

## MINIREVIEW

# Stress Wars: the Direct Role of Host and Bacterial Molecular Chaperones in Bacterial Infection

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Since 1962, when Ferruccio Ritossa discovered new puffing patterns in the polytene chromosomes of *Drosophila* incubated at an elevated temperature (153), we have been aware that stress at the cellular level is answered by the production of specific gene products. These products are variously termed heat shock proteins (Hsps) or cell stress proteins and were originally identified as molecules that are produced in response to the presence of unfolded proteins within the cell (2). However, it was not until the pioneering work of the groups of Laskey, Ellis, and Georgopoulos that the relationship between the generation of correctly assembled macromolecules and the proteins that function to ensure correct assembly was established. Laskey and coworkers studied the nuclear protein nucleoplamin, which ensures correct assembly of histones and DNA into nucleosomes. Laskey termed nucleoplamin a molecular chaperone as it mimicked the function of a human chaperone who ensures correct interactions between people (98). Ellis and Georgopoulos studied the protein which eventually was known as chaperonin 60 (Cpn60) and which was responsible for initiating the vast flood of papers on molecular chaperones over the past two decades (58). Currently, a molecular chaperone is defined by Ellis as “one of a large and diverse group of proteins that share the property of assisting the non-covalent assembly/disassembly of other macromolecular structures, but which are not permanent components of these structures when they are performing their normal biological functions” (58). A further refinement of terminology is necessary as certain molecular chaperones are produced constitutively and the concentrations within the cell do not increase in response to stress. These proteins are defined as molecular chaperones but are not Hsps or stress proteins. Molecular chaperones, whose concentrations increase in response to stress, are both chaperones and stress proteins/heat shock proteins.

The first protein-folding molecular chaperone to be discovered was Cpn60 (58). Since the identification of this protein as a molecular chaperone in 1988, many more proteins with actual or putative molecular chaperone functions have been discovered, and the term currently applies to 25 families of pro-

teins (Table 1). In all three kingdoms of life molecular chaperones are classified as essential proteins, and there is significant conservation of sequences between proteins used by prokaryotes and proteins used by eukaryotes (such as thioredoxin [Trx] family members, cyclophilins, chaperonins, Hsp70, and Hsp90). Eukaryotic cells have multiple compartments (cytosol, endoplasmic reticulum, mitochondria, nucleus), and in these compartments stress-induced protein folding is known as the unfolded protein response (158). The unfolded protein responses are an important element in the integrated biology of the cell, are linked to key intracellular signaling pathways, and are now being associated with human disease states (158). Implicit in the definition of molecular chaperones was that they were intracellular proteins involved in the folding of client proteins within cellular compartments, which, because of the high protein concentration (on the order of 200 to 400 mg/ml), favor inappropriate protein-protein interactions, resulting in significant protein denaturation (58). However, it is becoming clear that many molecular chaperones can exist outside the cell and participate in nonfolding actions.

Analysis of immune responses to bacteria in the 1970s identified what was termed a “common antigen” in many bacterial species (81). Patients infected with *Mycobacterium tuberculosis* or *Mycobacterium leprae* exhibit significant antibody responses to a 65-kDa antigen (189). Subsequent work identified this antigen (and the common antigen) as the molecular chaperone Cpn60 (193). It has now been established that a number of molecular chaperones from bacteria and protozoan parasites (Cpn60, Hsp70, and Hsp90) are (i) potent immunogens, (ii) active immunomodulators, and (iii) inducers of cross-reactive immunity and autoimmunity (176). The mammalian immune system recognizes the molecular chaperones of infecting parasites as particularly strong immunological signals, which is surprising in view of the significant homology between host and parasite proteins. This profound immune responsiveness should be useful in developing vaccines against pathogens. The Cpn60 (Hsp65) protein of *M. tuberculosis* has also proven to be an extremely powerful immunomodulator that is able to protect against a number of experimental autoimmune diseases in rodents, including diabetes. In a recent phase II study, immunization of individuals who had newly developed type I diabetes with a peptide derived from human Cpn60 proved to be effective in limiting the progression of this disease (146).

Most researchers studying molecular chaperones work

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TABLE 1. Eukaryotic and prokaryotic molecular chaperone and stress protein families

Family	Alternative name(s)		Function
	Prokaryotes	Eukaryotes	
Chaperonins	GroEL, GroES	Hsp60, TriC, CCT	Folding of proteins within cage structure
Thioredoxin	Trx, DsbA to DsbE, glutaredoxin	Trx, glutaredoxin, protein disulfide isomerase	Protein thiol-disulfide exchange
Small Hsps	IbpA, IbpB	Hsp25/27, $\alpha$ -crystallins	Adsorption of unfolded chains to prevent stress aggregation
Peptidyl-prolyl isomerases	Cyclophilins, FKBP, parvulins	Cyclophilins, FKBP, parvulins	Isomerization of peptide bond preceding proline
GrpE	GrpE	GrpE	ADP/ATP exchange factor interacting with DnaK/DnaJ
Hsp40/DnaJ	DnaJ, CbpA, RcsG	Hsp40, Hdj2, Mtj1	Hsp70 cochaperones regulating Hsp70 activity
Hsp70	DnaK, Hsc66, BiP, mitochondrial Hsp70, etc.	Many (e.g., Hsp70, Hsc70)	Prevention of aggregation of unfolded protein chains
Hsp90	HtpG	Hsp90, Gp96	Regulation of assembly of signal transduction proteins
Hsp100	ClpA, ClpB, ClpC, ClpX, ClpY	Hsp100	Disassembly of oligomers and aggregates
Prefoldin nascent chain-associated complex	Prefoldin	Nascent chain-associated complex	Binding to nascent chains as they emerge from ribosomes

within the paradigm that these proteins are present solely in intracellular compartments. However, in 1989, just 1 year after the identification of Cpn60 as a molecular chaperone, Japanese scientists found that the protein-folding catalyst and stress protein thioredoxin was secreted by T cells from patients with a certain form of leukemia and was able to induce T cells to express one of the subunits of the interleukin-2 (IL-2) receptor (167). Subsequently, the human protein was found to be a potent chemoattractant for neutrophils, monocytes, and T lymphocytes with a unique mechanism of action (124). Since this initial discovery, a growing number of mammalian molecular chaperones have been found to be secreted onto the cell surface or into the extracellular milieu, either tissue culture fluid (with cultured cells) or biological fluids such as blood, synovial fluid, or bronchoalveolar secretions (Table 2). Most of these secreted proteins have agonist activity with a mammalian cell population(s), normally myeloid and lymphoid cells and/or vascular endothelial cells (VECs) (62). Although less attention has been paid to the cell-cell signaling activity of bacterial molecular chaperones, bacterial Cpn10, Cpn60, and Hsp70 have been reported to stimulate or inhibit the proinflammatory actions of myeloid cells and VECs (62). Thus, it is becoming clear that molecular chaperones are examples of moonlighting proteins, that is, proteins which have more than one function. The enzymes of glycolysis are the prototypic moonlighting proteins. For example, secreted phosphoglucosyltransferase has been identified as three distinct cytokines: neuroleukin, autocrine motility factor, and differentiation and maturation mediator. This protein also acts as an implantation factor (76). Perhaps the most bizarre example of the moonlighting functions of molecular chaperones is the neurotoxin used by a hunting insect, the antlion or doodlebug, to paralyze its prey. This toxin is produced by a symbiotic bacterium, *Enterobacter aerogenes*, which lives in the insect's saliva. It has been determined that this toxin is a molecular chaperone, Cpn60. The *E. aerogenes* Cpn60 is almost identical to the *Escherichia coli* Cpn60 protein GroEL. Surprisingly, single-residue substitutions in GroEL can change it from an inactive protein into a potent insect neurotoxin (187).

In the last decade there have been a number of reports which support the hypothesis that inducible molecular chaperones, produced both by bacteria and by hosts, function as intracellular, cell surface, or extracellular signals which are involved in the control of the infectious process. This suggests that infection, among other things, is a contest of stress mechanisms with a multitude of unexpected evolutionary twists and turns. In this review we focus on bacterial infection but discuss examples from eukaryotic parasites that exemplify particular mechanisms. The molecular chaperones involved in the formation of the type III secretion system, while indirectly contributing to bacterial virulence, are not included here as they have been extensively reviewed elsewhere (48).

TABLE 2. Molecular chaperones found on the cell surface and/or secreted by cells and/or found in extracellular fluids

Protein	Cell surface	Secreted by cultured cells	Extracellular fluid
Hsp10			+
Trx80		+	+
Trx		+	+
Thioredoxin reductase		+	+
Glutaredoxin		+	+
TRANK <sup>a</sup>		+	
Peroxiredoxin		+	
Cyclophilin A		+	+
Cyclophilin B		+	
Hsp27	+		+
PDI	+		
Calnexin	+		
Calreticulin	+		
Hsp54	+		
Hsp60	+	+	+
Hsp70	+		+
Hsp72	+		
Grp74	+		
BiP	+	+	+
Hsp90	+		
Gp96	+		
Clusterin			+

<sup>a</sup> TRANK, thioredoxin peroxidase-related activator of NF- $\kappa$ B.

## INFECTION REGULATES STRESS PROTEIN SYNTHESIS IN PARASITE AND HOST

Alterations in any cell's environmental conditions result in changes in the profile of molecular chaperone synthesis. Infection is stressful both for the pathogen and for the host, and a key question that needs to be addressed is how pathogens and their host's target cells respond to the presence of each other in terms of stress protein production and function. The responses of bacteria to a variety of environmental stressors have been studied and reviewed previously (29, 138, 140, 179).

The initial event in a bacterial infection is the interaction of the bacterium with host epithelial cells or extracellular matrix. How does this interaction affect stress protein synthesis in the infecting organism? The interaction of *Neisseria gonorrhoeae* with epithelial cells induces *rpoH*, a homologue of  $\sigma^{32}$ , whose regulon contains the genes encoding Cpn60 and Cpn10. Attempts to inactivate *rpoH* were unsuccessful, but construction of a strain that conditionally expressed *rpoH* showed that although not necessary for adherence,  $\sigma^{32}$  is crucial for epithelial cell invasion by gonococci (28). Another gram-negative bacterium, *Porphyromonas gingivalis*, is a cause of periodontal disease. Microarray analysis of this bacterium, cultured on human epithelial cell line Hep-2, revealed significant increases in the levels of mRNA for Hsp40, Cpn60, Hsp70, Hsp90, a variety of peptidyl-prolyl isomerases (PPIs), and members of the thioredoxin family (70). This provided clues about the gene products that require further study. The gene encoding the *P. gingivalis* Hsp90 homologue, which was one of the most upregulated genes in this study, had been knocked out in a previous study and, unexpectedly, failed to show any phenotype with respect to bacterial adherence or invasion of cultured human epithelial cells (165).

Once past the epithelial barrier, bacteria encounter the immunological big hitters, such as the macrophages. Curiously, a number of organisms have adapted to an intracellular lifestyle within macrophages. This is obviously one of the most stressful environments for bacteria (138), even those that have evolved to survive within these cells. An example of the magnitude of the stress response induced in *M. tuberculosis* within macrophages is the report that the rate of transcription of the gene *acr*, encoding Hsp20, increases 800-fold in infected mice (159).

Thus, there is evidence that bacteria have enhanced cell stress responses when they encounter host cells. Do host cells respond in a similar manner to exposure to bacteria or their products? Surprisingly, this question has received little attention. In a whole-animal study, when the western painted turtle was infected with a *Citrobacter* sp., it exhibited tissue-specific upregulation of Hsp70 and Hsp90 (139). Exposure of the intestinal epithelial cell line Caco-2 to *Salmonella enterica* serovar Enteritidis stimulates production of Hsp70 and Hsp90. Interestingly, *Salmonella* lipopolysaccharide (LPS) did not replicate this effect (112). Lipopolysaccharide is the bacterial component that has received the most attention from biologists. All mammalian cells that have been studied respond to this amphiphilic molecule, and it has been found that a range of cellular systems are activated. There have been a few reports that LPS upregulates the expression of selected stress proteins. One of the earliest studies showed that within 15 min of exposure of human monocytes to LPS there was increased tran-

scription of the Hsp70 gene (36). This effect was also found in the circulating monocytes of mice inoculated intraperitoneally with LPS (192). Using subtractive hybridization to identify the genes transcribed in CD14-transfected Chinese hamster ovary cells exposed to LPS, it was found that 14 genes were differentially expressed, including the gene encoding the Hsp70-interacting protein Hop (56). One cell stress protein (although not a molecular chaperone) that is attracting much attention is heme oxygenase (Hsp32), which catalyzes the degradation of heme to biliverdin, iron, and carbon monoxide. It has been found that LPS is an inducer of Hsp32 (157). Bacterial superantigen (*Staphylococcus aureus* enterotoxin B) and LPS induce the synthesis of Hsp25 and Hsp72 in intestinal epithelial cells (87, 121). The effect of the induction of these various cell stress proteins is unclear. However, there is preliminary evidence that overexpression of Hsp70 (181) or hyperthermia in mice (71) inhibits the ability of cells to be activated by LPS. Does this suggest that the cell stress response increases the threshold for host cells to respond to bacteria or their products and that this is a protective measure?

## MOLECULAR CHAPERONES ACT AS HOST RECEPTORS FOR BACTERIA OR THEIR PRODUCTS

The late immunologist Charles Janeway presciently suggested a solution to one of the major problems in immunology: how does the innate immune system recognize and discriminate between pathogens? In an excellent example of Popperian hypothesis generation, Janeway conjured up pathogen-associated molecular patterns (PAMPs) and pattern recognition receptors (PRRs) as the yin and yang of the innate recognition system. Janeway defined the former as essential, evolutionarily stable molecules unique to the pathogen. Since such molecules are essential, their sequence or structure could not change significantly without a loss of biological function. This structural stability means that PAMPs should always be recognized by PRRs (20). We now recognize many PRRs in cell surface receptors, such as CD14, Toll-like receptors (TLRs), nucleotide binding sites, leucine-rich repeat receptors, mannose binding receptors, scavenger receptors, peptidoglycan recognition proteins, etc. (169). In this context, the situation of molecular chaperones is fascinating. They may be the only proteins involved in the infectious process that are both PAMPs and PRRs. Thus, a number of groups have reported that certain bacterial Cpn60 proteins bind to CD14 and/or to TLR2/4 on monocytes and vascular endothelial cells (60). Little is known about other bacterial molecular chaperones, but human Hsp60 (60), Hsp70 (4), and the Hsp90 homologue Gp96 (174) have been reported to bind to TLR2/4. Thus, these proteins can be classified as PAMPs, or should they be classified as human-associated molecular patterns? Therefore, the report in 1999 that the plant-derived antitumor agent taxol and *E. coli* LPS, which have similar actions on macrophages, bound to Hsp70 and Hsp90 was unexpected. Geldanamycin, a specific inhibitor of Hsp90, blocked the activation of NF- $\kappa$ B and the synthesis of tumor necrosis factor alpha induced in macrophages by LPS and taxol (11). A few years later, Kathy Triantafyllou and colleagues, using affinity chromatography and biophysical methods for measuring protein-protein interactions in lipid bilayers, reported that the cell surface "receptor" for LPS is actually a

TABLE 3. Macrophage activation induced by molecular chaperones

Protein	Eukaryotic protein <sup>a</sup>	Prokaryotic protein
Cpn10	Human protein inhibits caM $\phi$ activation	<i>M. tuberculosis</i> protein induces osteoclastogenesis
Trx80	Claimed to induce novel macrophage activation state	Not tested
Trx	Human Trx activates macrophages (state unknown)	<i>A. actinomycetemcomitans</i> protein does not activate macrophages
Cyclophilin A	Human protein activates macrophages (state unknown)	Bacterial proteins not tested
Peroxiredoxin	Eukaryotic protein induces aaM $\phi$	Bacterial proteins not tested
Hsp27	Human Hsp27 induces IL-10 but not tumor necrosis factor alpha	Bacterial proteins not tested
Hsp60	Human Hsp60 induces a caM $\phi$ -like state	Certain mycobacterial proteins block myeloid cell activation
Hsp70	Human protein induces a caM $\phi$ -like state	<i>M. tuberculosis</i> protein contains both inhibiting and activating domains
BiP	Inhibits monocyte activation and may induce aaM $\phi$	No bacterial equivalent
Gp96	Modified form of macrophage activation	Bacterial protein not tested
Clusterin	Induces what appears to be caM $\phi$	No bacterial equivalent

<sup>a</sup> caM $\phi$ , classically activated macrophages; aaM $\phi$ , alternatively activated macrophages.

receptor complex consisting of Hsp70, Hsp90, CXCR4, and the bone morphogenetic protein GDF5. These proteins interact with LPS and with CD14/TLR4. Neutralizing antibodies to Hsp70 or Hsp90 blocked LPS activation (171, 172). Cell surface-located chaperones are increasingly being identified as molecules that play a role in the recognition of infectious agents or their components and in the subsequent intracellular signaling processes. Thus, Hsp70 and Hsp90 are also components of the receptor for Dengue virus (149). JlpA, a surface lipoprotein of *Campylobacter jejuni*, is an adhesin for epithelial cells. This protein binds to cell surface Hsp90 $\alpha$  (there are two Hsp90 isoforms), and this binding can be blocked by geldanamycin or anti-human Hsp90 $\alpha$  antibody. Binding of JlpA to Hsp90 $\alpha$  results in activation of NF- $\kappa$ B and p38 mitogen-activated protein kinase (77). Another role for Hsp90 is facilitating the movement of bacterial toxins from the endosomal compartment into the cytosol (55, 145). Hsp90 is also involved in the induction of IL-8 synthesis by cells exposed to *Helicobacter pylori*. Cells exposed to *H. pylori* produced IL-8, but this production was completely blocked by geldanamycin due to deactivation of extracellular signal-regulated kinase 1/2 and NF- $\kappa$ B (186).

Another Hsp90 family member is the major endoplasmic reticulum chaperone, Gp96. This protein was identified during screening of a mutant library for genes involved in immune responses to endotoxin. Before this study, Gp96 was thought to be indispensable, but it is now recognized to be nonessential and involved in the maturation of a small number of client proteins. This includes the folding and export of the TLRs and some integrins (142). Thus, this protein is required for properly functioning innate immune responses to bacteria. A homologue of Gp96 has been identified as a receptor for the OmpA protein of *E. coli* and is involved in the binding to and invasion of vascular endothelial cells by *E. coli* (137). Does this suggest that bacteria have evolved to utilize Gp96 for their own purposes? However, in the case of the obligate intracellular bacterium *Orientia tsutsugamushi*, the causative agent of scrub typhus, there is a decrease in the number of Gp96 transcripts and protein in infected cells, which may contribute to the immunosuppression seen in infection by this bacterium (17).

In addition to Hsp70, Hsp90, and Gp96 it has been reported that Cpn60 acts as a cell surface receptor for *S. aureus* (30) and

for *Listeria monocytogenes* (177). The cognate bacterial ligands are the fibronectin binding protein and *Listeria* adhesion protein, respectively. Cell surface Cpn60 is upregulated in a range of murine cells in animals infected with *L. monocytogenes*, showing a potentially causal association between infection and surface expression of molecular chaperones (8). A range of other molecular chaperones, such as Hsp27, protein disulfide isomerase, calnexin, calretulin, BiP, etc., have been identified on the external surfaces of mammalian cells (64), and it is likely that these proteins play some role in the integrated biology of bacterial infection. For example, BiP has recently been identified as a high-affinity signaling receptor for  $\alpha$ 2-macroglobulin (119), an acute-phase protein which plays an as-yet-unidentified role in bacterial infection (67). Interestingly, BiP has also been found to be a cell surface receptor for coxsackievirus and Dengue virus (78).

#### BACTERIAL SIGNALING MOLECULAR CHAPERONES: HOMEOSTATIC DYSREGULATORS?

There have been a number of reports of mammalian (mainly human) molecular chaperones that are secreted by cells and appear in extracellular fluids (Table 2). These findings complement the reports over the past 16 years of molecular chaperones that have intercellular signaling activity (Table 3). The precise biological role(s) of human secreted molecular chaperones has not been formulated yet. One hypothesis is that these secreted proteins form part of a homeostatic regulatory network involved in the control of immune, and possibly other, cell populations (132). One recent report may be pertinent. This is the finding that the thioredoxin peroxidase of the sheep liver fluke, *Fasciola hepatica*, is an inducer of the macrophage alternative activation state (25). This introduces the concept of classically activated macrophages and alternatively activated macrophages (50, 113). The former is the well-known state produced in cultured macrophages by LPS and gamma interferon. Classically activated macrophages exhibit properties consistent with antibacterial activity and antigen presentation, including synthesis of IL-12, NO, and oxygen-derived free radicals (50). In alternative states of macrophage activation, which are induced by factors such as IL-4 and IL-13, IL-10, or immune complexes in combination with a TLR4 agonist, these

potent antibacterial agents are not induced, and cells produce IL-10 or specific patterns of chemokines. These alternative states were initially thought to be macrophages in an inactivated state. However, it is now believed that the alternatively activated states evolved to deal with multicellular parasites like flukes and worms (127). Although the results are preliminary, the data suggest that mammalian, mainly human, molecular chaperones are able to stimulate mammalian monocytes to become either classically or alternatively activated (Table 3). Thus, human Cpn10 has been reported to inhibit the activation of monocytes by LPS (79) and to be immunosuppressive in experimental animals (54). Other eukaryotic chaperones with the ability to induce what appear to be alternative activation states of macrophages include a natural truncated form of Trx termed Trx80 (134), Hsp27 (23), BiP (132), and Gp96 (147). The other human chaperones tested induce what appears to be a classically activated state in human macrophages (Table 3).

The obvious question is the biological relevance of these findings. One hypothesis favored by us is that the ability of cells, particularly immune cells, to respond to molecular chaperones is part of an unrecognized homeostatic regulation system which integrates cell stress with key cellular systems designed to cope with major stressors, such as infectious agents (132). One obvious inference is that bacterial homologues of mammalian molecular chaperones may have evolved to interfere with the signaling activities of the host proteins. This hypothesis has not been experimentally tested by adding human and bacterial chaperones to human cells and determining if the bacterial protein interferes with the signaling induced by the human protein. Given the marked homology between bacterial and mammalian molecular chaperones, the obvious assumption is that they have the same cell-cell signaling actions. However, the available information does not support this contention. Thus, human Cpn10, which was first identified as the immunosuppressive protein early pregnancy factor (12), has recently been shown to block the proinflammatory actions of LPS on murine monocytes and in mice (54). In contrast, the *M. tuberculosis* Cpn10 protein is a potent activator of murine myeloid cells, inducing their differentiation into osteoclasts (115). Human Trx functions as a chemokine (124) and as a costimulator for cytokine activity (188). One of us (B.H.) has cloned and expressed the Trx from an oral gram-negative bacterium that is the causative agent of localized aggressive periodontitis, *Actinobacillus actinomycetemcomitans* (61). The recombinant protein was enzymically active but was not able to activate human monocytes or lymphocytes (59). Human Cpn60 is a weak activator of human monocytes and VECs (111) and a potent activator of differentiation of myeloid cell into osteoclasts (116). It has been established that this protein activates cells by binding to CD14 and TLR4 (129). In general, bacterial Cpn60 homologues appear to be more potent and efficacious monocyte activators. However, the mechanisms of cell activation of bacterial proteins differ substantially. Thus, some proteins (e.g., those from *Chlamydia*) stimulate cells, like human Cpn60, by binding to CD14/TLR4 (88, 111). However, other proteins, such as the protein from *E. coli* (166) and Cpn60.2 from *M. tuberculosis* (103), do not use the CD14/TLR receptor complex. Around 25 to 30% of normal humans have significant levels of Cpn60 (>1 µg/ml and as high as 1 mg/ml) in their blood, and more than 50% have levels greater than 1 ng/ml

(102, 182). Thus, this molecule is a circulating "hormone" in a sizeable proportion of the population. At this time it is not clear what role liberated bacterial Cpn60 proteins have during bacterial infections. Comparison of human Hsp70 and the mycobacterial Hsp70 equivalent, DnaK, has revealed that they can both activate human myeloid cell cytokine synthesis. However, the human protein appears to bind to a range of receptors, including CD14/TLR2/4, LOX-1, and CD40 (99, 175). In contrast, the mycobacterial protein appears to bind only to CD40 (175). Of interest is the finding that the binding sites on the human and mycobacterial Hsp70 proteins for CD40 are distinct (99). Furthermore, the mycobacterial protein has been found to have two domains, one which is stimulatory and one which is inhibitory (99).

Thus, the data are tantalizing but so far have failed to show whether secreted bacterial and human molecular chaperones interact as part of the infectious process.

### BACTERIAL MOLECULAR CHAPERONES AS VIRULENCE FACTORS

As discussed above, infection is stressful to the infectious agent, as well as to the host, and it seems obvious that bacteria require increased production of molecular chaperones to survive the infectious process. Infection may be built on a foundation of molecular chaperones. However, there is also evidence which supports the hypothesis that the molecular chaperones of infectious agents moonlight as direct virulence factors. The ability of host stress proteins to act as receptors for bacteria was described above. As a corollary to this, a number of bacteria appear to use specific molecular chaperones as adhesins. In order to do this, the bacteria must secrete these proteins and they must attach to the cell surface. There is now considerable evidence that bacteria express a number of molecular chaperones on the cell surface and can release them into the extracellular milieu to act as signaling virulence factors (Table 4). This is a controversial concept, which is often claimed by critics to be due to (i) the release of proteins as a result of the small amount of bacterial lysis that occurs normally in growing bacteria and (ii) the selective binding of the released proteins to the bacterial surface. It is difficult to test the hypothesis that bacteria secrete molecular chaperones as it is often not possible to inactivate the genes encoding these proteins. There is a similar problem with the hypothesis that proteins of the glycolytic pathway are present on the surfaces of certain bacteria. It has been claimed that such proteins, like glyceraldehyde-3-phosphate dehydrogenase (GAPD), act as receptors for plasminogen and as adhesins for host cells (133). It is not possible to inactivate genes encoding glycolytic proteins. As an alternative, Pancholi and coworkers engineered the gene for GAPD in *Streptococcus pyogenes* by adding a nucleotide sequence coding for a hydrophobic tail on the protein. This resulted in an enzymically active form of GAPD that was not secreted. Bacteria expressing this mutant protein bound significantly less plasminogen, adhered poorly to target cells, and did not have the innate antiphagocytic activity (9). This was the first example in which it was possible to test directly the biological role of proteins found both intracellularly and on the outside of cells. The data clearly showed that cell surface GAPD is not the consequence of bacterial lysis.

TABLE 4. Cell surface and secreted bacterial molecular chaperones

Bacterium	Molecular chaperone	Cell surface or secreted	Ligand	Biological activity	Reference(s)
<i>A. actinomycetemcomitans</i>	Cpn60	Both	?	Osteolytic factor and cytolytic factor	51, 85
<i>Bartonella bacilliformis</i>	Cpn60	Secreted		Mitogen	118
<i>Borrelia burgdorferi</i>	Cpn60	Cell surface	Glycosphingolipid	Adhesin	83, 160
<i>Clostridium difficile</i>	Cpn60	Cell surface	?	Adhesin	65
<i>Coxiella burnetii</i>	Hsp70	Cell surface			110
Enteropathogenic <i>E. coli</i>	Hsp70	Cell surface	Sulfogalactosyl-ceramide	Adhesin	24
<i>Haemophilus ducreyi</i>	Cpn60	Cell surface	?	Adhesin	43
<i>Helicobacter pylori</i>	Hsp20	Cell surface	?		27
<i>Helicobacter pylori</i>	Cpn60	Cell surface	?	Adhesin	82, 183–185
<i>Helicobacter pylori</i>	Hsp70	Cell surface	Sulfatides	Adhesin	73
<i>Helicobacter pylori</i>	PPI	Secreted	?	Induces epithelial cell apoptosis	7
<i>Legionella pneumophila</i>	Cpn60, Hsp70	Cell surface	?	Adhesin	46, 47, 68
<i>Mycobacterium avium</i>	Cpn60	Cell surface	?	Adhesin	33, 143
<i>Mycobacterium avium</i>	Hsp70	Cell surface	?	Possible adhesin	144
<i>Mycobacterium smegmatis</i>	Cpn60	Cell surface	?		31
<i>Rickettsia prowazekii</i>	PPI	Cell surface	?		46
<i>Salmonella enterica</i> serovar Typhimurium	Cpn60	Cell surface	Mucus	Adhesin	32

The findings also open up the possibility of using such a control to determine the importance of exteriorized molecular chaperones.

At this time, nine bacteria (Table 4), including diverse organisms such as *Borrelia burgdorferi* (160), *Clostridium difficile* (65), *H. pylori* (73, 82, 183, 184, 185), and *Salmonella enterica* serovar Typhimurium (32), have been reported to utilize cell surface Hsp60 and Hsp70 as adhesins. The host molecules acting as the “receptors” for these ligands are, in general, not known. *B. burgdorferi*, the causative agent of Lyme disease, has a cell surface Cpn60 which binds to a glycosphingolipid (83, 160). Enteropathogenic *E. coli* Hsp70 binds to sulfogalactosyl-ceramide (24), *H. pylori* Hsp70 has been reported to bind sulfatides (73), and *S. enterica* serovar Typhimurium Cpn60 binds to undefined components of mucus (32). At first sight, it seems inherently unlikely that, for example, Hsp60 can have such a range of binding specificities. However, with three billion years of evolution behind it and with growing support for the hypothesis of protein moonlighting, it is not inconceivable that this molecular chaperone, and indeed other molecular chaperones, can display such a spectrum of biological activity. In addition to facilitating binding to the cell surface, the Cpn60 of *Legionella pneumophila* also promotes internalization of this bacterium (46).

In addition to acting as adhesins, bacterial Cpn60 and Hsp70 proteins have been shown to have cell-cell signaling properties and are able to modulate the activity of host cells. The molecular chaperones that are produced by bacteria and have extracellular signaling functions include (i) the Cpn60 proteins of *A. actinomycetemcomitans* (51, 85), *Chlamydia trachomatis* (88), *Chlamydia pneumoniae* (22, 111), *E. coli* (166), *H. pylori* (49, 111), *M. tuberculosis* (103), *M. leprae* (170), and *Rhizobium leguminosarum* (104); (ii) the Hsp70 proteins of *M. tuberculosis* (99), *Mycobacterium paratuberculosis* (97), and *E. coli* (128); (iii) the Hsp90 protein of *H. pylori* (186); the peptidyl-prolyl isomerase of *H. pylori* (7); and (iv) the Cpn10 protein of *M. tuberculosis* (115). It must be noted that while these proteins

can have potent and profound effects on host cells and while they may be known to be secreted (as determined mainly by proteome analyses of individual bacteria), the mechanism of secretion has not been defined. This lack of knowledge has been used to criticize the hypothesis that bacterial extracellular molecular chaperones play a role in infection. The same criticism has been directed to human secreted molecular chaperones. However, in the past few years one mechanism of release, membrane vesicles called exosomes, has been discovered (96). Also, Fossati et al. have demonstrated that *M. tuberculosis* Cpn10 is secreted when the bacterium is in the macrophage phagolysosome (39). Given the growing number of bacterial secretion pathways, it is likely that the answer to the question of secretion of bacterial molecular chaperones will be answered soon.

To summarize, the actions of bacterial stress proteins on host cells include inducing the synthesis of proinflammatory cytokines (99, 103, 115, 129, 166, 175, 186) and promoting apoptosis (7, 125). The former action is likely to result in a more efficient antibacterial response, whereas the latter is likely to inhibit host antibacterial responses. However, certain bacterial molecular chaperones have an apparent anti-inflammatory action. Thus, administering the recombinant Cpn60.1 protein of *M. tuberculosis* inhibits experimental asthma, an inflammatory lung condition, in the mouse (152). The *M. leprae* Cpn60.2 protein is also a potent inhibitor of this experimental disease (150). Note that the mycobacteria generally code for two or three Cpn60 proteins (106). However, in both of these studies (150, 152) other bacterial Cpn60 proteins, including the *M. tuberculosis* Cpn60.2 molecule, failed to show anti-inflammatory activity. This is surprising given that the *M. tuberculosis* and *M. leprae* Cpn60.2 proteins exhibit 95% sequence identity at the amino acid level. This supports the finding of the study of Yoshida et al. of the Cpn60 protein from *E. aerogenes* (187) that minor changes in the sequence of Cpn60 proteins can have profound effects on their biological actions. In addition to these effects, it has been reported that the Cpn60 proteins of *A.*

*actinomycetemcomitans* and *E. coli* promote epithelial cell migration and inhibit  $\beta_1$ -integrin expression in cultured keratinocytes. This may be a mechanism for altering epithelial cell function to enhance the infectivity of the bacterium (191).

Most of our knowledge of the roles of stress proteins such as Cpn60 has come from studies of pathogens. In mammals, commensal bacterial species outnumber pathogens by at least 10-fold. This suggests that there may be many stress protein-host interactions which facilitate the prokaryotic-eukaryotic contract that most multicellular organisms have entered into (114). The role of the Cpn60 from the symbiotic bacterium *E. aerogenes* as a neurotoxin for the antlion has been described by Yoshida et al. (187). A more complex bacterial Cpn60-host interaction is an interaction involving the plant-sucking insect *Lipaphis erysimi*. This insect is killed by a mannose-binding leaf lectin when it feeds on garlic (*Allium sativum*). Surprisingly, the insect receptor for this lectin is the Cpn60 protein of a symbiotic bacterium (a *Buchnera* species) in the insect gut. This protein, which is termed symbionin, also plays a role in viral transmission caused by the feeding of *L. erysimi* (6). This is a fascinating species interplay involving a single cell stress protein. Further information on the role of Cpn60 in bacterial endosymbiosis can be found in a recent review by Fares et al. (35).

#### ROLES OF SPECIFIC BACTERIAL MOLECULAR CHAPERONES IN VIRULENCE

Over the past 10 to 15 years attention has been focused on the best-studied cell stress proteins, chaperonins, Hsp70, and Hsp90. Recently, workers have begun to report on the roles of other, less-well-understood molecular chaperones and stress proteins in the bacterial virulence process. To avoid confusion, the functions of these individual proteins are described below and their roles in virulence are explained.

**Peptidyl-prolyl isomerases.** Peptide bonds can exist as *cis* or *trans* isomers, but the presence of the *cis* isomer in a polypeptide chain causes steric hindrance, and, as a consequence, most such bonds are in the *trans* form. With proline, because the side chain is a secondary amide forming a ring structure with the main chain, both isomers are approximately sterically similar and the peptide bond immediately preceding proline has a *cis/trans* ratio of around 4. As it is believed that the ribosome stereospecifically synthesizes peptide bonds in the *trans* configuration, some posttranslational modification must account for this unusual ratio. It has now been established that prokaryotes and eukaryotes have three highly conserved families of enzymes called peptidyl-prolyl isomerases, which catalyze these *trans/cis* isomerizations. Such an isomerization can be the rate-limiting step in protein folding. The three families have no sequence or structural homology, and the proteins are known as cyclophilins, FK506 binding proteins (FKBPs), and parvulins (34). There are many PPIs; humans have 11 cyclophilins, 18 FKBPs, and two parvulins. Surprisingly, with very few exceptions, the PPIs have not been found to be essential proteins, and there are serious questions about the contributions of their enzyme activities to their biological functionality. The PPIs came into prominence because in eukaryotic cells they are the binding receptors for the potent T-cell immunomodulatory

compounds cyclosporine, FK506, and rapamycin, which are used to treat transplant recipients (34).

There is evidence that PPIs are involved in the biology of infection. It has been known since the early 1990s that mammalian cyclophilins are secreted proteins with proinflammatory, potentially protective actions (3). The potential role of PPIs in bacterial virulence can be traced back to the identification of the macrophage infectivity promoter/potentiator (Mip) protein in *L. pneumophila* (18, 19, 38) and a homologue in *C. trachomatis* (107). These proteins, which are FKBPs, enhance the intracellular survival of these intracellular bacteria. Using monoclonal antibodies which either block or do not block the PPI activity of Mip, it has been shown that this isomerase activity is required for the biological activity of Mip (57). Recently, *N. gonorrhoeae* has been shown to have a surface-exposed lipoprotein with PPI activity and homology to the Mips. Inactivation of the gene encoding this protein results in a mutant which is more sensitive to macrophage killing (101).

One of the key protein-folding PPIs in bacteria is trigger factor (TF). This is the first chaperone to meet nascent polypeptide chains as they emerge from the ribosome. This protein is thought to function by scanning newly formed polypeptide chains and shielding hydrophobic regions to keep the proteins soluble (190). Inactivation of the gene encoding TF in *E. coli* resulted in no growth defects at temperatures between 15°C and 42°C (72). Although TF has PPI activity, this activity has been removed from the *E. coli* protein by mutation without affecting its protein-folding activity (91). This suggests that TF may have functions in addition to protein folding, possibly functions involving its PPI activity. Inactivation of the TF gene homologue in *Streptococcus mutans*, an oral bacterium and the major causative agent of dental caries, resulted in a bacterium that had decreased tolerance to acid killing and oxidative stress. This can be explained in terms of the role that this protein plays in protein folding. In addition, the isogenic mutant was significantly less competent (by 2 logs) for genetic transformation. *S. mutans* forms biofilms on tooth surfaces, and this behavior is linked to its pathogenicity. It was therefore of interest that inactivation of the gene encoding TF resulted in major alterations in the ability of the bacterium to form biofilms. This shows that TF must play a significant role in regulating multiple systems in this bacterium (178). Trigger factor is also involved in the maturation of the extracellular cysteine protease of *S. pyogenes* (108), a major virulence factor of this organism (105). Site-directed mutation to inactivate the PPI domain has shown that isomerase activity is essential for the maturation of this protease once it has been secreted. This is due to the failure to isomerize one proline residue in the protease. This study showed that while the PPI activity of trigger factor is not required for nascent protein folding, it is required for the maturation and activation of selected proteins (109). A cyclophilin has also been found to be associated with phenotypic variation in another streptococcal species, *Streptococcus pneumoniae* (131).

*H. pylori* promotes apoptosis of the gastric epithelial cell population, thus causing the atrophic gastritis and gastric dysplasia associated with the infection (80). A PPI (HP0175) is secreted by *H. pylori* (84) and is one of a small number of protein antigens of this bacterium recognized by patients with

gastrointestinal ulcers and not by patients with dyspepsia (5). The recombinant form of the protein has been produced and has been shown to induce apoptosis of gastric epithelial cells by binding to TLR4 and stimulating apoptosis signal-regulating kinase 1. Isogenic mutants with a disrupted HP0175 gene have an impaired ability to induce apoptosis (7). Thus, HP0175 is a secreted PAMP which is able to induce apoptosis and which is likely to contribute to the pathology of *H. pylori*. *Rickettsia prowazekii*, an obligate intracellular bacterium and the causative agent of epidemic typhus, has a cell surface PPI which acts as a virulence factor (31).

Inactivation of the PPI gene *fkpA* in *S. enterica* serovar Typhimurium biotype Copenhagen has been reported to result in an organism that is less able to survive within macrophages or epithelial cells in vitro (69). However, inactivation of the same gene in *S. enterica* serovar Typhimurium had only a minor effect on this organism (74). The reason for the difference in the responses of these two similar organisms to loss of the same enzyme is not understood.

To show the universal importance of chaperones in infection, there is evidence that PPIs are important in infection by the protozoan *Plasmodium falciparum* (93) and the fungus *Cryptococcus neoformans* (149).

**AAA+ proteins.** The AAA+ superfamily of proteins includes the Clp/Hsp100 family and the more extensive AAA family. This superfamily is characterized by a conserved segment consisting of roughly 220 amino acids normally referred to as an AAA domain or nucleotide binding domain (NBD). This domain contains several conserved motifs, including the Walker A and B motifs used for binding and hydrolyzing ATP. A number of the AAA+ family members contain specialized domains. Good examples are the Lon and FtsH proteins, which contain a protease domain. The AAA+ superfamily can be divided into two distinct classes on the basis of the number of AAA domains. Class I proteins (e.g., ClpA, ClpB, ClpC, and ClpE) contain two highly conserved NBDs separated by a linker region. Class II proteins (e.g., ClpX and ClpY) have only one NBD. The function of these proteins is to mediate ATP-dependent unfolding or disassembly of protein-protein or protein-DNA complexes. They are also involved in ATP-dependent regulation of protein degradation by targeting specific proteins for degradation by ClpP serine proteinases (26). As indicated above, there are a number of bacterial Clp proteins. In *Bacillus subtilis* ClpC is involved in allowing growth at elevated temperatures and in the control of cell division, competence, sporulation, and synthesis of degradative enzymes (92, 120, 126, 135). In *L. monocytogenes* ClpC is required for the survival of the organism inside host cells by promoting escape from the phagosome (155, 156). The same protein has also been reported to be required for adhesion to and invasion of hepatocytes by *L. monocytogenes* (123). In contrast, the homologue ClpE, although required for the virulence of *L. monocytogenes*, is not required for invasion (122, 123). Two other AAA+ proteins involved in the virulence of *L. monocytogenes* are the ClpP serine proteinase (44) and ClpB (14). Chasten et al. (14) found that ClpB plays no obvious role in stress tolerance, although it is involved in induced thermotolerance. Clearly, many of the members of this family aid in the virulence of *L. monocytogenes*.

Signature-tagged mutagenesis screening of *S. pneumoniae*

provided the first clue that ClpC is involved in virulence (136). A loss-of-function library of *S. pneumoniae* was screened for penicillin tolerance, and 1 of 51 mutants capable of surviving 10 times the MIC of penicillin contained an insertion duplication in the gene encoding ClpC. On the basis of this finding, a *clpC*-deficient mutant was constructed, which formed long chains and failed to undergo lysis in the presence of penicillin or vancomycin. Of note was the finding that the *clpC* mutant showed only a fraction of the adherence of the wild-type bacterium to human lung cells. The mutant also exhibited decreased transformation efficiency. The failure to produce ClpC was associated with a failure to produce the choline binding proteins LytA, CbpA, CbpE, CbpF, and CbpJ. This suggests that ClpC plays an essential and pleiotropic role in the homeostatic regulation of *S. pneumoniae* and in the interaction of this organism with its host (13). The role of ClpC in virulence was, however, not confirmed by another group (154). To clarify these divergent findings, Mitchell's group inactivated *clpC* in three strains of *S. pneumoniae* and, although finding strain differences, concluded that ClpC does contribute to virulence in vivo (75).

Pneumolysin is the major toxin produced by pneumococci, and a mutation in *clpP* has been shown to increase the level of mRNA for this protein after heat shock (94). This is due to posttranscriptional regulation of the mRNA by ClpP (95). The *clpP* isogenic mutant is more susceptible to macrophage killing and is defective in colonization of the murine nasopharynx and survival in the murine lung (95). The oral gram-positive organism *S. mutans* exhibited reduced growth under stress conditions and a reduced capacity to form biofilms when the *clpP* gene was inactivated, but this phenotype was not seen when *clpC* was inactivated (100).

The Clp ATPases have also shown up in searches for virulence genes in both *S. enterica* serovar Typhimurium (66) and *S. aureus* (117). In *S. enterica* serovar Typhimurium a *clpB* mutant has been found to be deficient in the ability to colonize the chicken alimentary tract (173). A number of reports have revealed the importance of ClpC (15), ClpP/ClpX (40, 41), and ClpY (42) in the virulence of *S. aureus*, including its ability to form biofilms.

Finally, the *clpB* gene of *Francisella novicida* was identified in a mutational screening analysis to identify mutants unable to grow in macrophages in vitro (52). Thus, there is no doubt that the AAA+ family of molecular chaperones and stress proteins with the associated proteases are involved in the survival and virulence behavior of a number of important pathogenic bacteria.

**DnaK/DnaJ.** DnaK belongs to the Hsp70 family and exhibits approximately 70% sequence identity with eukaryotic members of this family. In *E. coli*, it is an abundant cytoplasmic protein and accounts for approximately 1% of the total cellular protein at 30°C. DnaJ belongs to the Hsp40 stress protein family and acts synergistically with DnaK and another protein, the cochaperone GrpE, in the folding of nascent protein chains in the bacterial cytoplasm. DnaK interacts with TF as described by Fink (37). Like the TF mutants, *E. coli* in which the gene encoding DnaK has been inactivated are viable at temperatures between 20°C and 37°C (141). Inactivation of the gene encoding DnaK in *Brucella suis*, an organism adapted to intracellular replication and the causative agent of brucellosis



in pigs, resulted in a failure of the bacterium to grow within macrophages (86). Inactivation of DnaJ in *C. jejuni* resulted in a mutant which grew in culture but was unable to colonize chickens (89). Knockout of the *dnaK-dnaJ* operon in *S. enterica* serovar Typhimurium resulted in a mutant which did grow in culture, albeit at a lower rate. However, bacteria lacking DnaK/DnaJ did not survive and replicate in cultured macrophages or in cultured epithelial cells and failed to colonize mice. This is the first evidence that this operon is involved in invasion of epithelial cells (168).

**$\alpha$ -Crystallins.** The  $\alpha$ -crystallins or small heat shock proteins are a diverse class of cell stress proteins which share only certain short sequence motifs. They have low molecular masses and form complex quaternary structures containing up to 50 subunits (45). The *M. tuberculosis*  $\alpha$ -crystallin, Acr1, has been the subject of study since, unexpectedly, its synthesis increases dramatically in dormant cells in a hypoxic environment (161). A second  $\alpha$ -crystallin gene, *acr2*, is the most upregulated gene in *M. tuberculosis* subject to heat shock (162) or after uptake into macrophages (139). Despite this marked increase in synthesis, the *acr2* gene can be eliminated without affecting log-phase growth or the persistence of the organism in gamma interferon-activated macrophages. In addition, the Acr2 protein appears to be an Achilles heel of *M. tuberculosis* as it is a strong immunogen in both cattle and humans (180). In spite of these findings, when the *acr2* deletion mutant was used to infect mice, the disease caused was much milder than the disease caused by the wild-type organism, revealing that Acr2 plays some as-yet-unidentified role in the virulence of *M. tuberculosis* (163).

#### BACTERIAL MOLECULAR CHAPERONES AS ANTIBACTERIAL TARGETS

Cell stress proteins are clearly vital for the maintenance of living cells. Therefore, it is likely that these stress proteins are targeted both by pathogens and by host organisms in the infectious process. Some evidence for the targeting of host molecular chaperones by bacteria has been presented. Is there any evidence that the host targets bacterial molecular chaperones? It is still early, but there is evidence which supports the hypothesis that bacterial molecular chaperones are targeted by the host and may be interesting therapeutic targets for the development of novel antibiotics. Using labeled members of the proline-rich antibacterial peptide family (specifically, drosocin, pyrrolicorin, and apidaecin), it was found that these peptides specifically bound to DnaK and that the binding was associated with bacterial killing. Pyrrolicorin did not bind to the human equivalent of DnaK, Hsp70 (130). There is some controversy about the mechanism of action of these proline-rich peptides. The mechanism of DnaK protein folding involves hydrolysis of ATP. It has been reported that the antibacterial peptide pyrrolicorin is an inhibitor of the ATPase activity of DnaK and that it is this inhibition that prevents this Hsp70 homologue from assisting protein folding (90). In contrast, another group has reported that pyrrolicorin actually stimulates the ATPase activity of DnaK and has proposed that DnaK actually binds to the "substrate binding pocket," which would normally encompass unfolded protein sequences. It is this competition that blocks the activity of DnaK (16). Syn-

thetic more stable analogues of pyrrolicorin have been produced and have been shown to be active both in vitro and in vivo (21). Another class of bacterial molecular chaperones that has been deliberately targeted is the periplasmic chaperones that are required for the assembly of pili used by gram-negative bacteria to adhere to host tissues. These compounds can dissociate chaperone-pilus protein complexes (164). In addition to eukaryotic antibacterial peptides targeting molecular chaperones, it is emerging that bacteria also produce antichaperone antibiotics. The first report was that *Streptomyces* spp. produce stressgenin, which inhibits stress protein transcription in both bacteria and mammalian cells (1). More recently, *Streptococcus hawaiiensis* has been found to produce acyldepsipeptides, which have activity against streptococci and staphylococci both in vitro and in vivo. The cellular target of these peptides is ClpP, which is activated, causing uncontrolled proteolysis that leads to inhibition of cell division and bacterial death (10). It is expected that other antibiotic-chaperone interactions with the ability to inhibit bacterial growth or kill bacteria will be discovered in the near future.

#### CONCLUDING REMARKS

We are currently in paradigm transition with regard to molecular chaperones. The concept that these molecules are intracellular proteins whose only function is to help fold client proteins is evolving into the hypothesis that many, if not most, of the chaperones are moonlighting proteins that have functions in addition to protein folding. Furthermore, the nonfolding functions of the chaperones may be expressed on the surface of the cell or in the extracellular milieu. This new paradigm offers many more opportunities for molecular chaperones, from the host or from the infectious organism, to have interesting biological actions. Perhaps the most puzzling finding is the finding that the host can recognize both its own molecular chaperones and those from infecting bacteria as examples of PAMPs. Just as puzzling is the finding that the key PRR, the protein complex that recognizes LPS, the most stimulatory signal from bacteria, contains two host molecular chaperones, Hsp70 and Hsp90. In addition, human Cpn60 has recently been reported to bind to LPS (53). It is almost as if the host molecular chaperones are hardwired into the recognition processes that allow bacterial and other microbial pathogens to be identified. Indeed, a pattern emerging from what is still a small literature is that molecular chaperones are able to function as receptors for different ligands. Another emerging pattern is the ease with which small sequence alterations in molecular chaperones result in new biological functions. These proteins have a long evolutionary history. Could this have provided a greater sampling of protein hyperspace to produce the plethora of biological activities being identified for these proteins (63)? We are still in the infancy of the study of the role of molecular chaperones in bacterial virulence, and it is hoped that more funding will be provided for this still controversial area of study. For example, it has been suggested that bacterial periplasmic substrate binding proteins, in addition to their function in transport and chemotaxis, might be implicated in protein folding and protection from stress in the periplasm (151). Thus, many more chaperone proteins may be involved in bacterial and host homeostasis and in mutual interactions.

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