, Cln1,2/Cdc28, coupled with inhibition of the mitosis promoting cyclin/Cdk complex, Clb1,2/Cdc28p, results in hyperfilamentation (Ahn *et al.*, 2001). A delay in mitotic exit can also result in hyperfilamentation. In this case the mitotic cyclin complex, Clb1,2/Cdc28p, remains activated as a result of inhibition of the Anaphase Promoting Complex (APC), which is a negative regulator of Clb1,2 (Yeong *et al.*, 2000). These data indicate that perturbations in the cell cycle are important molecular determinants for pseudohyphal growth.

In this study a mammalian protein that shares homology with the yeast cell cycle regulator Cks1, the regulatory subunit for the Cdc28p cyclin-dependent kinase, was identified as a putative interacting protein for HtpB in a 2-hybrid assay. Cks1 is reported

to interact with Cdc28p in yeast cells to direct the proteosome to the promoter of the *CDC20*/APC gene (Morris *et al.*, 2003), leading to chromatin remodeling that may result in transcription from the *CDC20* promoter (Morris *et al.*, 2003). Since Cdc20p, which encodes a ubiquitin ligase that targets the mitotic cyclin Clb1,2 for destruction, its expression could induce yeast cell elongation through the inhibition of the mitotic cyclin Clb1,2 coupled with prolonged activation of the G1 cyclins Cln1,2 (Ahn *et al.* 2001). Hence one mechanism by which HtpB could induce yeast cell elongation in *S. cerevisiae* is through Cks1-mediated inhibition of the mitotic cyclins Clb1 and Clb2.

It is important to test by immune-coprecipitation using an HtpB PAb or MAb whether HtpB truly interacts with Cks1 inside yeast cells. This is of particular importance since Cks1 is a putative transcriptional activator of the *GAL1* gene in *S. cerevisiae* (Yu *et al.*, 2005) and; therefore, the mammalian HeLa cell homologue, CksHs1 could have similar function, and may have activated transcription from the two-hybrid assay *HIS3* reporter, which consists of the *HIS3* coding region fused to the *GAL1* gene promoter. This could increase the probability that CksHs1 could be a false positive as detected by the two-hybrid assay. However, the *ADE2* and *MEL1* reporters were fused to other gene promoters, from *MEL1* and *GAL2*, respectively and since these promoters could be insensitive to putative CksHs1 transcriptional activation, the identified interaction could still be true.

2.4. Fusel alcohol-induced pseudohyphal growth.

While diploid cells form invasive pseudohyphae on medium, with low nitrogen, and

sufficient carbon (in the form of a sugar), haploid cells do not (Lengeler *et al.*, 2000). In the case of haploid cells, invasive growth occurs after several days of growth, on rich medium, and may be triggered by the exhaustion of multiple nutrients (Roberts and Fink, 1994; Lengeler *et al.*, 2000). When yeast cells begin to deplete nutrients on a rich medium, cellular proteins and branched amino acids are catabolized to produce nitrogen; the by-products of this process being short chained alcohols called fusel oils (Lengeler *et al.*, 2000). Both haploid and diploid yeast strains can be induced to invade rich medium by adding short-chain alcohols (fusel alcohols) such as isoamyl alcohol (IAA) and butanol (Dickinson, 1996).

Phenotypically the activation of pseudohyphal growth by IAA resembles pseudohyphae formation in response to nitrogen starvation; however, some of the molecular mediators required to regulate the two processes are different. First, and not surprising, is that nitrogen response sensors such as the Gpr1p receptor and G-protein subunit, Gpa2α, are not required for fusel alcohol-induced filamentation (Lorenz, Cutler, and Heitman, 2000). Second, while butanol-induced and IAA-induced filament formation is dependent on Swe1p (Martinez-Anaya, Dickinson, and Sudbery, 2003), a *swe1Δ/swe1Δ* diploid strain is still able to form pseudohyphae in response to nitrogen deprivation (Ahn, Acurio, and Kron, 1999). Hyper-activation of Swe1p, achieved through disruption of the gene encoding its negative regulator, Hsl1p, enhances filamentous growth under nitrogen starvation conditions (La Valle and Wittenberg, 2001). However, the inactivation of Hsl1p has broader implications than simply hyper-activating Swe1p. Hsl1p is required for the localization of the Cdc5p kinase to the bud

neck (Asano *et al.*, 2005). In addition to its role as a negative regulator of Swe1p, Cdc5p is also a negative regulator of the GTPase activating complex comprised of Bfa-1 and Bub2 that functions in stalling mitotic exit (Hu *et al.*, 2001; Geymonat *et al.*, 2003). This is of particular interest, since a mammalian homologue of Bub2 called merlin-associated protein was identified as a putative interacting partner for HtpB in a two-hybrid assay. A model for Bub2 involvement in HtpB-induced yeast cell elongation is presented in Part 3 of this Discussion section.

In summary it would appear that either the cAMP/PKA or Ste/MAPK nitrogen responsive pathways must be functional for pseudohyphal growth to take place in response to nitrogen starvation. These pathways may function in parallel with Swe1p, which has been shown to be absolutely required for fusel alcohol-induced filamentation, to induce maximal differentiation in response to nutrient starvation (La Valle and Wittenberg, 2001). Of interest is that when HtpB was expressed in a *flo8*Δ/*ste12*Δ double deletion mutant (new data, pers.comm. Dr. L. Murray), the yeast cells did not elongate, which indicates that at least one of the two Ras2p-activated pathways, either the cAMP/PKA or the Ste/MAPK pathways must be functional for HtpB-induced pseudohyphal growth. To determine whether Swe1p is required for HtpB-induced cell would help to clarify the mechanism by which HtpB induces pseudohyphal growth in *S. cerevisiae* as well as further delineate the roles of different effectors in mediating individual pseudohyphal growth.

Fusel-alcohol-induced pseudohyphal growth that is dependent on Swe1p results in the phosphorylation of Cdc28p (Martinez-Anaya, Dickinson, and Sudbery, 2003). Swe1p

effects a delay in the G2/M transition by inhibiting the mitotic cyclin-Cdk complex, Clb1, 2-Cdc28p (Mendenhall and Hodge, 1998). Ectopic activation of the MAPK pathway that can also result in hyperfilamentation is bypassed by the hyper-expression of Clb1,2, indicating that activation of the MAPK pathway results in a delay in the progression through mitosis (Ahn, Acurio, and Kron, 1999). A stall at the G2/M transition is a characteristic of pseudohyphal growth, since mitosis and cytokinesis must be prevented for cells to form filaments. Furthermore, Cdc28p appears to play a pivotal role in regulating pseudohyphal growth. The *cdc28-127* mutant allele induces cell filamentation in diploid yeast grown in rich broth, as well as agar invasion by diploid yeast on rich medium, indicating that the mutant allele bypasses the need for nitrogen starvation and contact with the agar medium, to induce filamentation (Edgington *et al.*, 1999). Delayed cell separation, which is a characteristic of cells growing pseudohyphally in response to nitrogen starvation was not observed as a phenotype of the *cdc28-127* allele (Edgington *et al.*, 1999), indicating that *FLO11* transcription may not have been activated. This would indicate that Cdc28p may act downstream of the cAMP/PKA and MAPK/Ste pathways. Furthermore, the MAPK cascade can inhibit Clb1,2 Cdc28p activity independent of Swe1p (Ahn, Acurio, and Kron, 1999). Hence these data support the possibility that Cdc28p may integrate independent signals from Swe1p and the Ste/MAP kinase cascade to effect yeast cell elongation in response to different stimuli (Fig. 47). Exactly how Ras2p activity influences Cdc28p activity has not been reported. It would be interesting to express the *cdc28-127* allele in a *ras2*Δ strain to determine whether Ras2p acts upstream or downstream of Cdc28p. Cdc28p appears to represent

basal machinery that is essential to the process of cell elongation that results from the stall at G2/M in the cell cycle in pseudohyphal growth, and as such it seems plausible that Ras2p would act upstream to Cdc28p. These data could help to clarify the role of Ras2p in HtpB –induced pseudohyphal growth. Perhaps HtpB contributes to the formation of a molecular complex that consists of Cdc28p, Cks1 and Ras2p to facilitate pseudohyphal growth activation (Fig. 48).

2.5. Yeast-cell elongation can result from a stall in mitotic exit.

Chromosome segregation, the exit from mitosis, and cytokinesis must be coupled to one another to facilitate temporal and spatial coordination during mitosis (Seshan and Amon, 2004). For example, the exit from mitosis occurs after the completion of chromosome segregation. This is, in part, achieved through the inactivation of the B cyclins Clb1,2 that promote entry into mitosis (Ahn *et al.*, 2001). The inactivation of the Clb1,2-Cdc28p complex is achieved through a combination of inhibition (mediated by Swe1p), and ubiquitin-dependent degradation of Clb1,2 (mediated by components of the APC) (Hu and Aparicio, 2005; Wasch and Cross, 2002). In particular Cdc20p, a ubiquitin ligase, and a part of the APC, is essential for Clb1,2 degradation (Thornton and Toczyski, 2003). Of interest is that Cdc20p activity is down-regulated by the cAMP/PKA pathway (Bolte *et al.*, 2003; Searle *et al.*, 2004), indicating that the cAMP/TPK pathway may promote cell elongation associated with pseudohyphal growth by stalling mitotic exit, and this combined with the pathway's ability to activate *FLO11* transcription would coordinate the events necessary for pseudohyphal growth, namely agar invasion, increased cell-cell

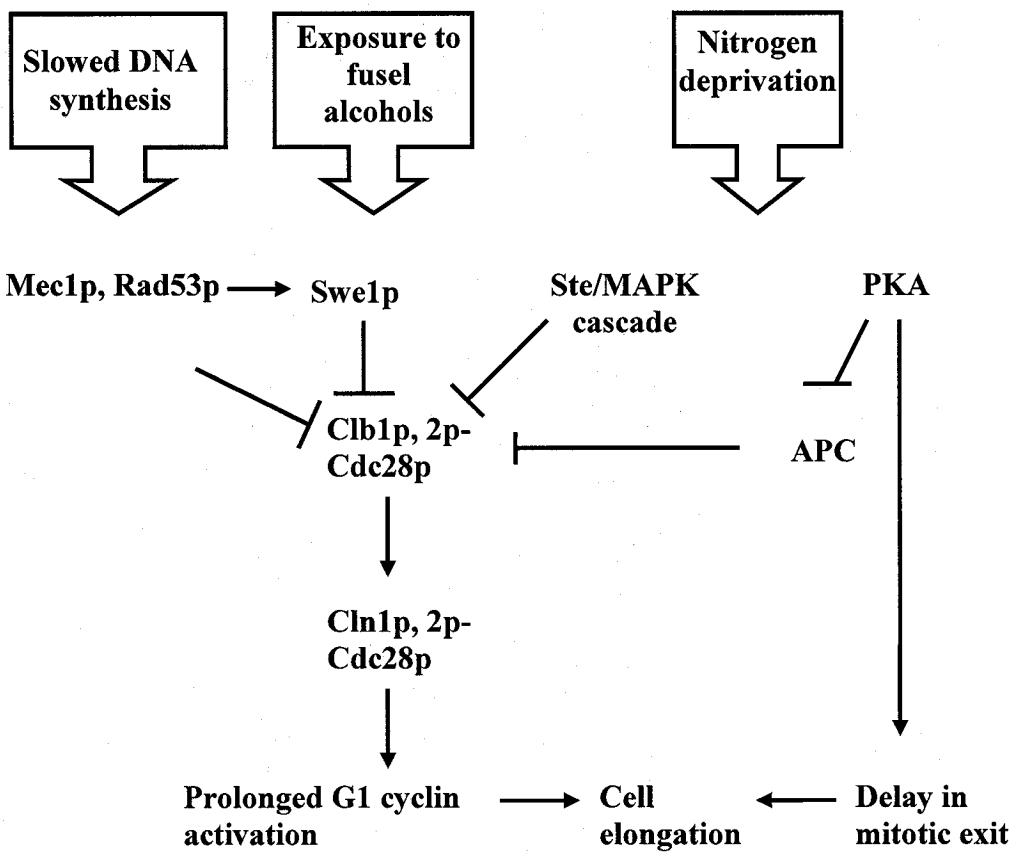


Figure 47. Factors that regulate cell elongation in *S. cerevisiae*. Kinases include the Ste/MAPK cascade, Swe1p, Mec1p, Rad53p, Cdc5, and Cdc28p; mitotic cyclins include Clb1p and Clb2p; G cyclins include Cln1p and Cln2p; transcription factors include, Fkh1p and Fkh2p; ubiqutin-dependent degradation complex: APC; arrows indicate activation; bars indicate inhibition; Δ = gene deletion.

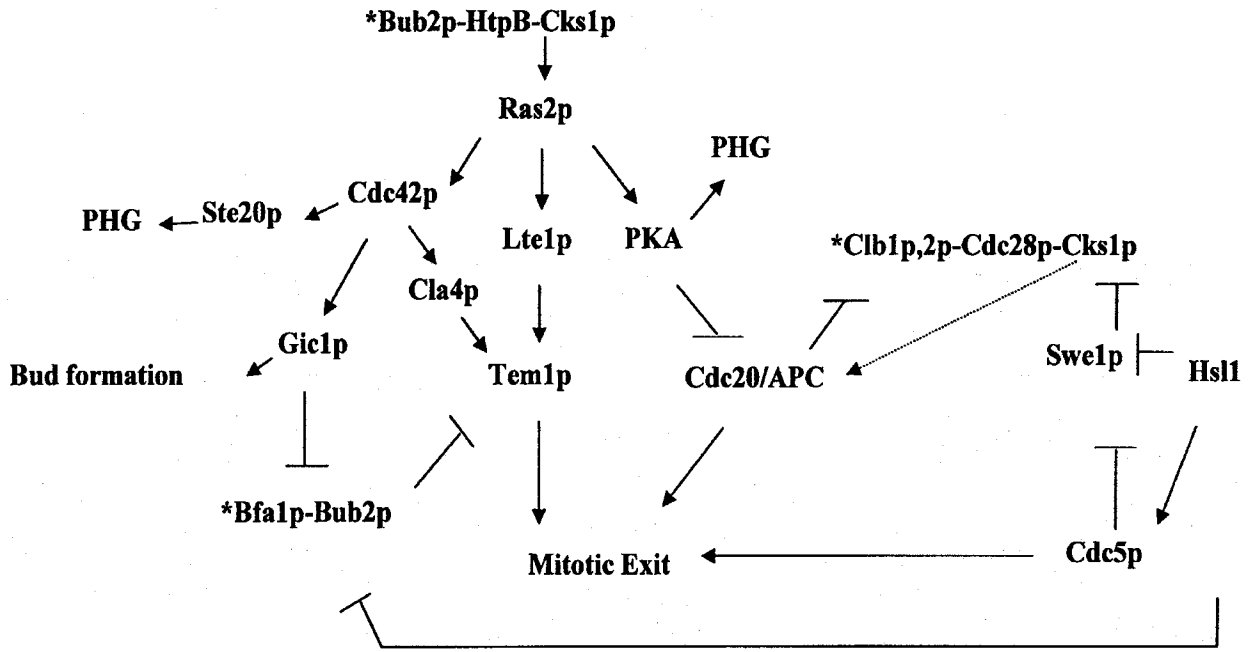


Figure 48. A possible role for HtpB in regulating key factors that control mitotic exit in *S. cerevisiae*. Factors include The GTP-binding proteins: Ras2p, Tem1p and Cdc42p; The kinases: Ste20p, Cla4p, Cdc5p and Swe1p; The GEF for Tem1p: Lte1p; AGTPase complex for Tem1p: Bfa1p-Bub2p; A Cdc42p effector and an inhibitor of Bub2: Gic1p; The anaphase promoting complex ubiquitin ligase: Cdc20p; The cyclins: Clb1 and Clb2; The cyclin-dependent kinase: Cdc28p; The regulatory subunit for Cdc28p: Cks1; A signal transduction pathway: PKA; PHG = pseudohyphal growth; .arrows indicate activation ; dashed arrow indicates regulation; bars indicate inhibition; dashes indicate direct protein interactions, * = protein complex.

adherence, and cell elongation. Therefore it would be possible for HtpB to induce yeast cell elongation through activation of the cAMP/TPK pathway (by effecting a delay in mitotic exit) or through activation of the Ste/MAPK pathway (by inhibiting the mitotic cyclins Clb1,2, thereby activating the G1 cyclins Cln1,2, in a fashion similar to Swe1p) (Fig. 47).

2.6. Effects of *S. cerevisiae* strain background on pseudohyphal growth.

Not all *S. cerevisiae* laboratory strains have the capacity to grow in a pseudohyphal manner in response to nitrogen starvation. Two laboratory strains, S288C and W303-1b, in particular, have been shown to lack the capacity to form filaments, and to invade media, in response to nitrogen starvation (Liu, Styles, and Fink, 1996). The S288C strain carries a *flo8* nonsense mutation that contributes to its inability to form pseudohyphae, in response to nitrogen limitation (Liu, Styles, and Fink, 1996). While for W303 complementation with *FLO8* could not restore the ability to form pseudohyphae, on nitrogen-limited medium (Liu, Styles, and Fink, 1996), indicating that there exists other mutation(s) that prevent the W303-1b from undergoing pseudohyphal growth when subjected to nitrogen starvation.

There also exits yeast strains that are hyperactive for filamentation in *S. cerevisiae*, and one example is the Σ1278b strain, which unlike W303-1b, is well known for its vigorous filamentation and invasion (Stanhill, Schick, and Engelberg, 1999). The replacement of the wild-type *RAS2* allele for a constitutive active allele encoding

$Ras^{Val19}p$ does not enhance invasive growth in the Σ1278b background, but greatly enhances invasive growth in the SP1 background, which is less pronounced for invasion (Stanhill, Schick, and Engelberg, 1999). These data indicate that the Σ1278b strain is hyperactive for filamentation and invasive growth. It is evident that different *S. cerevisiae* strains due to various genetic mutations have different capacities to form invasive pseudohyphae, hence it is of importance to consider strain background when interpreting data.

The capacity to form filaments is linked to the ability to flocculate, in that the activation of Flo8p results in the expression of both *FLO1* and *FLO11*, which are lectins that mediate cell-cell adhesion (Kobayashi, Yoshimoto, and Sone, 1999). Excessive flocculation can result in the clumping of cells in media; therefore, it is proposed that S288C and W303 became preferred laboratory strains, due to their lost capacity to flocculate, a characteristic that would allow for easy cell segregation (Liu, Styles, and Fink, 1996). Consequently, these strains lack the capacity to form pseudohyphae, in response to nitrogen starvation (Liu, Styles, and Fink, 1996).

Cell-cell adhesion and invasive ability are essential for the process of pseudohyphal growth, and is primarily mediated by Flo11p (Lo and Dranginis, 1996; Lo and Dranginis, 1998). Flo11p is a lectin-like surface protein that attaches to sugars that form part of glycoconjugates on the surface of yeast cells (Kobayashi, Yoshimoto, and Sone, 1999). Sugar-lectin binding is balanced with non-specific electrostatic interactions that repel the association of yeast cells; the result is the formation of a web-like structure

that resists sedimentation in broth (Kihn, Masy, and Mestdagh, 1988), a process referred to as flocculation. While Flo11p is required for the invasive growth phenotype, associated with pseudohyphal growth, it is not required for other pseudohyphal phenotypes, since yeast cells bearing a *flo11* deletion are still elongated in shape, bud in a unipolar fashion and display G2/M delay, in response to nitrogen starvation (Rua, Tobe, and Kron, 2001). Of interest is that Flo11p expression is high in diploid yeast cells when grown on nitrogen-poor medium, but low in diploid cells grown on rich medium (Lo and Dranginis, 1998). Conversely, Flo11p expression is high in haploid cells grown on rich medium, but low in haploid cells grown on nitrogen-poor medium (Lo and Dranginis, 1998). The differential expression of Flo11p by haploid and diploid cells when grown on different media may explain why it is that haploids preferentially invade rich medium, while diploids invade nitrogen poor medium (Lo and Dranginis, 1998).

In this study HtpB expression induced pseudohyphal growth in the haploid cells of the S288C and W303 strain backgrounds (Fig. 14 and Fig. 29), but did not induce pseudohyphal growth in the diploid strain MLD158 of the 21R strain background (Fig.15). Since HtpB was not expressed in a haploid cells of the 21R strain background it is not possible to determine whether the inability for HtpB to induce pseudohyphal growth in this strain was dependent on differences in gene regulation in haploid cells and diploid cells or due to strain background differences. In diploid yeast cells grown in rich medium (with sufficient nitrogen), the mating-type repressor **a**1/α2 has the capacity to suppress the expression of *FLO11*, and the related gene *STA1*; which does not occur in

haploid cells grown on the same medium (Lo and Dranginis, 1996; Dranginis, 1989). when cells were grown on medium containing sufficient nitrogen.

Since HtpB-induced pseudohyphal growth occurs on rich medium, it is plausible that this repressive effect of **a**1/α2 could have contributed to the inability for HtpB to induce agar invasion (which is dependent on Flo11p) in the diploid 21R cells (Lo and Dranginis, 1996). In the case of *STA1*, repression mediated by **a**1/α2 caused a reduction in *STA1* mRNA levels, indicating that the repressor may affect gene transcription. If this is also true for *FLO11* then it is possible that **a**1/α2 repression acts at a level high up in the signaling hierarchy to not only reduce *FLO11* gene transcription, but also to prevent yeast cell elongation by downregulating the cAMP/PKA and the Ste/MAPK pathways. This could explain why HtpB-induced cell elongation was repressed in diploid yeast strain MLD158.

Part 3. Is a delay in mitotic exit the cause for HtpB-induced cell elongation?

Qualitatively, pseudohyphal growth can be described as the result of actively growing cells that extend away from their parent cells, but remain attached to their parent cells. This is unlike vegetative cells that undergo cell separation. One would speculate that events which perturb the completion of mitosis and cell division may result in yeast filamentation, a phenotype of pseudohyphal growth. Insults to eukaryotic cells that result in mitotic spindle disorientation or the misalignment of chromosomes along the metaphase plate can prevent the completion of mitosis (Gorbsky, 1997). The cell cycle is

regulated by checkpoints such as the mitotic spindle checkpoint that prevent untimely cell segregation when cells are unable to accurately segregate chromosomes between mother and daughter cells (Elledge, 1996). When HtpB was used as bait in a two-hybrid assay to screen a HeLa cell cDNA library, one of the interacting partners identified was the Merlin Associated Protein (Map), which is a homologue of the yeast protein Bub2p (for budding uninhibited by benzimidazole) (Fig. 40). Bub2p and Bfa1 form a GTPase complex that inhibits the activity of Tem1p, a small GTP-binding protein that promotes the exit from mitosis, mediated through the degradation of the mitotic cyclins, Clb1,2 (Fig. 48, Pereira *et al.*, 2000 and Geymonat *et al.*, 2002; Visintin, Hwang, and Amon, 1999; Shou *et al.*, 1999). It is possible that HtpB may interact with Bub2p in *S. cerevisiae* to promote a delay in mitotic exit, and to prevent cytokinesis, since Bub2 activity is expected to cause prolonged Clb1,2 activity as a result of Tem1p inactivation (Pereira *et al.*, 2000; Geymonat *et al.*, 2002; Visintin, Hwang, and Amon, 1999; Shou *et al.*, 1999).

However it must be noted that for pseudohyphal growth to occur, cell growth must be active. This could explain why Ras2p is essential for HtpB-induced pseudohyphal growth (Fig. 24 and Fig. 48). Ras2p has been shown to play a major role in mediating mitosis. The deletion of genes encoding the three *S. cerevisiae* Ras homologues, Ras2p, Ras1p and Rsr1p results in yeast cell death, while single deletions in each *RAS* gene yields viable cells, indicating that there is redundancy in the regulation of cell cycle, as would be expected for such an essential event of life (Morishita *et al.*, 1995). Of interest is that cell death in the triple *RAS* knockout was suppressed by the

hyper-expression of *CDC5* and *TEM1*, which indicates that the Ras homologues are essential for the exit from mitosis (Morishita *et al.*, 1995), since Cdc5p and Tem1p are essential mediators of mitotic exit (Molk *et al.*, 2004; Hu *et al.*, 2001). Ras2p and Rsr1 can activate Cdc42p, which is an essential regulator of bud formation and the exit from mitosis (Mosch, Roberts, and Fink, 1996; Kawasaki *et al.*, 2003). Of particular interest is that Cdc42p activates Gic1,2 and Cla4p. Gic1p can bind directly to Bub2, thereby preventing the association of Bub2 with Tem1p, thus facilitating progression through mitosis (Hofken and Schiebel, 2004). Cla4p also functions with Ras2p to promote mitotic exit; by localizing Lte1 (the Tem1p activating GEF) to the bud cortex, its site of action (Seshan and Amon, 2005; Yoshida, Ichihashi, and Toh-e A, 2003). Taken together these data indicate that Ras2p-mediated activation of Cdc42p could antagonize a putative HtpB-mediated block in mitotic exit. However experiments performed to elucidate the role of Cdc42p in HtpB-induced pseudohyphal growth was inconclusive (Table 9). However, it is logical to expect that Ras-dependent activation of Cdc42p would promote the completion of mitosis, since the objective of cell cycle is ultimately cell multiplication. As such one would imagine that the spindle checkpoint must be kept in a dormant state during normal cell cycle events, and that perturbations that act to reduce the level or activity of inhibitory factors for the Bfa1-Bub2 complex could activate the checkpoint. Indeed multiple proteins are involved in suppressing Bfa-1-Bub2 activity including Cdc5 and Gic1 (Hu *et al.*, 2001; Geymonat *et al.*, 2003; Hofken and Schiebel, 2004). Perhaps HtpB expression promotes the activation of the spindle checkpoint

pathway via Bfa1-Bub2 to tilt the balance in favor of a stall in the process of mitotic exit. Perhaps HtpB acts to initiate the formation of a protein complex that mediates a stall in mitotic exit. Activated Bub2p would then have the ability to override mitosis-promoting factors.

Part 4. Possible roles for Map in *L. pneumophila* pathogenesis.

When *L. pneumophila* infects host cells it perturbs normal phagosome maturation events, such that *L. pneumophila* containing phagosomes do not fuse with lysosomes at least during early infection (up to 8 hours), after which time the *L. pneumophila* phagosome acidifies (Sturgill-Koszycki and Swanson, 2000). In contrast, phagosomes containing avirulent *L. pneumophila dotA* mutants acidify within 5 minutes post infection (Roy, Berger, and Isberg, 1998). Of interest is that during the intracellular phase of its lifecycle, replicative *L. pneumophila* differentiates into a more resilient form that may be more fit to tolerate the acidic environment of phagolysosomes (Garduno *et al.*, 2002). In order to disrupt the normal maturation of endosomes *L. pneumophila* may perturb cellular cytoskeletal elements or their regulatory proteins. *L. pneumophila* releases a large amount of HtpB into its replicative endosomes, and also expresses HtpB on its cell surface (Garduno, Garduno, and Hoffman, 1998; Garduno *et al.*, 1998). To lead an investigation into a possible role of HtpB in the infection of HeLa cells by *L. pneumophila*, a HeLa-cell cDNA library was screened with HtpB as bait to identify molecules that interact with HtpB. Of interest is that HtpB was found to interact with the

merlin-associated protein (Map), which is a protein that contains conserved Rab-GAP domains and a Src-homology 3 protein interaction domain (SH3 domain) (Lee *et al.*, 2004). Map interacts with Merlin, a tumor suppressor encoded by the Neurofibromatosis 2 (NF2) gene (Rouleau *et al.*, 1993). Merlin is closely related to the proteins ezrin, radixin and moesin (ERM), which are active actin reorganizers that link the cell membrane to the cytoskeleton (Takeuchi *et al.*, 1994). Merlin is unique in that it associates with motile regions of eukaryotic cells, at the leading edge of membrane ruffles (Gonzalez-Agosti *et al.*, 1996). Interesting is that *Salmonella spp.* cause membrane ruffling during pathogen-directed entry into its host cell (Zhou and Galan, 2001), indicating that processes involved in membrane ruffling may be key targets for pathogen control. Furthermore, merlin and moesin were found to inhibit N-WASP-mediated actin assembly in vitro (Manchanda *et al.*, 2005). Cdc42p is, in contrast, an activator of N-WASP-mediated actin assembly, and could possibly function as an antagonist to putative HtpB-induced merlin activation. This would support the hypothesis that in the yeast model HtpB and Cdc42p induce antagonistic effects on the progression through cell cycle, such that HtpB induces a stall in mitotic exit, while Cdc42p promotes the completion of mitosis. Cdc42p is a multipotent effector in *S. cerevisiae* and in mammalian cells. Aside from its involvement in N-WASP-mediated actin polymerization it is also involved in COP1-mediated vesicle transport and clathrin-mediated endocytosis (Erickson and Cerione, 2001). This is of interest since it was demonstrated that a dominant negative allele of the ARF GTPase (T31N), which

regulates CopI-mediated vesicle transport from the Golgi apparatus to the ER, delays the recruitment of the v-SNARE, Sec22b, to the surface of the *L. pneumophila* replicative phagosome, which reduces *L. pneumophila*'s capacity to replicate intracellularly (Kagan *et al.*, 2004).

In addition, merlin activity is influenced by the cAMP/PKA pathway in mammalian cells, which also parallels the role of cAMP/PKA in HtpB-induced pseudohyphae formation in *S. cerevisiae*. Furthermore, merlin is reported to act as an inhibitor of Ras-mediated activities (Tikoo *et al.*, 1994) including actin foci formation (Kim *et al.*, 2002) and Ras-induced ERK signaling (Lim *et al.*, 2003). This is particularly relevant because HtpB expression perturbed Ras2p activity in the yeast model. These conjectures are supported by studies performed by Audrey Chong, a graduate student with Dr. Garduno, who has demonstrated that the expression of HtpB in mammalian cells, the Chinese Hamster ovary cell-line, results in the disappearance of actin stress fibers (pers. comm. Dr. R. Garduno). Other studies indicate that *L. pneumophila* entry into host cells is actin mediated (Garduno, Quinn, and Hoffman, 1998; Goldoni *et al.*, 1998; Lu and Clarke, 2005). Therefore it seems reasonable to speculate that HtpB could interact with regulatory proteins of the actin cytoskeleton to induce aberrant regulation in actin assembly, and the generation of L. *pneumophila* replicative vacuoles.

In order to test the involvement of HtpB in mediating actin disruption via an interaction with the Map and/or merlin, I have suggested a few approaches. First, co-precipitation experiments, using cell lysates from HeLa cells expressing HtpB, should be

done to confirm by biochemical means the interaction of MAPand/or merlin with HtpB. Second, co-localization studies in uninfected HeLa cells and HeLa cells infected with *L. pneumophila* should be conducted to determine whether these interactions occur during infection. Third, dominant negative alleles for the genes encoding Map or its downstream effectors could be expressed in HeLa cells and then tested for their capacity to perturb *L. pneumophila* infection. If mutant versions of these proteins are found to perturb *L. pneumophila* intracellular replication, this would support the hypothesis that *L. pneumophila* uses HtpB to facilitate its intracellular lifestyle.

Part 5. Hsp60 Secretion Mechanisms.

In some bacteria Hsp60 homologues are transported to the cell envelope (inner membrane, periplasm, and/or outer membrane) (see Table 1), such as in *L. pneumophila* (Garduno *et al.*, 1998), *Brucella abortus* (Watarai *et al.*, 2003) and *Bordetella spp.* (Fig. 44 and Fig. 45), while in other cases such as in *E. coli* DH5α (Garduno *et al.*, 1998) and *Enteropathogenic E. coli* (EPEC) (Fig. 42 and Allan D.S., 2002) the protein is restricted to the cytoplasm. The mechanism(s) for the surface localization of Hsp60 homologues in bacteria is not fully understood. In Gram-negative bacteria there exit seven known mechanisms for protein secretion including type I, type II, type III, type IV, type V, the general secretion pathway, and Tat secretion (see introduction for a brief review of each). Since Hsp60 homologues share great sequence conservation, we anticipated that investigating Hsp60 secretion in different bacteria, for which secretion system deficient

mutants were available, would illuminate a generic mechanism for Hsp60 secretion. A discussion of putative mechanisms for Hsp60 secretion in bacteria with emphasis on *L. pneumophila* is discussed below in context of the properties required for protein mobilization by each secretion system.

5.1. Type I secretion.

Type I or ATP-binding cassette (ABC) transporters are ubiquitous, for example in *E. coli* at least 80 ABC transporters can be found (Andersen, 2003). Common defining features of this secretion system include that (i) substrates require a specific C-terminal motif for transport (Binet *et al.*, 1997), (ii) substrates are transported across the entire bacteria cell envelope with no periplasmic intermediates (Thanabalu *et al.*, 1998), and (iii) the system is usually comprised of three components, an ATPase that supplies the energy for the process, and two structural proteins that form the translocation component (Andersen, 2003). Although genes encoding a putative type I secretion system have been identified in *L. pneumophila* (Jacobi and Heuner, 2003), it is unlikely that such a system would be involved in transporting HtpB to the *L. pneumophila* cell envelope. HtpB lacks the .characteristic C-terminal motif required for transport (Hoffman, Houston, and Butler, 1990). In addition the HtpB protein can be found in the *L. pneumophila* periplasm as determined by immunogold electron microscopy (Garduno *et al.*, 1998; Allan D.S., 2002), indicating that it may be transported across the bacterial cell envelope in a two-

step process involving a periplasmic intermediate, whereas type I secretion transports protein in a one-step process with no periplasmic intermediates.

5.2. General secretion pathway.

The general secretion pathway (GSP) is widely used by Gram-negative bacteria for the secretion of molecules such as extracellular toxins, adhesins, invasins and proteases (Stathopoulos *et al.*, 2000). Defining features of the GSP include that (i) proteins are transported as semi-folded intermediates across the bacterial inner membrane, and into the periplasm, where they can then be mobilized by another system such as the main terminal branch of the GSP (type II secretion), by the autotransporter mechanism (type V secretion) or by type IV secretion across the outer membrane (Sandkvist, 2001), and (ii) substrates contain a characteristic N-terminal domain that is necessary for translocation (Stathopoulos *et al.*, 2000). *L. pneumophila* has almost the full complement of genes that encodes the GSP, with the exception of SecE, which forms a part of the three component translocase through which proteins are transported across the inner membrane (Lammertyn and Anne, 2004). Whether or not these genes represent a functional secretion system is not known. Based on analysis of the N-terminal region of HtpB, the protein appears to lack an N-terminal sequence that would allow transport by the GSP (Hoffman, Butler, and Quinn, 1989). This is in contrast to eukaryotic Hsp60s, which have N-terminal signal sequences that mediate transport into the mitochondria (Fig. 15) and (Singh *et al.*, 1990; Itoh *et al.*, 1995). In *E. coli*, GroEL functions in conjunction

with SecB, the GSP chaperone, to deliver proteins from the *E. coli* cytosol to the SecYEG translocase in the inner membrane (Danese, Murphy, and Silhavy, 1995; Muller *et al.*, 2001). GroEL has been reported to chaperone the assembly of its own monomers during the formation of the GroEL ring complex (Kusmierczyk and Martin, 2001); therefore, a mechanism could exist whereby the GroEL chaperonin complex transfers semi-folded GroEL monomers to the SecB chaperone for GSP transport. However, it should be noted that when HtpB was expressed from plasmid pSH16 in the *E. coli* cytoplasm, HtpB was restricted to the cytoplasm as is the endogenous GroEL protein (Garduno *et al.*, 1998), despite the fact that *E. coli* has a GSP system (Bieker and Silhavy, 1990). Hence it is unlikely that HtpB in *L. pneumophila* is transported by the GSP; however, it cannot be conclusively ruled out without experimental testing.

5.3. Type II secretion.

Type II secretion in Gram-negative bacteria is commonly referred to as the main terminal branch of the GSP, since substrates that are released by the GSP into the periplasm are often substrates for type II secretion across the outer membrane and into the extracellular environment (Sandkvist, 2001). Defining features of type II secretion include that (i) it is comprised of approximately 12 proteins that transport substrates using a piston-triggered mechanism through a pore in the outer membrane, (ii) it transports proteins that have matured through oligomerization or posttranslational modifications(mediated by oxidoreductases and disulfide bond isomerases), (iii) no common signal sequence has

been identified among known substrates, although it is speculated that a three-dimensional recognition domain in the folded protein may be the signal for transport (Izard and Kendall, 1994), and (iv) some of its components share homology with proteins that are involved in type IV pilus biogenesis (Nunn, 1999).

Since this secretion system has the capacity to transport protein complexes present in the periplasm across the outer membrane, it was an interesting candidate to test for its involvement in HtpB secretion, since chaperonins form tetradecameric, double-ringed functional structures. In *L. pneumophila* there exit proteins that are involved in type II secretion and/or type IV pilus biogenesis including homologues to the PilD peptidase, the PilB nucleotide binding protein, the PilC transmembrane protein, the $PilE_L$ type IV pilin subunit, the LspD ATPase, the LspE secretin that forms a pore in the bacterial outer membrane, and the LspF-K pseudopilins (Hales and Shuman, 1999; Liles, Viswanathan, and Cianciotto, 1998). However, by immunogold electron microscopy and trypsin degradation assay it was determined that the association of HtpB with the bacterial periplasm and outer membrane in two type II secretion mutants, PilD- (peptidase deficient), LspDE- (ATPase deficient and secretin deficient) was not inhibited as determined by comparison to the parent strain, 130b (pers. comm. Dr. R. Garduno). In fact the type II secretion mutants were found to have more surface-exposed HtpB as compared to the parent strain, as indicated by the amount of surface associated HtpB available for trypsin degradation (pers. comm. Dr. R. Garduno). These data indicate that

type II secretion is probably not required for the association of HtpB with the *L. pneumophila* outer membrane.

5.4. Type III secretion.

Type III secretion systems are well known for their capacity to transport bacterial virulence factors from inside the bacterial cell, across the bacterial cell envelope, across the host cell membrane, directly into the host cell (Cornelis, 2000). Type III secretion is activated upon host cell contact, and occurs in a one-step process (Cornelis and Van Gijsegem, 2000). Since *L. pneumophila* does not have genes encoding a type III secretion system in its genome (Cazalet *et al.*, 2004; Chien *et al.*, 2004), this system is not an option for HtpB transport. Furthermore, HtpB translocation involves an intermediate periplasmic stage, which would not be possible if it were transported by a type III secretion mechanism.

The role of type III secretion in the membrane association of Cpn60 (the *Bordetella spp.* Hsp60 homologue) in *Bordetella bronchiseptica* was investigated, since a type III defective mutant (RB50) that harbored a disruption in the *bscN* gene, which encodes an essential energy supply protein for type III secretion was available (Yuk, Harvill, and Miller, 1998). As determined by immunogold electron microscopy, Cpn60 association with the bacterial cell envelope in RB50 was not affected by the *bscN* disruption, such that immunogold labeling of Cpn60 in RB50 was indistinguishable from labeling in the wild-type parent strain, WD3 (Fig. 44). Therefore, type III secretion is not

required for the association of Cpn60 with the *B. bronchiseptica* cell membrane.

Enteropathogenic *E. coli* (EPEC) and other enteric pathogens have type III secretion systems that are involved in transporting virulence factors from the bacterial cytosol directly into the cytosol of host cells (Zaharik *et al.*, 2002). Since EPEC has this secretion system that is not present in *E. coli* DH5α (previously shown to restrict GroEL and HtpB to the cytoplasm(Garduno *et al.*, 1998), the capacity for EPEC to mobilize GroEL and HtpB to the bacterial cell envelope was explored. When EPEC expressing HtpB from plasmid psh16 was exposed to heat shock (to upregulate GroEL expression), and was placed in contact with HeLa cells (to activate type III secretion) (Fig.42 and Allan D.S., 2002), GroEL and HtpB remained restricted to the cytoplasm, indicating that the type III secretion mechanism in EPEC does not mobilize GroEL or HtpB to the bacterial cell envelope.

5.5. Type IV secretion.

5.5.1. General information.

Type IV secretion systems are defined by their relatedness to DNA conjugation systems, and have not only the capacity to conjugate DNA, but are also able to transport proteins even as macromolecular complexes across the bacterial outer membrane (Ding, Atmakuri, and Christie, 2003; Sagulenko *et al.*, 2001; Lammertyn and Anne, 2004; Pizza *et al.*, 1990; Nencioni *et al.*, 1991). Type IV secretion systems are classified into two

groups, type IVA and type IVB depending on whether they are most closely related to the *Agrobacterium tumefaciens* VirB system (required for T-DNA transfer) (see Fig. 6) or the DNA transfer (*tra*) locus found in the IncI plasmid family (required for plasmid conjugation between bacterial cells) (see Fig. 7 and Ding, Atmakuri, and Christie, 2003). Type IV secretion systems can have different mechanisms for protein translocation. In *A. tumefaciens*, pathogenic DNA and accessory proteins are transferred in a one-step process from inside the bacterial cytosol to inside the host cell (Christie, 1997). In contrast, in *B. pertussis*, pertussis toxin (ptx) is transported by the Ptl system (which shares homology with the *A. tumefaciens* VirB system, see Figure 6) from the bacterial periplasm to the extracellular environment as a part of a two-step process, in concert with the GSP (Christie, 2001). Therefore the type IV secretion systems are a diverse group, which display unique characteristics.

5.5.2. *L. pneumophila.*

In *L. pneumophila* there exist two functional type IV secretion systems, the type IVA Lvh system, and the type IVB Dot/Icm system (Segal, Russo, and Shuman, 1999; Berger and Isberg, 1993; Brand, Sadosky, and Shuman, 1994). Both systems have the capacity to conjugate plasmid DNA. Deletion of the entire *lvh* locus (*lvh*), results in a 10-fold reduction in the capacity of *L. pneumophila* to conjugate DNA (but did not abolish it), indicative of the existence of redundancy in the system (Segal, Russo, and Shuman, 1999). In an *icmE-lvh* mutli-locus mutant, the capacity to conjugate DNA was

completely abolished, indicating that the Dot/Icm and Lvh type IV secretion systems, are redundant systems for DNA conjugation (Segal, Russo, and Shuman, 1999). Of interest is that, in a *dotB-lvh* muti-locus mutant, the capacity to conjugate DNA was dramatically reduced, but not abolished (Segal, Russo, and Shuman, 1999), indicative of redundancy for DotB function. Indeed there exist at least three proteins (one encoded within the lvh locus) that could account for such redundancy (see below for details)(Segal, Russo, and Shuman, 1999). It is important to note that the Dot/Icm and Lvh systems have unique function as well, since the Dot/Icm system is essential for *L. pneumophila* intracellular replication, while the Lvh system is not (Segal, Russo, and Shuman, 1999).

In *L. pneumophila*, the Dot/Icm system has the capacity to mobilize plasmid DNA (Segal, Purcell, and Shuman, 1998) and proteins including, DotA (Nagai and Roy, 2001), RalF (Nagai *et al.*, 2002), LidA for lowered viability in the presence of dot (Conover *et al.*, 2003), and Sid for substrate of Icm/Dot (Luo and Isberg, 2004) proteins. These proteins with the exception of DotA (which is essential for Dot/Icm system function) are not alone essential to the process of *L. pneumophila* intracellular replication. However, RalF and LidA have the capacity to alter the process of eukaryotic vesicle transport (Nagai *et al.*, 2002; Derre and Isberg, 2005), which may contribute to the process of infection. In contrast, a functional Dot/Icm system is essential for the process of intracellular replication, indicating that the system itself may promote intracellular replication (perhaps DotA and or other Dot/Icm proteins have a direct role in the process

of intracellular replication), there exits functional redundancy among Dot/Icm substrates, or an as yet unidentified substrate is responsible for mediating intracellular replication.

HtpB is a surface-localized virulence factor in *L. pneumophila* that mediates the attachment to and the invasion of host cells (Garduno, Garduno, and Hoffman, 1998), and the Dot/Icm system is a protein secretion system that is required especially during the early phases of infection for successful intracellular replication by *L. pneumophila* (Roy, Berger, and Isberg, 1998). Therefore it was of interest to investigate whether the Dot/Icm system is involved in mediating the association of HtpB with the bacterial cell membrane. By immunogold electron microscopy of *L. pneumophila* thin sections, probed with an anti-HtpB polyclonal antibody (HtpB Pab), it was determined that in a *dotA*$^-$ mutant there was an accumulation of HtpB in the cytoplasm as compared to the wild-type parent strain Lp02 (3% more, expressed as the difference between of the total % of gold particles counted in the cytoplasm for *dotA*$^-$ and Lp02, which was increased to 18% when the system was overloaded with HtpB, as expressed from plasmid pTrcKm), and a reduced amount of HtpB found on the outer membrane (18% less, which slightly increased to 20% with HtpB overexpression) (pers. commun. Dr. R. Garduno and Allan D.S., 2002). In a *dotB*$^-$ mutant an accumulation of HtpB was only seen in the cytoplasm when HtpB was overexpressed in *L. pneumophila* from plasmid pTrcKm (8% more as compared to wild-type Lp02), and a reduced amount of HtpB was present on the outer membrane of the *dotB*$^-$ mutant as compared to Lp02 (2% less, which increased to 8% when HtpB was over-expressed) (pers. commun. Dr. R. Garduno and Allan D.S., 2002). The reduced

levels of HtpB present on the outer membrane of *L. pneumophila dotA*$^-$ and *dotB*$^-$ mutants were confirmed by trypsin digestion assays, which demonstrated that there was less HtpB accessible to trypsin on the cell surfaces of these mutants, and also a *dotG*$^-$ mutant, as compared to wild-type Lp02 cells (pers. commun. Dr. R. Garduno). These data indicate that the Dot/Icm system is involved in the membrane-localization of HtpB in *L. pneumophila*, but that components of another secretion mechanism may also be involved in HtpB transport, since HtpB association with the bacterial cell envelope was not abolished in the *dotA*$^-$ or *dotB*$^-$ mutants.

Since HtpB localization to the outer membrane was not completely abolished in the Dot/Icm mutants, it was of interest to examine the role of the Lvh system in mobilizing HtpB to the bacterial outer membrane. Based on trypsin digestion assays, it was determined that in an *lvh*$^-$ mutant there was no difference in the amount of surface-associated HtpB accessible to trypsin, as compared to wild-type cells (pers. commun. Dr. R. Garduno). This data indicates that the Lvh system is not necessary for the association of HtpB with the outer membrane. However, since it is reported that *lvhB10* (see Fig.6) shares homology with *dotB*$^-$ (Segal, Russo, and Shuman, 1999), it is possible that under conditions where *dotB* is deleted, the *lvhB10* gene product could support Dot/Icm function (Segal, Russo, and Shuman, 1999). In addition possibly two other proteins in the *L. pneumophila* proteome are ATPases (like DotB) that may also serve as functional replacements including PilB (the ATPase for pilus biogenesis) (Liles, Viswanathan, and Cianciotto, 1998) and LspD (the ATPase for type II secretion) (Rossier and Cianciotto,

2001). Since HtpB surface association is most greatly impaired in a *dotA*⁻ mutant, and there exits much functional redundancy for the *dotB* gene, it would be ideal to assess the distribution of HtpB in a multi-locus *dotA*⁻*dotB*⁻*pilB*⁻*LspD*⁻ *lvh*⁻ mutant, and to also overexpress HtpB in this mutant, since differences in HtpB association with the bacterial cell membrane was most apparent (in *dotA*⁻ and *dotB*⁻ mutants) when HtpB was overexpressed from plasmid pTrcKm (Allan D.S., 2002). It is apparent that secretion systems are very complex and diverse in terms of their cooperativity and mechanisms of action respectively, which can make studying their biology cumbersome. Perhaps, once key secretion system components necessary for HtpB surface localization have been identified, the genes encoding these components could be expressed in *E. coli* (previously shown to restrict GroEL and HtpB to the cytoplasm) to conclusively determine whether they are sufficient to mobilize HtpB and GroEL to the bacterial cell surface.

5.5.3. *Bordetella pertussis.*

In this study the Hsp60 homologue, Cpn60, was localized by immunogold electron microscopy in *B. pertussis*, since a mutant defective for a functional type IVA secretion system, called the pertussis toxin liberation (Ptl) system that is required to transport the pertussis toxin across the *B. pertussis* bacterial outer membrane, was available (Weiss, Johnson, and Burns, 1993). It was demonstrated first by immunogold electron microscopy that Cpn60 in wild-type *B. pertussis*, strain BP388, was present in association with bacterial cell envelope (Fig. 45). Then it was demonstrated by the same method that

in a Ptl-negative mutant (Weiss, Johnson, and Burns, 1993), Cpn60 was still present in association with the bacterial cell surface (Fig. 45), indicating that the Ptl system is not required for Cpn60 mobilization. Since Type IVA secretion in *B. pertussis* is regulated by a two component system composed of BvgS (a histidine kinase that serves as an environmental sensor located in the cytoplasmic membrane) and BvgA (a response regulator which activates transcription from virulence gene promoters) (Roy, Miller, and Falkow, 1989; Kotob, Hausman, and Burns, 1995), Cpn60 was also localized in a BvgS-negative mutant that was available (Weiss and Falkow, 1984). In this mutant Cpn60 was restricted to the cytoplasm (Fig.45), indicating the association of Cpn60 with the *B. pertussis* cell envelope is positively regulated by BvgS. The BvgAS system also regulates type III secretion in *B. bronchiseptica*, which is nonfunctional in typical *B. pertussis* strains, but as indicated above, type III secretion was not essential for Cpn60 localization in *B. bronchiseptica*, and is; therefore, not likely involved in BvgS-mediated Cpn60 membrane localization in *B. pertussis*. It is intriguing that, in *B. pertussis*, Cpn60 membrane localization is a controlled process that is co-regulated with virulence gene expression, and it would be interesting to express the Cpn60 in the cytoplasm of yeast to determine whether it has a physiological effect on the eukaryotic cell. Perhaps Cpn60 is a virulence factor in *B. pertussis*.

5.5.4. *Brucella abortus*.

The *B. abortus* Hsp60 homologue is a virulence factor that mediates *B. abortus*

swimming internalization into host cells (Watarai *et al.*, 2003). By immunofluorescence of whole bacterial cells, probed with an anti-Hsp60 antibody, it was determined that a functional type IVA secretion system (that shares homology with VirB system in *Agrobacterium tumefaciens*, Fig. 6) is required for Hsp60 surface localization (Watarai *et al.*, 2003). It would be informative to visualize the location of the *B. abortus* Hsp60 homologue in thin sections of *B. abortus* wild-type and *virB* mutants, by immunogold electron microscopy, to assess the distribution of the Hsp60 protein in the cytoplasm, inner membrane and periplasm. This data could illuminate whether the VirB system mediates Hsp60 localization in a one- or a two- step process. As indicated above, in *L. pneumophila* and *B. pertussis* the homologous systems, the Lvh and Ptl systems respectively (see above) were not involved in the localization of HtpB or Cpn60, respectively, to bacterial cell membranes. These data highlight that bacteria may utilize different mechanisms for Hsp60 localization to the cell envelope, and that a generic mechanism for Hsp60 mobilization to the cell surface may not exist.

5.6. TypeV secretion (autotransporters).

Autotransporters are composed of (i) an N-terminal signature sequence, (ii) a passenger domain that is either retained at the cell envelope or released, (iii) an intramolecular chaperone domain that holds the protein in an unfolded state, (iv) and a C-terminal translocation unit that forms a pore through which the protein can be transported (Oliver, Huang, and Fernandez, 2003; Oliver, Huang, and Fernandez, 2003; Desvaux, Parham,

and Henderson, 2004; Henderson, Navarro-Garcia, and Nataro, 1998). Therefore, the autotransporters have an inherent capacity to transport themselves across bacterial membranes. When recombinant HtpB was expressed from plasmid pSH16 in *E. coli*, HtpB was localized to the *E. coli* cytoplasm (Garduno *et al.*, 1998). In contrast, when GroEL was expressed in the *L. pneumophila* cytoplasm from plasmid pTrcKm, it was localized to the *L. pneumophila* cell surface (Allan D.S., 2002). Taken together these data indicate that HtpB and GroEL are transported across the *L. pneumophila* cell envelope by secretion mechanism(s) that is present in *L. pneumophila*, but absent in *E.coli*. Therefore, it is unlikely that HtpB has capacity to transport itself across biological membranes, since HtpB and GroEL remain cytoplasmic in *E. coli*. Also, in a *B. pertussis* BvgS-negative mutant, Cpn60 was localized to the cytoplasm, indicating that Cpn60 probably cannot transport itself across the cell envelope, and must be dependent on other factors for mediating its association with the *B. pertussis* cell envelope.

5.7. Tat secretion.

The Tat secretion system is unique in that it transports folded proteins across the bacterial inner membrane, as opposed to unfolded proteins as is the case for transport by the GSP (Palmer and Berks, 2003). Features that define the Tat system are that (i) substrates have a specific N-terminally located "twin-arginine motif (SRRxFLK), (ii) energy is supplied by a proton electrochemical gradient, and (iii) the system is composed of three components TatA, which forms a channel in the cell envelope, TatB, and TatC,

which are involved in substrate recognition (Berks, Palmer, and Sargent, 2003). Some organisms have an additional protein, TatE that can serve as a functional replacement for TatA (Berks, Sargent, and Palmer, 2000).

The fact that the Tat system has the capacity to transport folded proteins across the bacterial inner membrane, makes this secretion system attractive as a putative transporter for HtpB, since one would imagine that HtpB would assume its oligomeric structure in the cytoplasm, where it functions to fold proteins. Also, while no obvious Sec-dependent signal peptides are present in HtpB as determined by the analysis of HtpB's amino acid sequence (Hoffman, Butler, and Quinn, 1989), a possible twin-arginine motif may be present. The Tat recognition sequence consists of an N-terminal amino acid consensus sequence, S/T-R-R-x-F-L-K, where the two twin arginine residues are essential and the other amino acids occur at a rate of >50% (Berks, 1996). In HtpB there appears to reside a putative Tat-like signal sequence, D-R-R-K-A-M-L, which commences at position 282 (NCBI Accession No.: AAA25299). Although Tat recognition sequences have not been reported to exist away from the N-terminal region of proteins, one would imagine that since the Tat secretion system mobilizes folded proteins, it could recognize a 3-dimensional signature domain. It would seem plausible then that the Tat system could detect a non-N-terminus-located motif. As compared to the Tat consensus sequence, and Tat recognition sequences for known Tat substrates (Berks, 1996), the amino acids that make up the putative Tat recognition sequence in HtpB share similarity or similar biochemical properties (Fig. 49). Since *L. pneumophila*

Consensus		**S/T-R-R-X-F-L-K** **p p pp/hh h p**
L. pneumophila	**HtpB**	**D-R-R-K-A-M-L** **p p p p h h p**
W. succinogenes	**FdhA**	**D-R-R-K-F-L-K**
M. maze	**VhoG**	**D-R-R-T-F-M-K**
T. ferrooxidans	**Iro**	**T-R-R-D-A-L**

Figure 49. A comparison of the Tat consensus sequence with a putative Tat recognition sequence in HtpB and Tat recognition sequences from known Tat substrates. S = serine, T = threonine, R= arginine, F = phenylalanine, L = leucine, K = lysine, D = aspartic acid, A= alanine, M = methionine, p = amino acid with a polar side-chain, h = amino acid with a hydrophobic side chain. The Tat recognition sequences for the Tat substrates shown were obtained form Berks, 1996, and those that are also found in the putative Tat recognition sequence in HtpB are underlined.

has the capacity to localize GroEL to its cell surface (Allan D.S., 2002), one would expect that GroEL should also have the signal sequence required for mobilization by the putative HtpB translocation system. Indeed GroEL (NCBI Accession No.: AAC77103) has the D-R-R-K-A-M-L amino-acid motif present at position 283. The *B. pertussis* Cpn60 (NCBI Accession No.: P48210) also has the identical amino acid sequence at position 283. Although *E. coli* has a Tat system, it has been reported that Tat secretion machineries are specific with regard to the substrates they transport (Blaudeck *et al.*, 2001); therefore it is plausible that the *L. pneumophila* Tat system has the capacity to mobilize HtpB and GroEL to the *L. pneumophila* inner membrane, and for the *E. coli* Tat system to lack this capability. It would be informative to mutate the arginine residues in HtpB, Cpn60 and/or GroEL to determine whether the residues influence their association with the *L. pneumophila* cell envelope.

5.8. Summary.

It is apparent that there may not exist a generic system for Hsp60 surface localization in bacteria, since *B. abortus* uses a system different from that of *L. pneumophila* or *B. pertussis* for mobilizing its Hsp60 homologue to the bacterial cell envelope. Therefore, Hsp60 secretion will have to be considered on a case by case basis. In *L. pneumophila* it appears that the Dot/Icm system is involved in localizing HtpB to the bacterial cell membrane, however, the existence of functional homologues for Dot/Icm secretion system components in *L. pneumophila* complicates the conclusive identification of the

predominant secretion mechanism for HtpB. Perhaps the use of multi-locus mutants (*dotA⁻dotB-lspD⁻pilB⁻lvh⁻*) combined with strategies that involve the identification of putative secretion-promoting domains in HtpB such as the potential twin arginine motif will lead to the more conclusive identification of a secretion mechanism for HtpB. In parallel genes deemed necessary for HtpB secretion in *L. pneumophila*, such as those encoding the Dot/Icm system, could be introduced into *E.* coli (previously shown to restrict HtpB to the cytoplasm), to determine whether they are sufficient to mobilize HtpB to the *E. coli* cell membrane. Alternatively, a high-throughput assay could be developed to detect HtpB on the *L. pneumophila* cell surface, which would facilitate the screening of transposon mutants to identify mutants that are not able to transport HtpB to the *L. pneumophila* cell surface. For example an ELISA assay could be developed using anti-HtpB polyclonal antibodies to test for HtpB surface localization in the transposon mutants. Since a *B. pertussis* mutant strain, BvgS-negative, expresses Cpn60 in the cytoplasm it could be used as negative control. A similar approach could be used to identify critical gene products that are necessary for Cpn60 cell-membrane association in *B. pertussis*.

Part 6. An HtpB Purification Strategy.

The bacterial chaperonins, Cpn60 and HtpB, Hsp60 homologues from *B. pertussis* and *L. pneumophila* respectively, were purified as recombinant proteins, expressed from plasmids in *E. coli*. The method of purification used in this study involved the use of

anion exchange chromatography followed by gel filtration chromatography, which allow for the separation proteins based on their differences in charge and size respectively. Buffer conditions and the apparatus for purifying the *B. pertussis* chaperonin, Cpn60, and its cofactor Cpn10 were developed in this study. However, an assay for the biological activity of the chaperonins purified by this method was not further pursued as a part of this work, since another graduate student, Vanessa Landry, undertook the project. An assay that could measure the capacity of the purified chaperonins to hydrolyze ATP such as the EnzChk® phosphatase assay kit (Invitrogen Canada Inc., Burlington ON) could be considered an option for testing the quality of the chaperonins purified by this method.

The purification method utilized in this study as outlined in the Material and Methods section represents a starting point for the development of a purification procedure for the bacterial chaperonins. Due to the nature of chaperonins, their tendency to oligomerize, and to associate with other proteins, it is recommended that a denaturation step be added to the purification procedure followed by gel filtration, so that the chaperonins may dissociate from other protein contaminants (Kusmierczyk and Martin, 2001). The GroEL chaperonin when denatured in 4M urea can spontaneously oligomerize to form functional complexes in the presence of 20 % sucrose (Kusmierczyk and Martin, 2001). Denaturation in 8M urea results in the production of monomers that can be stimulated to oligomerize in the presence of magnesium ions and ATP (Kusmierczyk and Martin, 2001), indicating that this could prove a viable method for purifying chaperonin monomers that can be used for downstream assays.

Part 7. HtpB Expression and Organelle Association.

Since *L. pneumophila* can delay the fusion of its replicative endosome with host lysosomes for up to 8 hours post infection (Sturgill-Koszycki and Swanson, 2000), it was of interest to examine whether HtpB expressed in yeast could alter the organization of lysosomes within the yeast cell. Yeast cells expressing HtpB, as well as, cells expressing no protein were stained with a lysosome-specific dye (LysoTracker™, Invitrogen Canada, Burlington, ON). In wild-type yeast cells expressing no protein, stained structures appeared as punctuate dots distributed around the yeast cell circumference in both budded and unbudded cells (Fig. 13A and 13B). In yeast cells expressing HtpB, staining appeared concentrated in the center of budded and unbudded cells (Fig.13C and 13D). Therefore, HtpB expression does appear to influence the distribution and integrity of lysosome structures in *S. cerevisiae*. It would be interesting to test whether this finding is relevant to the infection of HeLa cells by *L. pneumophila*, since a delay in phagosome-lysosome fusion is essential for the process of *L. pneumophila* infection (Sturgill-Koszycki and Swanson, 2000). As a method to assess whether lysosome-phagosome fusion is affected by HtpB expression, a mammalian cell-line expressing HtpB in its cytoplasm could be infected with a virus that requires endosome-lysosome fusion for the infection process. Plaque assays could then be utilized as a quantitative means to determine whether HtpB expression affects virus replication, which would be an indication of whether phagosome-lysosome fusion is perturbed.

In a two-hybrid assay, the HeLa Ribosomal protein L4, a component of the 60s

ribosome subunit (Uechi, Tanaka, and Kenmochi, 2001) was identified as a putative interacting protein for HtpB. This is interesting because 2-4 hours post-infection of a host cell, *L. pneumophila* is found within a ribosome-studded vacuole (Horwitz, 1983; Sturgill-Koszycki and Swanson, 2000). It is suggested that a part of *L. pneumophila*'s strategy to escape degradation by host lysosomes is to escape the endocytic pathway and to instead reside in the exocytic pathway in association with ER-derived vesicles (Kagan and Roy, 2002). Whether or not *L. pneumophila*'s association with the ER is a strategy to acquire nutrients in the form of peptides synthesized on ribosomes in the ER or is simply a result of its escape from intracellular digestion has not been determined. It will be interesting to determine whether this putative association between RPL4 plays a role in *L. pneumophila* infection of mammalian cells.

8.0. Functional Evolution of Hsp60 homologues.

8.1. General information.

Hsp60 homologues, also known as chaperonins, are primarily known for their role in assisting peptides to assume a functional conformation (Lund, 1995). In this capacity the chaperonins function as multi-subunit, double-ringed complexes that form a barrel-like structure with a central cavity that provides an environment, where peptides may fold into functional proteins; this is an ATP-dependent process (Sigler *et al.*, 1998). Therefore the chaperonins share homology especially within regions that are essential for maintenance

of the ring structure, and for ATP-hydrolysis, but may be divergent in domains that facilitate substrate binding (Kim, Willison, and Horwich, 1994). This observation is supported by the finding that chaperonin homologues in the eukaryotic cytosol, the CCTs, have a much more limited substrate repertoire, as compared to eubacterial chaperonins, such as the *E. coli* GroEL protein (Dunn, Melville, and Frydman, 2001; Houry *et al.*, 1999). In addition chaperonin homologues present within in a single eukaryotic organism such as yeasts (eukaryotic cells can have eight or nine different CCT homologues that form a single ring structure) can share less than 50% sequence homology (Stoldt *et al.*, 1996). Therefore it should not be surprising that chaperonins have unique functions aside from their role as protein folders (Table 1). For example, the Hsp60 homologue of *Enterobacter aerogenes* (a symbiont found in the saliva of *Myrmeleon* larvae), is a potent insect toxin (Yoshida *et al.*, 2001). During my doctoral experimental work I showed that the Hsp60 homologue of *Legionella pneumophila*, called HtpB, activates signal transduction cascades that triggers pseudohyphal growth , when expressed in the cytoplasm of *S. cerevisiae*. In contrast, the *E. coli* Hsp60 homologue (GroEL), when expressed in the cytoplasm of *S. cerevisiae* could not induce pseudohyphal growth.

8.2. Strategies to identify key residues for HtpB-induced pseudohyphal growth.

To identify amino acids in HtpB that are involved in triggering pseudohyphal growth in yeast, I would suggest a couple approaches. First, a molecular evolutionary

approach that would involve a comparison of HtpB amino acid sequences from within the *Legionella* genus, as opposed to a comparison of Hsp60 sequences among different genera, as was done in this study, could be undertaken. The advantage of this strategy is that it would be expected for *L. pneumophila spp.*, still living the intracellular lifestyle, to have maintained a similar functionality due to its requirement for the adaptation of the ancestral *L. pneumophila* organism to its new niche, perhaps the intracellular environment of an amoeba. Residues found to be functionally conserved among the HtpB homologues, but not involved in protein folding, would be most interesting candidates to test for a role in inducing pseudohyphal growth in *S. cerevisiae*, and for *L. pneumophila* intracellular pathogenesis. To identify these residues of interest the HtpB homologues could be aligned using Clustal W, and then compared for amino acid conservation using GeneDoc. The conserved residues involved in protein folding can then be identified based on the information available for other Hsp60 homologues, and excluded from further analysis. Site-directed mutagenesis could then be used to alter the residues of interest, so as to test whether they are required for the survivability of their host organism under a defined set of growth conditions, such as growth in amoebae and in mammalian cell lines.

As a second approach to identify residues in HtpB that are key for pseudohyphal growth, domain swaps can be made between HtpB and GroEL and/or *S. cerevisiae* Hsp60p to generate hybrid proteins that could be expressed in *S. cerevisiae* to identify regions in HtpB that are necessary for pseudohyphal growth. Once identified in HtpB, at

the protein level, the corresponding regions of the HtpB *gene* may be mutagenized, and the resulting alleles (if they still encode proteins that have chaperone function) could then be exchanged for the wild-type copy of *htpB* in *L. pneumophila*. These strain(s) could then be tested for its ability to attach to, to invade, and to replicate inside, host cells, and to cause disease in a guinea pig infection model. In this way, the importance of the domains in HtpB that induce pseudohyphal growth in *S. cerevisiae* can be tested for it relevance in *L. pneumophila* virulence.

8.3. How did sequence diversity arise in the chaperonins?

One cannot help but wonder how sequence diversity in the chaperonins arose. An especially interesting example is the chromosomally encoded eukaryotic *HSP60* homologue, which possess a 72 nucleic acid sequence that encodes a 24-amino acid N-terminal, mitochondrial targeting sequence that is not present in the bacterial Hsp60 homologues (Singh *et al.*, 1990). Based on the comparison of amino acid sequences between eukaryotic mitochondrial Hsp60 homologues (not the CCTs, which function in the eukaryotic cell cytosol) and amino acid sequences from eubacterial Hsp60 homologues, it is postulated that eukaryotic mitochondrial Hsp60 homologues are most closely related in sequence to present day *Rickettsia spp.*, and that mitochondria and the *HSP60* gene were derived from an ancestor common to *Rickettsia spp.*, reviewed in (Viale and Arakaki, 1994). Based on this assumption and on the covenants of natural selection, one could propose that the mitochondria were once free-living organisms, and

that the *HSP60* gene that is found in the eukaryotic genome today was once present in the mitochondrial genome. Hence the mitochondrial targeting sequence would not be a selective advantage until after the pre-mitochondrial organism had integrated with its host organism and had lost its genomic copy of *HSP60*. This situation unearths the very interesting question of by what mechanism of natural selection is it possible for a protein to acquire 24 amino acids? Instead, it is possible that N-terminal signal sequence was originally present in ancient bacterial ancestor, but how would this have been a selective advantage for this organism, and how is it that free-living bacteria today do not have the N-terminal mitochondrial targeting sequences? Alternatively it is possible that many different forms of Hsp60 proteins were present from the beginning of life and that each is developed uniquely to undertake its specific task. In support of this it is of interest that the chaperonins in different bacteria are localized to different cellular compartments (the cytoplasm or the cell envelope), and that different secretion mechanisms are possibly employed in each organism for the process of cell envelope localization (see above for a discussion of secretion mechanisms for Hsp60 proteins). In the case of *B. pertussis* it would seem that the localization of Cpn60 to the bacterial cell envelope is perfectly coordinated with the expression of virulence factors in the organism, through BvgS control. This again brings to the forefront the question of whether these secretion systems were originally designed to deliver these proteins or whether this intricate relationship was weaved through natural selection, indicating that other yet undescribed mechanisms could be playing a role in shaping the interaction between biological entities.

In other examples of sequence variation among Hsp60 homologues, only a few amino acids are required for the acquisition of new function. For example the Hsp65 protein from *Mycobacterium leprae* and the Hsp60 from *E. aerogenes* each requires only three specific amino acids for protease activity (Portaro *et al.*, 2002) or four specific amino acids for toxin activity (Yoshida *et al.*, 2001) respectively. In these cases it could better be envisioned how the process of selective pressure combined with errors in DNA replication could result in these minor changes. In the case of the *E. aerogenes* Hsp60 homologue only 16 amino acids differ from the GroEL protein, of which only four critical amino acids (Val 100[valine at the 100^{th} residue in the *E. aerogenes* Hsp60 homologue], Asn 101,Asp 338 and Ala 471) are required for insect toxicity (Yoshida *et al.*, 2001). *E. aerogenes* is an endosymbiont present in the saliva of antlions, which are larvae of the *Myrmeleontidae* family that feed on other insects; therefore the antlions benefit greatly from its endosymbiotic relationship with *E. aerogenes*, which assists the antlions by providing the Hsp60 protein that acts as a paralyzing agent for prey such as cockroaches. It is evident that the Hsp60 homologues are a versatile group of proteins that have made possible the adaptation of organisms to their specific niches.

Because the chaperonin family is essential for the survival of organisms, and because members of this family can tolerate variations in certain domains, this family of proteins may act as facilitators for the adaptation of organisms to new or changing environments. Hsp60 protein monomers are structurally and functionally divided into modules that facilitate the formation and subsequent activity of chaperonin complexes

(Sigler *et al.*, 1998). The configuration of such modules is dependent on conserved amino acid sequences; they serve as contact points for the interaction of subunits that make up the folding ring complex, and as residues that form the ATP binding pocket (Brocchieri and Karlin, 2000). However, the amino acids that make up the substrate recognition domain (the apical face) of the chaperonin complex are highly divergent, since they are not essential for the formation of a functional protein folding complex (Kim, Willison, and Horwich, 1994). Indeed it is within domains not necessary for protein folding that the amino acids that confer new functions reside as is the cases for the *E. aerogenes* and *M. leprae* chaperonins, which have toxin activity and protease activity respectively (Yoshida *et al.*, 2001; Portaro *et al.*, 2002). In a similar fashion amino acid differences may be present in HtpB that has proven to be advantageous specifically for *L. pneumophila*'s survival inside amoeba. Hence perhaps a functional genomic approach could be used to identify HtpB residues *in silico* that are under positive selection.

Part 9. Conclusion.

The HtpB protein is present on the surface of *L. pneumophila*, and is released into vacuoles where this bacterium replicates. Using the yeast model I determined that HtpB expression activates signal transduction cascades that result in the phenotype of pseudohyphal growth. It was demonstrated that the latter event is dependent on Ras2p, the Ste/MAPK pathway, and cAMP pathway. Consistent with the latter observations is

that HtpB interacts with HeLa cell proteins, as determined by the two-hybrid assay, that are homologous to yeast proteins Bub2 and Cks1. These two proteins are involved in regulating mitotic exit, the inhibition of which causes yeast cell elongation, a phenotype of pseudohyphal growth. As such it is highly probable that the two-hybrid hits represent a true biological function for HtpB. Of interest is that the mammalian homologue for Bub2, called Map interacts with merlin, a tumor suppressor that affects Ras-dependent processes, such as actin foci formation. Indeed this study supports the more recent findings of Audrey Chong, who has demonstrated that HtpB expression in CHO cells results in a loss of actin stress fibers. Future studies should be directed toward investigating the relevance of these findings for *L. pneumophila* infection, since *L. pneumophila* cell entry has been shown to be actin-mediated, and since HtpB has been shown to be an invasion factor for HeLa cells. This work illuminates the biological potency of HtpB, and supports the hypothesis that HtpB may have a biological effect in eukaryotic cells that may be relevant for *L. pneumophila* pathogenesis.

The uniqueness of function for individual members of the Hsp60 family is becoming more apparent. In this study I demonstrated that HtpB, but not the yeast Hsp60p or *E. coli* GroEL induced pseudohyphal growth in *S. cerevisiae*. Tied to protein function is the localization of proteins to their sites of action within cells. In this study I demonstrated that enteropathogenic *E. coli* restricts GroEL to its cytoplasm, while *Bordetella spp.* mobilize their Hsp60 homologues to the bacterial cell envelope. In the case of *B. pertussis*, I demonstrated that Cpn60 cell envelope-association may be

regulated in concert with the expression of bacterial virulence factors. These observations support the development of a new image for the chaperonin family, as not only essential factors for protein folding inside the cell, but also as versatile facilitators for the adaptation of organisms to their dynamic environments.

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APPENDIX.

The reagents used in this study were purchased from SIGMA-ALDRICH Canada, Ltd., Oakville, ON, unless otherwise indicated.

Part1. Media.

Components designated filter sterilized must be added post autoclaving to media.

BCYE agar
10 g yeast extract
1 g ACES
1g α-ketoglutaric acid
1.5 g charcoal
16 g agar
dd$H_2$0 to 1L
pH adjusted to 6.6 with 6N KOH
0.4 g L-cysteine (dissolved in a 4 ml volume, pH adjusted to 6.6 with KOH, and filter sterilized)
1 ml, 25% (wt/vol) iron pyrophosphate (filter sterilized and stored in the dark at 4 °C)

BYE broth
The formulation for BYE broth is the same as BCYE with the following exceptions: no charcoal or agar is added; furthermore, the BYE formulation is made without L-cysteine and iron pyrophosphate and can be stored at room temperature until needed, while BYE complete should be stored at 4 °C and in the dark for a maximum of 2 weeks.

Luria-Bertani (LB) broth
5 g yeast extract
10 g tryptone
10 g NaCl
ddH_2O up to 1 L
Add 15 g agar to the LB broth mixture prior to autoclaving for LB agar.

NZY^+ broth
10 g NZ amine (casein hydrolysate)
5 g yeast extract
5 g NaCl
ddH_2O to 1 L
pH adjusted to pH 7.5 with NaOH

Supplemented with the following filter sterilized components:
20 ml, 20 % (wt/vol) dextrose
12.5 ml, 1 M $MgCl_2$
12.5 ml, 1 M $MgSO_4$

M9 minimal medium
200 ml, 5X M9 salts
100 ml, 20 % dextrose solution (filter sterilized)
10 ml of a 0.4 % (wt/vol) solution of each of the following L-amino acids: arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, tryptophan (filter sterilized), threonine (filter sterilized), and valine
100 ml, 0.04 % (wt/vol) tyrosine (filter sterilized)
40 ml, 0.05 % (wt/vol) of the purine base, adenine (filter sterilized),
10 ml, 0.2% (wt/vol) solution of the pyrimidine base, uracil
1 ml, 1M thiamine-HCl (filter sterilized)
4 ml, 10 mg/ml proline (filter sterilized)
15 g agar
ddH_20 to 1L

5X M9 salts
64 g $Na_2HPO_4.7H_2O$
15 g KH_2PO_4
2.5 g NaCl
5.0 g NH_4Cl
ddH_20 to 1L

Stainer-Scholte broth (Casamino acid formulation)
10 ml 100X SS supplement
10 g Casamino acids
10.7 g L-glutamic acid
0.24 g L-proline
2.5 g NaCl
0.5 g KH_2PO4
0.2 g KCl
0.1 g $MgCl_2$
0.2 0.02 g $CaCl_2$
6.1 g Tris base
pH adjusted to 7.5
ddH_2O to 1L

100X SS supplement
40 mg L-cysteine
10 mg $FeSO_4.7H_2O$

4 mg niacin
150 mg glutathione
0.4 g ascorbic acid
ddH_2O to 10 ml
filter-sterilized and stored at –20 °C

Synthetic complete (SC) broth
10 g succinic acid and 6 g NaOH dissolved in 650 ml ddH_20
5 g $(NH_4)_2SO_4$
1.7 g YNB
10 ml of a 0.4 % (wt/vol) solution of each of the following L-amino acids: arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, tryptophan [filter sterilized], threonine [filter sterilized], and valine
100 ml, 0.04 % (wt/vol) tyrosine (filter sterilized)
40 ml, 0.05 % (wt/vol) adenine (filter sterilized)
10 ml 0.02% (wt/vol) uracil
100 ml 20 % (wt/vol) dextrose (autoclaved separately)

For SC agar add 15 g agar prior to autoclaving.

Synthetic defined (SD) media
Similar to SC medium except that the appropriate amino acid(s) or purine and pyrimidine base(s) are omitted from the medium formulation in accordance with strain auxotrophies, so as to support the maintenance of plasmids by nutritional selection.

YEPD agar
1.7 g YNB
5 g $(NH_4)_2SO_4$
10 g yeast extract
20 g peptone
20 ml, 0.05 % (wt/vol) adenine (filter sterilized)
5 ml, 0.04 % (wt/vol) uracil
100 ml, 20 % (wt/vol) dextrose (autoclaved separately)
15 g agar
ddH_20 to 1 L

YM-1 broth
10 g succinic acid
6 g NaOH
1.7 g YNB
5 g $(NH_4)_2SO_4$
5 g yeast extract
10 g peptone

20 ml, 0.05 % (wt/vol) adenine (filter sterilized)
5 ml, 0.4 % (wt/vol) uracil
100 ml, 20 % (wt/vol) dextrose
15 g agar
ddH_20 to 1 L

YPDA
10 g yeast extract
20 g peptone
30 ml, 0.05 % (wt/vol) adenine (filter sterilized)
100 ml, 20 % dextrose (autoclaved separately)
ddH_20 to 1 L
15 g agar

Part 2. Buffers, Solutions and Emulsions.

Tris-acetate (TAE, working solution)
0.04 M Tris-acetate
0.001 M EDTA

50 x TAE
242 g of Tris base (GIBCO®)
57.1 ml glacial acetic acid (Fischer Scientific)
100 ml 0.5 M EDTA, pH 8.0

1 % agarose gel
1g agarose (GIBCO® now at Invitrogen, Burlington, ON)
100 ml 1x TAE buffer
Weigh 1 g agarose and add to 100 ml1X TAE. Microwave until agarose has dissolved and allow to cool enough to touch, then add 4 µl, 10 mg/ml ethidium bromide.

Plasmid extraction solution I
50 mM dextrose (BDH Inc.)
25 mM Tris-HCl pH 8.0
10 mM EDTA pH 8.0
autoclaved and stored at 4 °C

Plasmid extraction solution II
0.2 N NaOH (EMD Biosciences, San Diego, CA), freshly diluted from a 10 N stock
1 % SDS

Plasmid extraction solution III
For 100 ml:
60 ml, 5 M potassium acetate
11.5 ml glacial acetic acid (Fisher Scientific Ltd., Nepean, ON)
28.5 ml ddH_2O
The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate.

10X TE buffer
0.1 M Tris-HCl, pH 7.5
0.01 M EDTA, pH 8.0

10X Lithium acetate solution
1M lithium acetate, pH 7.5 (adjusted with acetic acid)

40 % PEG 3350 solution
40 % PEG
1X TE
1X lithium acetate
(made fresh from sterile 50 % PEG, 10 x TE and 10X lithium acetate stock solutions)

STET solution
8 % (wt/vol) sucrose (BDH Inc., Toronto, ON)
50 mM Tris-HCl, pH 8.0
50 mM EDTA
5 % (vol/vol) Triton X

Phenol/chloroform/isoamyl alcohol emulsion
Chloroform (Fisher Scientific Ltd.) and isoamyl alcohol are mixed in a proportion of 24:1 respectively, then the chloroform/isoamyl alcohol solution is mixed with buffer-saturated phenol (GIBCO®) at 1:1.

Yeast breaking buffer
2 % (vol/vol) Triton X
1 % (vol/vol) SDS
100 mM NaCl (EMD Biosciences)
10 mM Tris-HCl, pH 8.0
1 mM EDTA, pH 8.0

Z buffer
16.1 g $Na_2HPO_4 \cdot 7H_2O$
5.5 g $NaH_2PO_4 \cdot H_2O$
0.75 g KCL
0.246 g $MgSO_4 \cdot 7H_2O$
ddH_2O up to 997.3 ml, adjusted to pH 7.0
2.7 ml/L β-mercaptoethanol (added fresh to the required volume)

Laemmli sample buffer
50 mM Tris-HCl, pH 6.8
2 % (wt/vol) SDS

10 % (vol/vol) glycerol
0.1 % (wt/vol) bromophenol blue
ddH_20
10 % β-mercaptoethanol is added as required for the specified application.

Resolving gel buffer
1.5 M Tris-HCl pH 8.8
0.4 % (wt/vol) SDS

Stacking gel buffer
0.5 M Tris-HCl pH 6.8
0.4 % SDS

30 % Acrylamide (Bio-Rad laboratories Inc.)
29.2 % acrylamide
0.8 % bis-acrylamide

12 % polyacrylamide gel
For two gels (approx. 8 ml/gel):
4 ml resolving gel buffer
6.4 ml 30 % (wt/vol) acrylamide
100 µl 10 % (wt/vol) ammonium persulfate (the 10 % APS stock must be prepared fresh),
5.6 ml ddH_2O
24 µl tetramethylethyldiamine (TEMED, BioShop Canada Inc., Burlington, ON)

5 % stacking polyacrylamide gel
840 µl stacking gel buffer
566 µl 30 % (wt/vol) acrylamide
23.4 µl 10 % APS
1.94 ml ddH_2O
6.6 µl TEMED

Acrylamide-gel running buffer (Tris-glycine working solution)
25 mM Tris
250 mM glycine (electrophoresis grade)
0.1 % (wt/vol) SDS (electrophoresis grade)

Coomaise stain
Per 500 ml:
250 ml methanol
50 ml acetic acid
1 g Coomasie blue powder
ddH_2O up to 500 ml

Destain solution I
Per liter:
500 ml methanol
100 ml acetic acid
ddH_2O up to 1 L

Destain solution II
Per Liter:
50 ml methanol
70 ml acetic acid
ddH_2O up to 1 L

Acrylamide-gel preservation solution
20 % vol/vol methanol
3 % vol/vol glycerol

Protein transfer buffer	Per Liter:
39 mM glycine	2.9 g glycine
48 mM Tris base	5.8 g Tris base
0.037 % SDS (electrophoresis grade)	0.37 g SDS
20 % methanol	200 ml methanol
	ddH_2O up to 1L

10X Ponceau S stock
Per 100 ml:
2 g Ponceau S powder
30 g trichloroacetic acid
30 g sulfosalicylic acid
ddH_2O up to 100 ml

10X phosphate-buffered saline (PBS) stock used for making 1X PBS working solution for destaining Ponceau S-stained blots

	Per liter:
87 mM Na_2HPO_4	12.36 g Na_2HPO_4
15 mM	1.8 g NaH_2PO_4
1.45M NaCl	85 g NaCl
	ddH_2O up to 1 L

PBS formulation used for staining HeLa cells with the TAT antibody
140 mM NaCl
3 mM KCl
8 mM Na_2HPO_4
1 mM KH_2PO_4

The solution is adjusted to pH 7.4 with HCl.

PBST
140 mM NaCl
3 mM KCl
8 mM Na_2HPO_4
1 mM KH_2PO_4
0.5 % (wt/vol) BSA
0.5% (vol/vol) Triton X-100
The pH of the solution is 7.4.

Tris-buffered saline (TBS, working solution)
20 mM Tris-HCl, pH 7.6
500 mM NaCl

TTBS (working solution)
20 mM Tris-HCl, pH 7.6
500 mM NaCl
0.05 % (vol/vol) Tween 20

Alkaline phosphatase developing buffer (AP buffer working solution)
0.1 M Tris-HCl, pH 9.5
0.1 M NaCl
0.05M $MgCl_2$

Nitrobluetetrazolium (NBT) solution
75 mg/ml NBT in 70 % dimethylformamide and 30 % ddH_2O)

Developing solution
In 10 ml developing buffer, 0.0016 g 5-bromo-4-chloro-3-indolylphosphate (BCIP, Diagnostic Chemicals, Ltd., Charlottetown, PEI) and 44 µl nitrobluetetrazolium solution are dissolved.

Anion exchange column buffer
35 mM KCl
25 mM NH_4Cl
50mM Tris-HCl pH 7.6
5mM EDTA
1 mM dithiothreitol (DTT)
1 mM phenylmethy-sulfonyl fluoride (PMSF)
DTT and PMSF are added just prior to use.

Lowry reagent A
copper/tartrate/carbonate solution per liter:
10 g Na_2CO_3
0.5 g $CuSO_4 \cdot 5H_2O$
1 g sodium tartrate
Ten grams sodium carbonate is dissolved in 500 ml distilled water. One half gram copper sulfate pentahydrate and 1 gram sodium tartrate are dissolved in 500 ml distilled water. The copper/tartrate solution is stirred slowly while the carbonate solution is slowly added. This solution is kept at 4 °C and is stable for a year.

Lowry reagent B
1 volume 2 N Folin-Ciocalteau phenol reagent plus 5 volumes ddH_2O
This solution is stored in an amber bottle at room temperature and is stable for a couple months.

Immunogold sample fixative solution
4 % parafomaldehyde
0.1 % gluteraldehyde
The fixative solution was prepared in 0.1 M sodium cacodylate (BDH, Poole, England) buffer, pH 7.3.

Immunogold wash buffer
10 mM Tris-HCl, pH 8
0.3 M NaCl
0.1 % BSA

Immunogold antibody buffer
10 mM Tris-HCl, pH 8.0
0.2M NaCl
0.2 % BSA

Part 3. Equations.

Calculating annealing temperature.
The melting temperature is first approximated for each primer using the equation 4 °C [# of dGTP + dCTP nucleotide residues] + 2 °C [# of dATP + dTTP nucleotide residues]. Five degrees is subtracted from the approximate melting temperature of each primer and the lower of the two values is selected as the primer annealing temperature.

Calculating β-galactosidase units.
$OD_{420}/(OD_{600}$ of assayed culture x volume assayed x time [minutes])

Calculating mating efficiency and number of clones screened in a two hybrid assay.

1. Count the colonies (cfu) growing on the SD/-Leu, SD/-Trp, and SD/-LEU/-Trp dilution plates that have 30 – 300 cfu.
2. Calculate the viable cfu/ml on each type of SD medium: cfu x 1000 µl/ml / (volume plated (µl) x dilution factor) = # viable cfu/ml
   # cfu/ml on SD/-Leu = viability of Y187 partner
   # cfu/ml on SD/-Trp = viability of the AH109 partner
   # cfu/ml on SD/-Leu/-Trp = viability of diploids
3. Compare the viable cfu/ml of the two mating partners. The strain with the lower viability is the limiting partner. In this library screening protocol, the Y187 strain is the limiting partner to ensure that the maximum number of library cells find a mating partner.
4. Calculate the mating efficiency (i.e., % Diploid): (# cfu/ml of diploids / # cfu/ml of limiting partner) x 100 = % Diploid
5. Estimate the number of clones screened: # cfu/ml of diploids x resuspension volume = # of clones screened.