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CHAPTER 12
Bacterial Moonlighting Proteins and Bacterial Virulence

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Abstract

Implicit in the central dogma is that each protein gene product has but one function. However, over the past decade, it has become clear that many proteins have one or more unique functions over-and-above the principal biological action of the specific protein. This phenomenon is now known as protein moonlighting and many well known proteins such as metabolic enzymes and molecular chaperones are now known to moonlight. A growing number of bacterial species are being found to express moonlighting proteins and the moonlighting activities of such proteins can contribute to bacterial virulence behaviour. The glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase (GAPD) and enolase and the cell stress proteins: chaperonin 60, Hsp70 and peptidyl prolyl isomerase are among the most common of the bacterial moonlighting proteins which play a role in bacterial virulence. It is likely that only the tip of the bacterial moonlighting iceberg has been sighted and the next decade will bring with it many new discoveries of bacterial moonlighting proteins with a role in bacterial virulence.

12.1. Introduction

Francis Crick's Central Dogma which states

GENE (DNA) ——————— RNA——————— PROTEIN

and defines (incorrectly) the direction of information flow in gene-to-protein synthesis, also carries with it the unspoken assumption that each protein has a single function. The human genome is now believed to encode 23-25,000 proteins and up until the 1980s there was no evidence for the alternative hypothesis - that individual proteins can have more than one biological activity. However, in 1988, Joram Piatigorsky, working at the National Eye Institute in Bethesda, USA, reported that the lens crystallin protein in the duck was the metabolic enzyme argininosuccinate lyase (Piatigorsky et al., 1988). He proposed that this phenomenon, in which one gene product has more than one function, be called *gene sharing* (Piatigorsky et al, 1988). Subsequent work showed that a whole range of metabolic enzymes, and other proteins, can generate transparent lens structures in a variety of animal species. Piatigorsky also realised that this gene sharing had consequences for the transcriptional control of the shared genes (Piatigorsky, 1998). Although the pioneer of this field of protein molecular biology (see Piatigorsky, 2007), Piatigorsky's gene sharing hypothesis failed to attract the attention it deserved and the term 'gene sharing' is rarely seen in the current literature.

The term 'protein' moonlighting can be traced back to two scientists working for Hoffman-La-Roche who reported that the well established neuropeptides, somatostatin and growth hormone releasing hormone (GH-RH), also exhibited immunological activity and termed this activity 'moonlighting' (Campbell and Scanes, 1995). The second use of the term 'moonlighting' was in an editorial describing a paper that reported that yeast enzymes involved in repairing double strand breaks in DNA are also involved in the maintenance of telomeres (Weaver, 1998). However, it has been Constance Jeffery who has publicised the concept of protein moonlighting and attempted to bring some definition to this novel area of protein biology (Jeffery, 1999). While this is the simplistic version of the history of protein moonlighting, it should be noted that there are earlier descriptions of protein moonlighting, but with the phenomenon not being explicitly recognised. Glyceraldehyde-3-phosphate dehydrogenase (GAPD) is one of the most accomplished moonlighting proteins, now well recognised

for having functions in the nucleus (Sirover, 2005). That GAPD could bind single, but not double, stranded DNA was reported as early as 1980 (e.g. Perucho et al, 1980).

The term moonlighting means to have a second job, at night, in addition to the daytime activity. In Jeffery's first review (1999) she attempted to categorise the various functional facets of moonlighting proteins and to limit their definition. Thus proteins generated by gene fusions, homologous but non-identical proteins, splice variants, protein decoration variants, protein fragments and proteins operating in different locations or utilising different substrates are not considered to be moonlighting proteins (Jeffery, 1999). Enzymes which have two metabolic functions or utilise two different substrates are categorised as bifunctional enzymes (Moore, 2004). The term 'catalytic promiscuity' has also been applied to the situation of an enzyme which has an active site able to catalyse two different reactions (Copley, 2003). This form of 'moonlighting' will not be discussed in this article. The key attributes of moonlighting proteins are the expression of clearly distinct biological activities. This may be an attribute of the original active site of the protein or may be due to the evolution of other sites on the protein with biological function (Fig 1). Often this is dependent on where the protein is found. Many proteins have one originally-defined activity within a cell or a cell compartment and another biological action when the protein is present in another cell compartment or, indeed, is secreted from the cell. Thus moonlighting activity is partially dependent on the environment the protein finds itself in and this may be thought of as 'geographical' moonlighting. As will be seen, as this review develops, a growing number of proteins have not just one moonlighting activity but multiple such activities and there is evidence that moonlighting can contribute to pathology in eukaryotes. In prokaryotes it is emerging that an increasing number of moonlighting proteins are involved in bacterial virulence. One of the most fascinating aspects of protein moonlighting is its apparent spanning of the Kingdoms of life. It is now accepted that mitochondria originated from a eubacterium (Gray et al, 1999). Two examples of moonlighting proteins involving mitochondria/eukaryotic cell interactions are: (i) the role of the cytokine-induced transcription factor Stat (Signal Transducer and Activator of Transcription) 3, which can enter into mitochondria and control mitochondrial respiration (Wegrzyn et al, 1999) and; (ii) the role of the mitochondrial F_1F_o ATP synthase as a cell surface high affinity receptor for apoA-I, which is the main protein in high density lipoproteins (HDL) (Vantourout et al, 2010). It is even speculated that this cell surface ATP synthase could be a therapeutic target in heart

disease. The reader should be aware that if protein moonlighting proves to be a property of the majority of proteins it has major ramifications for evolutionary theory, cellular network complexity, genetics and medicine. This will be briefly discussed at the conclusion of this review.

12.2. Eukaryotic Moonlighting Proteins and Human Disease

This article will focus on prokaryotic moonlighting proteins but, at the moment, more is known about moonlighting in eukaryotes, and many of the eukaryotic moonlighting proteins have homologues in bacteria. Thus it is possible that these bacterial proteins might be able to mimic the moonlighting actions of eukaryotic proteins. This could be important, as a number of human moonlighting proteins have pathological activity.

There are probably well over a hundred eukaryotic moonlighting proteins described in the literature. One of the surprising findings is that many of these proteins are well known metabolic proteins such as the glycolytic and the tricarboxylic acid (TCA) cycle enzymes. Another growing family of moonlighting proteins are the molecular chaperones and protein-folding catalysts involved in protein folding and the cell stress response. These are all ancient gene products, and this leads to the question – are all moonlighting proteins early-evolved gene products. Unfortunately, it is too early to answer this question definitively. Now one of the major surprises in protein moonlighting is the number of moonlighting actions that individual proteins can acquire. For example, the glycolytic enzyme, phosphoglucoisomerase (PGI) has four distinct extracellular functions which have been identified and individually named: (i) a neurotrophic activity termed neuroleukin (Chaput et al., 1988); (ii) a factor which promotes cell motility, and is involved in tumour malignancy, called autocrine motility factor (AMF – Watanabe et al., 1996); (iii) differentiation and maturation mediator, which promotes myeloid cell differentiation and may play some role in leukaemia (Xu et al., 1996) and (iv) an implantation factor activity in the ferret (Schulz and Bahr, 2003). Most studies of PGI's moonlighting activity have concentrated on its AMF activity. It is now established that AMF is significantly correlated with breast cancer progression and with a poor prognosis of this condition. This appears to be due to the ability of secreted PGI/AMF to promote epithelial-to-mesenchymal transition (EMT), which is a precursor to tumor metastasis (Funasaka et al., 2009). More recently, another potential moonlighting function of AMF has been described – regulation of endoplasmic reticulum (ER) stress by a novel mechanism involving controlling ER calcium levels (Fu et al., 2011). Now one of the

fascinating aspects about the biology of PGI, when it functions as AMF, is the nature of the receptor. It turns out to be another moonlighting protein called gp78, an endoplasmic reticulum (ER) membrane-anchored ubiquitin ligase involved in endoplasmic reticulum-associated protein degradation (Fairbank et al., 2009). Secreted PGI clearly has important pathophysiological moonlighting actions which could be mimicked by secreted bacterial homologues of this enzyme and contribute to bacterial pathogenicity. The available evidence is contradictory, with one group, who cloned, expressed and crystallised the PGI from *Bacillus stearothermophilus* (a Gram-positive environmental thermophile) finding the bacterial protein to have both AMF and neuroleukin activity (Sun et al., 1999). In contrast, another group, using a commercially available source of this same enzyme, found the protein enzymically active but lacking in moonlighting activity (Amraei and Nabi, 2002). The most sensible explanation for these opposite findings is that the commercially available enzyme has some alteration in the PGI moonlighting site, or perhaps a change in post-translational modifications. A recent study has proposed that the *Mycobacterium tuberculosis* PGI structure is similar to that of the human enzyme (Anand et al., 2010) suggesting this enzyme could mimic human PGI in patients with tuberculosis. Other well known eukaryotic proteins with moonlighting functions are listed in Table 12.1. In case the reader is sceptical about the possibility that glycolytic enzymes could have biological actions other that driving glycolysis the recent report that cancer cells ulitilise an alternative glycolytic pathway (Vander-Heiden et al, 2010) reveals that we do not know everyting about this pathway. To strengthen this message, it was a well known finding that tumour cells secreted a reductase that reduced and activated plasmin allowing the production of the blood vessel inhibitor, angiostatin. It is now known that this secreted reductase is the glycolytic enzyme, phosphoglycerate kinase, which is secreted in large amounts by tumour cells (Lay et al, 2000).

Now it is easy to make unwarranted assumptions in biology. This applies to moonlighting proteins where it would be sensible to suppose that if one protein exhibits particular moonlighting actions then all homologues of this protein should also have these additional activities. This turns out not to be the case. Only one example of this will be provided at this stage and others will be briefly mentioned later in the text. Sperm have to undergo physiological and biochemical changes after ejaculation to be able to fertilise the oocyte. Collectively, these changes are termed ‘capacitation’ and result in functionally competent sperm. It would be natural to assume that the crucial importance of the capacitation

phenomenon would limit evolutionary change in this process. It was surprising to find that capacitation of mouse sperm required the active participation of two molecular chaperones – Hsp60 and Hsp90 (Asquith et al., 2004) with a potential additional involvement of Hsp10 (Walsh et al., 2008). The Hsp60 and Hsp90 proteins are surface located on sperm and require to be tyrosine phosphorylated to achieve capacitation. An even bigger surprise is the report that human sperm have none of these molecular chaperones on their surface and there is no evidence of cell surface tyrosine phosphorylation (Mitchell et al., 2007). Thus the evolutionary development of these moonlighting chaperones in the mouse has clearly occurred within the past 75 million years since the mouse diverged from the precursors of *Homo sapiens* (Stillman and Stewart, 2004). This reveals a fairly rapid evolutionary dynamic in the evolution of these molecular chaperone moonlighting sites. Studies of bacterial moonlighting proteins should be able to probe, in more detail, the evolutionary dynamics of moonlighting sites.

12.3. An Introduction to Protein Moonlighting in Bacteria

The first moonlighting bacterial protein described was the glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPD), which was found on the cell surface of group A streptococci (*Streptococcus pyogenes*) in studies that were conducted before the moonlighting concept was introduced (Pancholi and Fischetti, 1992). This protein was shown to be tightly attached to the cell surface by a mechanism which is still not defined. The isolated purified GAPD from the surface of this organism was found to bind to a variety of ligands including the major host adhesive glycoprotein, fibronectin and to lysozyme, as well as the cytoskeletal proteins, myosin and actin. Thus GAPD is truly is the prototypic bacterial moonlighting protein, as many bacterial moonlighting proteins are cytoplasmic proteins found on the cell surface and endowed with adhesive functionality. Indeed, Jeffery (2005) has proposed that proteomic analysis of proteins in ‘unusual’ sites is one way of identifying moonlighting proteins. Clearly, the mere identification of a protein on, say, the cell surface, does not mean that it is necessarily a moonlighting protein. However, it is a start for such analysis. Over the past decade a number of bacteria have been subject to proteomic analysis and a surprising number of cytosolic proteins have been found to exist on the outer bacterial surface. Various different streptococci have been studied in this regard and there is interesting variation and similarity in the populations of secreted cell surface proteins. In *Streptococcus agalactiae* the following cytosolic

proteins were identified on the outer surface of the cell: PGI, non-phosphorylating GAPD, enolase, purine nucleotide phosphorylase, ornithine carbamoyltransferase, cysteine synthase, superoxide dismutase, chaperonin 60, and DnaK (Hughes et al, 2003). In *Strep. oralis,* 27 cell surface proteins were identified including the glycolytic enzymes: fructose bisphosphate aldolase, triosephosphate isomerase (TPI), GAPD, enolase and phosphoglycerate kinase. However chaperonin 60 and DnaK were absent. In addition, the population of proteins on the cell surface changed when bacteria were grown under acidic conditions (Wilkins et al, 2003). Another streptococcal species, the swine pathogen, *Strep. suis,* has another set of cell surface proteins including: phosphoglycerate mutase, 6-phosphofructokinase, phosphopentomutase, amylase-binding protein, acetylserine lyase, chaperonin 10, chaperonin 60 and GrpE (Hsp90) (Wu et al, 2008). *Strep. pneumoniae* also has a number of glycolytic enzymes on its cell surface including lactate dehydrogenase. Interestingly, antibodies to many of these pneumococcal proteins are found in healthy individuals (Ling et al, 2004).

Surprisingly, the papers on the cell surface location of enzymes in the streptococci suggest that the whole of the glycolytic pathway enzyme cohort could be present on the bacterial cell surface. This raises the speculation that some bacteria may be able to drive glycolysis at the cell surface. To do this would require a source of glucose and ADP/ATP. It is possible that levels of these essential glycolytic substrates, present within specific environments of the bacterial host, may be high enough to drive this pathway. Indeed, a recent study has concluded that tethering glycolytic enzymes closely together on two-dimensional matrices dramatically increases the efficiency of glycolysis (Mukai et al, 2009). What would be the advantage to cell surface glycolysis? The products of glycolysis include a range of sugar metabolites and ATP. It is now appreciated that ATP is a potent signal, acting through ionotropic P2X receptors and metabotropic P2Y receptors (Corriden and Insel, 2010). In addition, it is possible that extracellular glycolytic intermediates may have intercellular signalling actions. If extracellular glycolysis does exist, and has cell signalling activity, this would be the first example of a moonlighting pathway rather than a moonlighting protein.

It is expected that the glycolytic pathway and its individual enzymes will present us with many surprises in the years to come and some of these surprises will be due to moonlighting actions. As an example, the glycolytic pathway in the model organism, *Bacillus subtilis*, is revealing an interesting pattern of protein-protein interactions. Two hybrid analysis has revealed interactions between

phosphofrrctokinase, phosphoglyceromutase and enolase suggesting these enzymes may form a complex within the cytoplasm. In addition, such interactomic analysis has established interactions between glycolytic enzymes and proteins involved in RNA degradation, suggesting the presence of additional moonlighting functions for these proteins (Commichau et al, 2009).

It is still early days in the study of bacterial protein moonlighting. However, it is already clear that a theme is emerging in the role of such proteins. Most of them seem to play some role in bacterial pathogenesis, either by acting as secreted cell signalling agents and/or cell surface bacterial adhesins. A confusing factor in the literature is the separation between the adhesive function and the signalling function of individual moonlighting proteins and it appears to be that many bacterial moonlighting proteins can both act as ligands for host receptors and as adhesins for either host matrix components and/or receptor proteins on the host cell. The remainder of this article will deal separately with the signalling and the adhesive actions of bacterial moonlighting proteins and the contribution such moonlighting makes to bacterial virulence behaviour. Bacteria are not the only microbes to utilise moonlighting proteins and so examples will be given of moonlighting in eukaryotic microbes where appropriate.

12.4. Bacterial Moonlighting Proteins that Signal to the Host

It is now well-recognised that signalling between commensal or pathogenic bacteria and their hosts is essential for mutual bacterial-host survival and for bacterial pathogenesis. Understanding such signalling has given rise to the discipline of Cellular Microbiology (Henderson et al, 1999). One of the most prevalent classes of bacterial moonlighting proteins, which function as extracellular signals for the host, are molecular chaperones and protein-folding catalysts. As will be seen, this type of protein is also implicated in moonlighting bacterial adhesion and cell invasion.

12.4.1.Molecular Chaperones and Protein-Folding Catalysts

Each cell contains a huge amount of protein (between 80 and 400 g/L (Ellis and Minton, 2006)) concentrated into a small volume. Such large protein concentrations favour the misfolding of nascent proteins and the aggregation of proteins generally. Such protein misfolding and protein aggregation is enhanced when cells are subject to stress. The evolutionary solution to protein misfolding and protein aggregation is the molecular chaperone (Gregersen and Bross, 2010) and protein-folding catalyst (PFC) (Gething, 1997). These proteins aid in the correct folding of proteins, in the inhibition of protein

aggregation and the solubilisation of existing protein aggregates. Moreover, many of these proteins (collectively termed stress proteins) dramatically increase in concentration in stressed cells (Richter et al, 2010). There are now around 25 families of molecular chaperones and PFCs (Gething, 1997; Makarow and Braakman, 2010) with probably a few hundred proteins (many being homologues) now identified (Table 12.2). New molecular chaperone types are still being found. For example, analysis of protein folding in *E. coli* has discovered a novel molecular chaperone termed Spy (Quan et al, 2011). Many molecular chaperones and PFCs are essential for cell viability.

The signalling activity of molecular chaperones and PFCs, which will, collectively, be termed cell stress proteins in this review, was first discovered with eukaryotic proteins, and a growing number of these proteins have been reported to be secreted by cells and function as intercellular signalling proteins or cell surface receptors (Henderson and Pockley, 2010). While there are many individual prokaryotic molecular chaperones only four of these proteins, in order of increasing molecular mass: chaperonin (Hsp)10, peptidylprolyl isomerase (PPI), chaperonin (Hsp)60 and DnaK (Hsp70) have been reported to have the ability to signal to human cells. This does not mean that other bacterial cell stress proteins do not act as cell signals, only that they have not been tested for this ability. This compares with around eighteen eukaryotic cell stress proteins with cell signalling activity (Henderson and Pockley, 2011). Most studies of moonlighting cell stress proteins in bacteria have focused on three pathogens: *Mycobacterium tuberculosis*, *Chlamydia pneumoniae* and *Helicobacter pylori* with a few other organisms studied in far less detail. These signalling actions of the cell stress proteins of these individual organisms will be dealt with in turn. The different bacteria that utilise moonlighting proteins in bacteria-host interactions are delineated in Table 12.3.

12.4.1.1.*Mycobacterium tuberculosis*

Chaperonin (Hsp)10 is a heptamer composed of 10kDa subunits and forms a cap for the chaperonin (Cpn)60 protein to aid in protein folding (Horwich et al, 2009). The human Cpn10 protein was originally identified as an immunosuppressant protein termed early pregnancy factor (Noonan et al, 1979), has been shown to inhibit both macrophage (Johnson et al, 2005) and T cell function (Zhang et al, 2003) and is being used in clinical trials for a variety of diseases including rheumatoid arthritis (Vanags et al, 2006) and psoriasis (Williams et al, 2008). It has also recently been reported to also be

an endothelial-derived differentiation factor (Dobocan et al, 2009). Thus the human homologue has some interesting moonlighting actions.

The mycobacterial Cpn10 protein is a powerful inducer of Th1 responses in patients with leprosy (Launois et al, 1995). Administration of *M. tuberculosis* Cpn10 to rats with adjuvant arthritis (Ragno et al, 1996) or mice with experimental allergic arthritis (Riffo-Vasquez et al, 2004) caused inhibition of these experimental lesions. Curiously, in spite of these various findings, it has also been reported that *M. tuberculosis* Cpn10 is a potent inducer of osteoclast formation and bone resorption in *in vitro* bone cell and organ cultures, with activity being associated with the flexible loop and residues 65-70, suggesting there is a single conformational unit which encompasses the bone-resorbing activity (Meghji et al, 1997).

Most information on moonlighting cell stress proteins in *M. tuberculosis* comes from the study of the chaperonin 60 proteins of this bacterium. Most mycobacteria encode two Cpn60 proteins termed Cpn60.1 and Cpn60.2 (Kong et al, 1993). The Cpn60.2 protein was the first such chaperonin discovered in *M. tuberculosis* and is also known as Hsp65. One of the first reports that molecular chaperones have extracellular signalling activity with human leukocytes was of the stimulation of human monocyte cytokine synthesis by *M. tuberculosis* Cpn60.2 (Friedland et al, 1993). This has led on to the study of a range of Cpn60 proteins from other bacteria and from rodents and humans (Henderson and Pockley, 2010). The assumption from Friedland's study was that *M. tuberculosis* Cpn60.2 stimulated so-called 'classic activation' of monocytes. This is the type of activation induced by bacterial lipopolysaccharide (LPS) or the cytokine, gamma-interferon (γ-IFN), and primes macrophages to ingest bacteria and present bacterial antigens to T lymphocytes on MHC II proteins. Markers of this form of activation include increases in cell surface MHC II and Fc receptor expression and upregulation of macrophage free radical production. The possibility that macrophages could undergo other patterns of activation, now termed alternative macrophage activation, was only introduced by Siamon Gordon in 1992 (Stein et al, 1992 see review by Martinez et al, 2009). When macrophages exposed to *M. tuberculosis* Cpn60.2 were examined for markers of classic macrophage activation, such markers were not present. Thus the influence of this molecular chaperone on human macrophages is to induce pro-inflammatory cytokine synthesis without, at the same time, inducing other cellular changes found in classically-activated macrophages (Peetermans et al, 1994). The

exact alternative activation state induced by *M. tuberculosis* Cpn60.1 has not been identified. Thus this particular Cpn60.2 protein appears to be inducing an alternative macrophage activation state. Indeed, it has been proposed that many molecular chaperones are able to induce such alternative macrophage activation states (Henderson and Henderson, 2009). As will be discussed in the next section, *M. tuberculosis* Cpn60.2 also acts as an adhesion. binding to CD43 on the macrophage surface (Hickey et al, 2009,2010). Such receptor binding may be responsible for this particular form of macrophage activation induced by Cpn60.2.

There is a growing realisation that the normal and pathological turnover of the largest organ system of the body, the skeleton, is controlled by cells of the immune system. This has given rise to a new discipline termed osteoimmunology (Takayanagi, 2009). Work from the principal author's lab revealed that the Cpn60 protein from the oral pathogen, *Aggregatibacter actinomycetemcomitans*, was a potent inducer of bone destruction *in vitro* (Kirby et al, 1995). The highly sequence identity homologous *E. coli* protein, GroEL, was also a potent stimulator of bone resorption (Kirby et al, 1995) and turned out to be a very active promoter of the formation of the multinucleated osteoclast population of bone – the cells responsible for bone breakdown (Reddi et al 1998). Curiously, the *M. tuberculosis* and *M. leprae* Cpn60.2 proteins failed to stimulate bone breakdown (Kirby et al, 1995) as did the recombinant *M. tuberculosis* Cpn60.1 protein (Meghji et al., 1997). The two *M. tuberculosis* Cpn60 proteins have >60% sequence identity and it was assumed they would have identical biological activity. Direct comparison of their effects on human monocytes revealed significant differences in their potency as cytokine inducers (Lewthwaite et al, 2001) and direct competition studies have shown that neither protein competes with the other for binding to human monocytes (Cehovin et al, 2010). Thus in spite of their sequence similarities, these proteins act as distinct cellular ligands and signalling proteins (Henderson et al, 2010). This was clearly shown when the effects of the two *M. tuberculosis* chaperonin 60 proteins were re-examined for their influence on bone resorption and on the formation of osteoclasts. This showed that the Cpn60.2 protein had no positive or negative effects on bone breakdown or osteoclast formation. In contrast, the Cpn60.1 protein proved to be a potent inhibitor of bone breakdown and osteoclast formation *in vitro* and blocked the massive osteoclast-driven bone destruction found in the joints of rats with adjuvant arthritis, without inhibiting joint inflammation. This

inhibition of osteoclast formation was associated with the inhibition of transcription of the key osteoclast transcription factor NFATc1 (Winrow et al, 2008).

The Cpn60.1 protein from *M. tuberculosis* is also a potent inhibitor of the eosinophilia and bronchial hyperreactivity found in mice with experimental allergic asthma, while the Cpn60.2 protein was inactive (Riffo-Vasquez et al, 2004). This has proved to be a very intriguing finding as an earlier study had shown that the *M. tuberculosis* Cpn60.2 protein was also inactive in this model, yet the *M. leprae* Cpn60.2 protein was a potent inhibitor of this allergic asthma model (Rha et al, 2002). What makes this finding intriguing is that the *M. leprae* and *M. tuberculosis* Cpn60.2 proteins exhibit >95% sequence identity and comparison of the Cpn60.1 and Cpn60.2 sequences from these two bacteria reveals only a handful of residues that could account for this major difference in biological activity of the Cpn60.2 proteins. These few different residues presumably point to the sequence of the moonlighting site that accounts for the anti-asthmatic effects of these proteins. The *M. tuberculosis* Cpn60.1 protein has also been shown to inhibit macrophage activation by the pro-inflammatory component of this bacterium, PPD. Such inhibition involves modulation of cell activity via TLR2 signalling (Khan et al, 2008).

The evidence suggests that the chaperonins of *M. tuberculosis* are important signalling proteins with some role to play in the interaction between the bacterium and the host macrophage. Curiously, the *M. tuberculosis* Cpn60 proteins behave very differently from the prototypic Cpn60 protein, the *E. coli* GroEL. GroEL is a tetradecamer comprising two 7-membered rings stacked back to back and with a molecular mass of around 860kDa (Krishna et al, 2007). In contrast, the Cpn60 proteins from *M. tuberculosis* appear not to form tetradecameric structures at the concentrations at which GroEL does and their ATPase activity is very low (Qamra et al., 2004). Further, the crystal structure of the *M. tuberculosis* Cpn60.2 protein is a dimer and not a tetradecamer (Qamra and Mande, 2004). It is unclear how these 60kDa chaperonins will fold proteins if they cannot form at least one ring structure.

To address the importance of the chaperonins in the virulence of *M. tuberculosis* efforts were made to inactivate the genes encoding Cpn10, Cpn60.1 and Cpn60.2 in the virulent *M. tuberculosis* strain H37Rv Hu et al, 2008). As *cpn10* and *cpn60.1* appeared to form an operon, it was assumed that these were the proteins essential for survival and therefore that only the gene encoding Cpn60.2 would be able to be inactivated. In fact, the only one of the three chaperonin genes able to be

inactivated was *cpn60.1.* The *Δcpn60.1* isogenic mutant grew normally in culture and within quiescent and activated macrophages and responded to major stresses in an identical manner to the wild-type organism. Thus it looked like the Cpn60.1 protein was not acting as a stress protein. This was confirmed in complementation experiments where it was shown that the *cpn10* and *cpn60.2* genes would complement an *E. coli* mutant with conditional inactivation of the *groES* (*cpn10*) and *groEL* (*cpn60*) genes. However, the *cpn60.1* gene failed to complement this mutant, supporting the hypothesis that this protein has evolved away from protein folding to some other function. This is also the conclusion from study of the *M. smegmatis* Cpn60 proteins (Rao and Lund, 2010) and may be a more general finding (reviewed by Lund 2009). With no *in vitro* phenotype it was assumed that the *Δcpn60.1* mutant would behave normally when used to infect animals. However, in both infected mice and guinea pigs, although the *Δcpn60.1* mutant grew as well as the wild-type organism, it failed to induce a granulomatous inflammation in the lungs (Hu et al, 2008). This finding was supported by *in vitro* studies of human granuloma formation from whole blood. Again, the *Δcpn60.1* mutant failed to induce the formation of multinucleate giant cells from monocyte precursors (Cehovin et al, 2010). This reveals some very interesting effects of the *M. tuberculosis* Cpn60.1 protein on myeloid cell differentiation. Thus this protein prevents the formation of osteoclasts but its absence is associated with the failure to generate multinucleate giant cells suggesting that this protein is somehow inducing giant cell formation and the formation of granulomas (Fig 2). As the granuloma is the hallmark of mycobacterial infection this finding suggests that the Cpn60.1 protein is an important contributor to this form of inflammation.

In the present context the differences in the biological actions of the Cpn60 proteins of the plant symbiotic bacterium *Rhizobium leguminosarum*, are relevant. This bacterium encodes three Cpn60 proteins of which the Cpn60.1 is the main housekeeping chaperonin and present in the highest amount while the other two are expressed at low levels and are not required for protein folding. Comparison of the human monocyte cytokine inducing activities of the Cpn60.1 and Cpn60.3 proteins of *Rh. Leguminosarum* revealed that in spite of around 80% sequence identity, only the Cpn60.3 protein was able to induce monocyte cytokine synthesis. The major chaperonin, Cpn60.1 was without any monocyte activating activity. It is not known what sequence of structural differences between these two proteins are responsible for this major difference in biological activity. It also shows that

even bacteria which have no normal interactions with mammals may have proteins able to influence immune functions (Lewthwaite et al, 2002).

The other major molecular chaperone that functions as a signalling molecule in *M. tuberculosis* is Hsp70 or DnaK. There are at least 13 human Hsp70 genes, of which at least three have been shown to act as intercellular signals, and one of these Hsp70 proteins, BiP (an immunosuppressive protein), is now in clinical trial for the treatment of rheumatoid arthritis (Henderson and Pockley, 2010). As has been touched upon, tuberculosis can be thought of as a disease of the macrophage/dendritic cell populations (Mortellaro et al, 2009) in which the production of specific chemokines is important in the immunopathology of the disease (Mendez-Samperio, 2008). Lehner's group in London, UK, were the first to show that the *M. tuberculosis* Hsp70 protein signalled to leukocytes, in this case primate CD8 lymphocytes, causing the release of the CC chemokines CCL3-5 (Lehner et al, 2000). Studies of the effects of *M. tuberculosis* Hsp70 on myeloids cells has shown that this protein stimulates monocytes to secrete CCL5 through a mechanism involving the transmembrane receptor of the TNFα gene superfamily, CD40 (Wang et al, 2001). This is an interesting finding in light of the recent report that CCL5 participates in protection against *M. tuberculosis* in the early phase of infection (Vesovsky et al, 2010). Most reports of Hsp70 binding to monocytes supports the hypothesis that signalling is through TLR4, although a number of other receptors have been implicated (Henderson and Pockley, 2010). There is one study of the human Hsp70 protein that concludes that this protein does bind to CD40. However the receptor binding site in the human Hsp70 protein is within the N-terminal ATP-binding domain (Becker et al, 2002), which is distinct from the identified receptor binding site in the *M. tuberculosis* Hsp70 protein (Wang et al, 2002) This clearly indicates that the moonlighting activity of these two Hsp70 proteins evolved independently. Truncation mutagenesis and peptide mapping identified the receptor binding site of *M. tuberculosis* Hsp70 to a 20-mer peptide sequence within the C-terminus (Wang et al, 2002,2005). Later studies from the same group also revealed that *M. tuberculosis* Hsp70 also bound the HIV co-receptor CCR5 (Whittall et al, 2006; Floto et al, 2006). Given the known synergy between infection with tuberculosis and HIV this is a very interesting finding. This raises the question as to whether this protein can block HIV binding to target cells? This experiment has been done and the answer is that *M. tuberculosis* Hsp70 can block HIV uptake, suggesting that this protein has some therapeutic potential (Babaahmady et al, 2007).

Since these studies were conducted it has been shown that *M. tuberculosis* releases large amounts of Hsp70 (Hickey et al, 2009). Indeed, an earlier paper had reported that *Mycobacterium bovis* released two ATPases which could inhibit ATP-induced monocyte apoptosis. One of these two enzymes turned out to be the Hsp70 protein of this bacterium (Zaborina et al, 1999).

12.4.1.2. *Helicobacter pylori*

This organism colonises the stomach, which must be one of the most stressful environments for any bacterium to occupy, thus one may expect that it would have an active cell stress response. It is therefore not surprising that *H. pylori* Hsp60 is a dominant antigen in patients with gastric disease due to this bacterium and that this is a useful diagnostic marker (Macchia et al, 1993; Yunoki et al, 2000). As with a number of other bacteria (to be discussed) Cpn60 is also found on the cell surface of *H. pylori*, which may account for its immunogenicity (Yamaguchi et al, 1996). Surprisingly, a monoclonal antibody to *H. pylori* Hsp60, when cultured with *H. pylori*, inhibits the growth of this organism, suggesting that, somehow, the surface location of this molecular chaperone controls intracellular mechanisms involved in cell division (Yamaguchi et al, 1997a). It is not known if this is related to the report that the cell surface Cpn60 of *H. pylori* binds to lactoferrin, which might have effects on cell growth (Amini et al, 1996).

Like the *M. tuberculosis* Cpn60 proteins, it has been reported that *H. pylori* Cpn60 can stimulate monocytes (Lin et al, 2005) and epithelial cells (Yamaguchi et al, 1999) to secrete cytokines. There is confusion in the literature in respect of the receptors required for such activation and the nature of the activating ligand. Most studies claim that the recombinant *H. pylori* Cpn60 protein works by binding to TLR2 or TLR4 (e.g. Takenaka et al, 2004; Zhao et al, 2007). However, using a non-recombinant form of the *H. pylori* Cpn60 protein no requirement for TLR2/4 or myeloid differentiation factor (MyD)88 was identified (Gobert et al, 2004). It is unclear what is responsible for the differences in these results. The *H. pylori* Cpn60 protein, like that of the *M. tuberculosis* Cpn60 proteins, in isolation, is a dimer or tetramer and not a tetradecamer. The activity of this protein in stimulating cytokine synthesis is reported to depend on the oligomeric status of the chaperone (Lin et al, 2009) so this may contribute to these differences in receptor requirement. This conflicts with the finding that the isolated equatorial domain of the *M. tuberculosis* Cpn60.1 protein is as active as the native protein (Tormay et al, 2005) and, again, reveals that moonlighting actions of homologous proteins can exhibit promiscuity of

mechanism. In addition to acting as a pro-inflammatory stimulus, the *H. pylori* Cpn60 protein is proposed to be involved in the process of gastric carcinoma formation (e.g. Lin et al, 2010).

The C-terminus of the *M. tuberculosis* Cpn60.1 protein contains 6 histidine residues and this protein can be purified on a Ni-NTA affinity column (Kong et al 1993). It has recently been reported that *H. pylori* contains a protein, HspA, that is a homologue of *E. coli* GroES. It has now been found that HspA contains a unique histidine-rich C-terminal extension that binds nickel. Deletion of the gene encoding HspA caused a decrease in intracellular nickel content and reduced nickel tolerance suggesting this Cpn10 homologue is a moonlighting protein involved in nickel sequestration and detoxification (Schauer et al, 2010).

The other molecular chaperone which acts as a *H. pylori* moonlighting signalling protein is the peptidyl prolyl isomerase (PPI 1075). Like Hsp60, this protein is secreted and is immunogenic in patients with gastric ulceration (Atanassov et al, 2002). There is growing evidence that human PPIs of the cyclophilin class are secreted and function as pro-inflammatory factors or cell growth factors (Henderson and Pockley, 2010). In contrast, the *H. pylori* PPI (1075), when cultured with a gastric epithelial cell line, induced apoptosis of these cells by a mechanism involving TLR4 and apoptosis signal-regulating kinase 1 (Basak et al, 2005). Inactivation of the gene resulted in a mutant with impaired ability to induce epithelial cell apoptosis. In addition to gastric epithelial cell destruction, the gastropathy associated with *H. pylori* infection involves an inflammatory response with overexpression of cytokines, particularly IL-6. Again, the PPI of *H. pylori* is a major inducer of monocyte-induced IL-6 production. Inactivation of the gene encoding this PPI results in an isogenic mutant with attenutated IL-6-inducing activity (Pathak et al, 2006).

The so-called high-temperature requirement A (HtrA) proteins of eukaryotes and prokaryotes are chaperones and serine proteases which monitor intracellular protein quality control. It has been found that *H. pylori* secretes this protein, which can proteolytically cleave E-cadherin allowing it to be shed and causing epithelial cell dissociation and, it is assumed, changes in cell signalling. This presumably can allow the bacterium to access the intercellular space in the gut (Hoy et al, 2010).

12.4.1.3. *Chlamydia spp*

The Chlamydia are obligate intracellular bacteria and the two best known in terms of human disease are *C. pneumoniae,* which causes approximately 10% of cases of community-acquired pneumonia

and 5% of cases of bronchitis (Burrilo and Bouzo, 2010) and *C. trachomatis* which is best known for its infection of the genital tract (Darville and Hiltke, 2010) and for trachoma (Burton and Mabey, 2009). The Chlamydia are also implicated in the pathogenesis of atherosclerosis (Watson and Alp, 2008). Most attention has focused on the moonlighting actions of the Cpn60 of the Chlamydia due to the perceived role of this protein in the pathogenesis of atheroma. It has already been pointed out that some bacteria have more than one Cpn60 protein. The Chlamydiae have three *cpn60* genes (Karunakaran et al, 2005) with all work on the signalling actions of these proteins being done on the Cpn60.1/GroEL1 protein. Of interest, in this context, is the report that the gene encoding Cpn60.2 in one serovar of *C. trachomatis* is under the response of the extracellular iron content, being greatly increased when the bacterium is maintained in iron-deficient conditions (LaRue et al, 2007). The first report of the signalling actions of *C. pneumoniae* Cpn60.1 was the ability of this protein to stimulate monocytes to secrete pro-inflammatory cytokines and metalloproteinases (Kol et al, 1998). It was then demonstrated that recombinant *C. pneumoniae* Cpn60.1 stimulated murine monocytes and human microvascular endothelial cells through, what is perceived to be, a conventional TLR4/MD-2/Myd88-dependent pathway (Bulut et al, 2009). Activity was heat labile and blocked by antibodies to *C. pneumoniae* Cpn60.1, thus controlling for LPS contamination. Recombinant *C. pneumoniae* Cpn60.1 also stimulated maturation of murine bone-marrow-derived dendritic cells in a TLR-2/4-dependent manner (Costa et al, 2002). A similar effect has been reported with *C. pneumoniae* Cpn60.1 as an inducer of human monocyte-derived dendritic cell maturation, which involved induction of expression of IL-12 and IL-23 (Ausiello et al, 2006).

It has already been described that administration of mycobacterial Cpn60.1 proteins can have therapeutic effects. However, these proteins have not been administered to healthy animals. *In vivo* administration of purified chlamydial Cpn60.1 to the periotoneal cavities of mice resulted in increased serum levels of the CXC chemokines CXCL1 and CXCL2 and marked accumulation of neutrophils. Significantly, Cpn60.1 was a more potent neutrophil attractant than was endotoxin or the CpG oligonucleotide 1668 (Da Cost et al, 2004). Intra-tracheal administration of recombinant *C. pneumoniae* Cpn60.1 in wild type mice resulted in local accumulation of inflammatory cells and up-regulation of cytokine levels (Bulut et al, 2009). These findings support the hypothesis that at least this bacterial Cpn60 protein is pro-inflammatory.

In addition to stimulating cellular cytokine synthesis, it has been reported that *C. pneumonia* Cpn60, but not Cpn10, is capable of inducing the oxidation of low density lipoprotein (LDL) (Kalayoglu et al, 2000). It also promotes the proliferation of human vascular smooth muscle cells by a mechanism dependent on TLR4 binding and the activation of p44/42 MAP kinase (Sasu et al, 2001).

Finally, a peptidyprolyl isomerase of *C. trachomatis* has been shown to be involved in the invasion of the targets cells by this bacterium (Lundermose et al, 1993).

12.4.1.4. Other Bacteria

There are a number of sporadic reports of molecular chaperones with various cell-cell signalling actions not covered in the previous sections. Most of these have used bacterial Cpn60 proteins. Some of these have multiple effects such as the Cpn60 protein of the oral bacterium *Aggregatibacter actinomycetemcomitans* which promote bone breakdown (Kirby et al, 1995), has effects on epithelial cell viability and cell turnover which may be relevant *in situ* (Zhang et al, 2004a) and stimulates epithelial cell migration (Zhang et al, 2004b). The Cpn60 of *Bartonella bacilliformis* is reported to induce apoptosis in cultured vascular endothelial cells (Smitherman and Minnick, 2005). The probiotic organism *Lactobacillus johnsonii* La1 (NCC 533) has Cpn60 on its cell surface and like some other bacterial Cpn60 proteins it is able stimulate epithelial cell IL-8 synthesis (Bergonzelli et al, 2006). With most Gram-negative bacteria the LPS is the most pro-inflammatory component of the organism. However, the causative agent of Tularaemia, *(Francisella tularensis*) a potentially fatal systemic disease, has an LPS with virtually no pro-inflammatory activity. In this organism it is the Cpn60 protein that is the major pro-inflammatory signal and this chaperone actually synergises with the LPS to activate human macrophages (Noah et al, 2010).

There are a small, but growing, number of examples of bacterial moonlighting proteins being involved in bacterial invasion and post-invasion events. The gene encoding a DnaJ-like protein (DjlA) in *Legionella dumoffi* was found in a transposon mutagenesis analysis to identify genes involved in intracellular growth. This has identified DjlA as a molecular chaperone involved in inhibiting lysosome-phagolysosome fusion in macrophages invaded by this organism (Ohnishi et al, 2004).

In addition to bacterial molecular chaperones signalling to human or rodent cells, there are a few examples of signalling to lower organisms. One of the most fascinating reports is of the insect known as the antlion or doodlebug (*Myrmeleon bore*) which bites and paralyses its prey through the action of

an insect neurotoxin. This neurotoxin was isolated and turned out to be the Cpn60 protein of the bacterium *Enterobacter aerogenes* which is a commensal in the saliva of this insect. Curiously, the sequence of this Cpn60 protein was virtually identical to that of *E. coli* GroEL and single residue mutations were sufficient to turn GroEL, which has no neurotoxic properties, into a potent insect neurotoxin (Yoshida et al, 2001). Another member of the enterobacteriaceae is *Xenorhabdus nematophila*, a virulent insect pathogen and also a symbiotic organism (Herbert and Goodrich-Blair, 2007). This bacterium also secretes a Cpn60 protein with insecticidal activity. Structure:function studies suggest that all three domains of the protein are needed for insecticidal activity and that this can be blocked by N-acetylglucosamine and chito-oligosaccharides. Generation of protein mutants identified the surface-exposed residues Thr347 and Ser356 as essential for binding to the target insect gut epithelium and for insecticidal activity (Joshi et al, 2008). However nothing is known of the mechanism through which Cpn60 exerts its neuro-toxic effect. Clearly, these two Cpn60 turned insect neurotoxins are dramatically different in their structure:function relationships and, again, this exemplifies the enormous variation that can occur in moonlighting proteins that have evolved the same moonlighting functions.

Another example of the role of the Cpn60 protein in cell-cell communication concerns biofilm formation in *Mycobacterium smegmatis.* Most bacteria form biofilms which have to be seen as equivalent to eukaryotic organ systems as they develop particular three-dimensional shapes to optimise oxygen and nutrient uptake and depend upon cell-cell interactions as well as secretd soluble signals (Hall-Stoodley et al, 2004). In *M. smegmatis,* biofilm formation does not occur in an isogenic mutant in which the *cpn60.1* gene has been inactivated (Ohja et al, 2005). In the absence of Cpn60.1, cells show normal planktonic growth but fail to form biofilms due to the role that this chaperonin plays in the formation of cell wall mycolic acids. In contrast, inactivation of the same gene in *M. tuberculosis* has no influence on biofilm formation (Hu et al, 2008). Another mycobacterial molecular chaperone involved in biofilm formation is the small (18kDa) heat shock protein of *Mycobacterium ulcerans* (Pidot et al, 2010).

12.4.2.Metabolic Enzymes

In eukaryotes the preponderant proteins showing protein moonlighting activity are enzymes involved in metabolic processes, such as the glycolytic pathway or the TCA cycle. However, there are only a

few such moonlighting proteins that act as cell signals. The same is true for prokaryotes with only a small number of examples of metabolic enzymes functioning as intercellular signals. The potential cell signalling activity of the PGI of *B. stearothermophilus* has already been described (Sun et al, 1999). Another possible signalling glycolytic enzyme is the fructose-bisphosphate aldolase of *Strep. pneumoniae* which binds to a member of the cadherin superfamily member, flamingo (Blau et al, 2007). The cadherins are a well established class of cell surface signalling receptor involved in a variety of cellular effects including leukocyte extravasation. Whether binding of this bacterial aldolase induces cell signalling remains to be determined.

The best example of a metabolic enzyme acting as a cellular signal is the glyceraldehyde 3-phosphate dehydrogenase (GAPD) of the group A streptococci. Indeed, the signalling properties of this streptococcal surface enzyme were identified before the introduction of the term protein moonlighting. A major surface protein of group A streptococci, termed streptococcal surface dehydrogenase (SDH), was identified as having homology with high sequence similarity to GAPD (Pancholi and Fischetti, 1992). This protein was tightly bound to the cell surface, being unable to be removed by 2M NaCl or 2% SDS and, as will be described, bound to a number of host proteins. This cell surface GAPD is found on virtually all streptococcal groups and in all group A streptococcal M strains tested (Pancholi and Fischetti, 1992). It was then shown that this streptococcal GAPD could auto-ADP ribosylate itself at a cysteine residue, resulting in inhibition of the GAPD activity (Pancholi and Fiscetti, 1993). This ADP ribosylation is also catalysed by eukaryotic GAPD enzymes (e.g. Dimmeler et al, 1992). ADP ribosylation was one of the first enzymic actions identified as being caused by bacterial toxins (Henkel et al, 2010) and it is now recognised that ADP ribosylation, caused either by endogenous or exogenous proteins, is a key mechanism in controlling cell functionality (Hottiger et al, 2010).

Did the cell surface GAPD on group A streptococci function to control the activity of target cells? Incubation of a human pharyngeal cell line with purified GAPD from *Strep. pyogenes* led to particular patterns of protein tyrosine phosphorylation in what is assumed to be membrane proteins. Such phosphorylation was related to the ability of group A streptococci to invade pharyngeal cells suggesting that the cell surface GAPD plays a major role in the invasiveness of this organism (Pancholi and Fischetti, 1997). It turns out that streptococcal GAPD is a rather sticky protein and it

took until 2005 for Pancholi to identify that there is a specific cell surface receptor for this protein – urokinase plasminogen activator receptor (uPAR Smith and Marshall, 2010) – on the surface of pharyngeal cells (Jin et al, 2005). Binding to uPAR can promote a wide range of cell signalling events and thus this could account for the significant tyrosine phosphorylation seen in earlier studies.

While these findings are extremely interesting, they really failed to capture the collective bacteriological imagination. This is largely due to the general feeling that GAPD on the cell surface is simply an artefact due to the death and dissolution of bacteria and the sticking of the released GAPD onto the surface of living bacteria. Indeed, this is often the general opinion of the finding of proteins 'where they should not be' – to ignore the finding. One of the great strengths of moderm molecular microbiology is the capacity, in most organisms, to inactivate selected genes for the purpose of testing hypotheses about the said gene product. However, in group A streptococci, the gene encoding GAPD is essential and therefore cannot be inactivated, and so the hypothesis that GAPD is an evolved cell surface enzyme could not be tested. In what can only be described as a brilliant alternative, Pancholi devised an alternative 'knockout strategy'. Instead of inactivating the *gapd* gene, his group generated a modified gene encoding a GAPD protein with a hydrophobic C-terminus in the hope that this protein would be unable to be secreted (Boel et al, 2005). This gene replaced the wild type gene and the isogenic mutant was shown to grow normally and contain a functionally active GAPD. However, levels of cell surface GAPD were extremely low as assessed by enzyme activity or with immunofluorescence. Now this cell surface mutant bound much less to pharyngeal cells, but the level of invasiveness was not reported. What was surprising was that this GAPD cell surface isogenic mutant was extremely sensitive to killing by culturing the bacteria in whole blood. In other words, the isogenic mutant had lost its innate anti-phagocytic capacity. How this anti-phagocytic activity was induced by the cell surface GAPD was not clear. However, in 2006 it was shown that the GAPD of group A streptococci interacts with the key chemotactic complement component and anaphylotoxin, C5a. Such interaction can form a non-functional complex or the GAPD can enhance the cell surface proteolysis of C5a (Terao et al, 2006). Such inhibition of C5a is an unexpected moonlighting action of this cell surface GAPD and reveals the richness of the moonlighting landscape of this one protein. Another example of the cell signalling actions of GAPD is the immunological activity of the *Streptococcus agalactiae* protein (Madureira et al, 2007). The recombinant GAPD from this bacterium

stimulated the formation of B lymphocytes in mice in a non-antigenic manner. Moreover, a *Strep. agalactiae* strain overexpressing GAPD was more virulent than the wild type strain in mice and this virulence was minimised in IL-10-deficient mice and in mice treated with an anti-GAPD antiserum. Thus this particular GAPD is an interesting immunomodulatory factor contributing to the immune pathogenesis of infection (Madureira et al, 2010).

Further support for the evolution of specific binding of GAPD to the outer bacterial cell wall comes from studies of the extracellular GAPD of *Lactobacillus plantarum*. Thus soluble GAPD from this bacterium does not bind to the organism's surface. Moreover, the presence of GAPD on the surface of this bacterium relates to the cell wall permeability of the organism (Saad et al, 2009).

There are only a few other examples of GAPD being involved in controlling microbial cellular function or in microbe-host interactions. For example, the ADP ribosylating activity of GAPD is involved in controlling spore germination in the fungus *Phycomyces blakesleeanus* (Deveze-Alverez et al, 2001). Phagocytosis of bacteria which normally survive intracellularly requires control over the fusion of the phagosome and the endosomal/lysosomal compartments. A key element in such control is the small GTPase Rab5, and this can be subverted by the action of the intracellular pathogen, *Listeria monocytogenes* (Alvarez-Dominguez et al, 1996). The *Listeria* protein controlling Rab5 has been found to be GAPD and this protein showed similarity in activity to the ExoS toxin of *Pseudomonas aeruginosa.* This reveals another putative novel virulence activity of the GAPD protein (Alvarez-Dominguez et al, 2008).

One of the key virulence protein families of *L. monocytogenes* are the internalins. This organism uses internalin (Inl)A and InlB to bind in a species-specific manner to the adhesion molecule E-cadherin and the hepatocyte growth factor receptor (HGFR) Met, respectively, to aid internalisation (Bonazzi et al, 2009). It is known that InlB mimics the intracellular action of Met in causing triggering of clathrin-dependent endocytosis and lysosomal degradation of Met. In other words InlB can be thought of as a moonlighting form of Met (Li et al, 2005).

12.5.Bacterial Moonlighting Proteins that Function as Adhesins

Colonisation of host organisms with commensals or pathogens requires that the bacteria selectively adhere to some component of the host or to some other bacterium, which is itself attached selectively to the host. In the latter case the bacteria are forming a mixed species biofilm. It is now recognised

that bacteria have evolved a very large number of molecules which have affinity for binding to one or other components of the host. These are generically termed adhesins and can be: sugars, lipids, peptides, proteins, protein aggregates and so on (Ofek et al, 2003). However, most of the high affinity adhesins of bacteria are proteins and the story is emerging that many bacterial adhesins are moonlighting proteins. There is now a growing literature on bacterial moonlighting adhesins and it is rather difficult to subdivide the growing number of such proteins into any sort of order. So there will be a somewhat arbitrary division of these proteins into groupings such a molecular chaperones, glycolytic enzymes, other metabolic enzymes, moonlighting fibronectin adhesins, and so on.

12.5.1. Molecular Chaperones Moonlighting as Bacterial Adhesins

At the present time a large number of bacteria (and other microbes), both Gram-negative and Gram-positive have been reported to have one or other molecular chaperones or protein-folding catalysts on their cell surface (Table 12.4). For a number of these organisms it has been shown that the cell surface molecular chaperone/protein folding catalyst functions as an adhesin which is important in the colonisation of the microorganism. For a growing number of such cell surface cell stress proteins the identity of the host receptor is becoming known. The early literature suggested that Cpn60 and Hsp70 recognised a variety of glycolipids (Tabe 12.4). Indeed, it has been shown that both prokaryotic and eukaryotic Hsp70 proteins contain a specific sulphogalactolipid binding site (Mamelak et al, 2001). In the more recent literature there is evidence for these surface cell stress proteins acting to bind to known cell surface protein receptors. These receptors include CD43, DC-SIGN, CCR5, CD40 etc.

For some important pathogenic bacteria there is evidence that cell surface molecular chaperones and protein-folding catalysts contribute significantly to colonisation and bacterial invasion of host cells. Thus *Legionella pneumophila*, which causes Legionnaire's disease (Cianciotto, 2001) has a key virulence factor termed macrophage infectivity potentiator (MIP) which is a member of the FKBP family of the peptidyl prolyl isomerases. Interestingly, the active site of this PPI is required for the ability of this bacterium to infect the target cells (Helbig et al, 2003). In addition to binding to cells and aiding invasion, the *L. pneumophila* PPI can also bind to a variety of collagens and this binding has been shown to be important in *in vivo* tissue invasion (Wagner et al, 2007). These findings make MIP

an important therapeutic target and small molecule MIP inhibitors are starting to be produced (Juli et al, 2010).

The chaperonin 60 protein is increasingly being recognised as a bacterial/microbial adhesin as well as being a signalling ligand. Early studies suggested that the causative agent of chancroid, *Haemophilus ducreyi*, used a cell surface bound Cpn60 as an adhesin for binding target cells (Frisk et al, 1998). More detailed study of this binding phenomenon (Pantzar et al, 2006) revealed that the Cpn60 protein from *H. ducreyi* can bind to a variety of glycosphingolipids including lactosylceramide, gangliotriaosylceramide and GM3 ganglioside. In Chlamydia species there are three genes encoding Cpn60 proteins (Karunakaran et al, 2003) with the Cpn60.1 protein appearing to be the major cell stress protein. This protein is also found on the cell surface of the organism and mediates the adhesion of this bacterium to host cells. Indeed, coating recombinant latex beads with *C. pneumoniae* Cpn60.1 allows these beads to attach to target cells. Surprisingly, the other two Cpn60 proteins of this bacterium have no cell adherent properties (Wuppermann et al, 2008). This, yet again, reveals the specific nature of moonlighting protein activity.

There is increasing interest in the moonlighting roles of the molecular chaperones of the mycobacteria and *M. tuberculosis* uses both Cpn60 proteins and Hsp70 as virulence factors. The Cpn60.2 protein of *M. tuberculosis* is well recognised as a powerful immunogen and also a monocyte cytokine-inducing protein (Henderson et al, 2010). In addition to these functions it is now recognised that the Cpn60.2 protein is also found on the surface of *M. tuberculosis* where it functions as an adhesin for the binding of this bacterium to human monocytes (Hickey et al 2009). The receptor on the host macrophages recognising the Cpn60.2 protein is CD43 (Hickey et al, 2010), a protein which has been recognised to be involved in the control of the intracellular growth of *M. tuberculosis* (Randhawa et al, 2008). This raises the question of whether binding of *M. tuberculosis* Cpn60.2 to CD43 controls the intracellular growth of this pathogen?

Like the glycolytic enzymes, it is generally difficult to inactivate the genes encoding molecular chaperones and protein-folding catalysts and so direct testing of the hypothesis that cell surface cell stress proteins are involved in adhesion and subsequent virulence is rarely possible. However, with the fungus, *Histoplasma capsulatum*, the causative agent of histoplasmosis, it has been reported that monoclonal antibodies to the Cpn60 protein of this organism significantly prolong the survival of mice

infected with this fungus (Guimarães et al 2009). This fungus has a cell surface Cpn60 protein which binds to CD11/CD18 on target cells (Long et al, 2003). Treated animals revealed reduced intracellular fungal survival, revealing that the Cpn60 protein of this organism is important in the pathogenesis of infection.

It is therefore clear that many bacteria (and other microbes) contain a number of molecular chaperones and protein-folding catalysts on their cell surfaces and that these proteins can play important roles in adhesion of the organism to host matrices and host cells and such binding can promote virulence. It will now be important to identify, structurally, just how these molecular chaperones function as host receptor ligands.

12.5.2. Glycolytic Enzymes as Bacterial Adhesins

A surprising finding is that most of the proteins of the glycolytic pathway in bacteria have some adhesive actions. Most attention has thus far focused on bacterial glycolytic enzymes but there are some reports of eukaryotic glycolytic proteins having adhesive properties and these may be relevant to the prokaryotic situation.

Hexokinase is the first enzyme of the glycolytic pathway and so far it has not been shown to have specific adhesive activity in bacteria. However, in the protozoan, *Leishmania donovani*, it moonlights as a receptor for haemoglobin (Krishnamurthy et al, 2005). In mammals there are four hexokinase isozymes of which types I and II bind to the mitochondria and can enhance mitochondrial oxidation and decrease mitochondrially driven apoptosis. This is due to the interaction of hexokinase with the Voltage Dependent Anion Channel 1 (VDAC1) and enhancing such binding may have anti-cancer potential (Rosano et al 2011). As the mitochondrion was originally a bacterium, such binding to hexokinase may also occur with true bacteria but may simply not have been looked for, and so, not reported.

The next two enzymes of glycolysis, phosphoglucoisomerase and phosphofructokinase, have not been reported to function as adhesins although, as described earlier, phosphoglucoisomerase as AMF is involved in tumour cell invasion and so may also interfere with cell adhesion. However, the next enzyme in the pathway, fructose-bisphosphate aldolase (FBA), which is a cell surface protein in streptococci (Wu et al, 2008), has been reported (in *Strep. pneumoniae*) to bind to the human cadherin homologue of the *Drosophila* Flamingo cadherin receptor (Blau et al, 2007). A number of

bacteria utilise these cadherins for cell binding. Of interest, a newly identified group of environmental bacteria that metabolise complex polysaccharides contains a member, *Saccharophagus degradans* strain 2-40, which encodes a number of very large proteins which contain multiple cadherin domains and these proteins are thought to contribute to cell-cell interactions within these organisms (Fraiberg et al, 2010). *Neisseria meningitidis* also contains a cell surface FBA and generation of a mutant deficient in this glycolytic enzyme revealed that there was no effect on bacterial growth but the ability of the organism to bind to target cells was significantly affected revealing that this protein is acting as an adhesin (Tunio et al, 2010a). A cell surface aldolase is also involved in the invasion of the protozoan *Toxoplasma gondii* and mutational analysis has shown that enzymic activity it not required for cell invasion (Starnes et al, 2009).

The next enzyme of glycolysis is triose-phosphate isomerase (TPI). The only reported example of this enzyme being surface located and involved in adhesion is that the TPI of *Staphylococcus aureus,* which is reported to be on the surface and to recognise glycan components of the fungus *Cryptococcus neoformans.* This work started with the discovery that *S. aureus* would kill *C. neoformans* if these organisms were co-cultured and that capsular polysaccharide from the fungus could inhibit such killing (Saito and Ikeda, 2005). The hypothesis is that TPI is a cell surface bound enzyme in *S. aureus* (Yamaguchi et al, 2010) which recognises and binds to glycans in the capsule of the fungal pathogen (Ikeda et al, 2007; Furuya and Ikeda, 2009)

12.5.2.1. Glyceraldehyde-3-phosphate Dehydrogenase

The discussion now returns to the subject of GAPD. In the previous section on GAPD as a cell signalling ligand, it was obvious that this protein also enabled group A streptococci to bind to target cells (e.g. Boel et al, 2005). There are also reports of other bacteria using cell surface GAPD to bind to host cells. For example, *Mycoplasma suis* binds to and colonises erythrocytes from a variety of vertebrates. The adhesin for such binding has been identified as GAPD (Hoelze et al, 2007). In *Neisseria meningitidis* there are two genes encoding GAPD proteins (Gap-A-1 and -2). Only one of these, GapA-1, is present on the cell surface. Inactivation of the gene encoding this protein resulted in an isogenic mutant with markedly decreased ability to bind to human epithelial or endothelial cells (Tunio et al, 2010b).

In addition to binding to cells, GAPD from a growing number of bacterial species, and from other microorganisms, has been shown to bind to a variety of host ligands including transferrin, plasminogen and fibronectin. One of the first such ligands identified was the iron-binding protein, transferrin and it was reported that a cell surface GAPD on *S. aureus* and *Staphylococcus epidermidis* bound to transferrin, and also to plasmin (Modun and Williams, 1999). This initial report of the transferrin-binding actions of GAPD was refuted by another group who claimed that the cell surface transferrin binding protein of *S. aureus* was a completely different protein (Taylor and Heinrichs, 2002). However, in support of the initial claim, other workers have reported that GAPD is a cell surface transferrin receptor in *Trypanosoma brucei* (Tanaka et al, 2004) and human and mouse macrophages (Raje et al, 2007). Clearly, further work is required to determine if GAPD is a general transferrin binding protein in bacterial species.

The GAPD from group A streptococci was first identified as a plasminogen binding protein in the early 1990s (Lottenberg et al, 1992). Recruiting plasminogen to a bacterial surface can promote the formation of the active protease, plasmin, allowing the bacterium to degrade the host's extracellular matrix and gain entry into the tissues (Lähteenmäki et al, 2005). This has been shown with the lung pathogen *Strep. pneumoniae* (Attali et al, 2008) and *Strep. agalactiae* (Magalhães et al, 2007) and in both organisms binding to plasminogen enhances virulence. Since this initial discovery a number of bacteria and other microorganisms have been shown to utilise cell surface GAPD to bind plasminogen. Binding of the *Stretococcus equisimilis* GAPD to plasmin(ogen) has been assessed by surface plasmon resonance and has revealed a Kd of 220nM for binding to plasminogen and 25nM for the binding to plasmin (Gase et al, 1996). These data reveal that the binding between GAPD and plasmin(ogen) is of relatively high affinity and is not simply some non-specific interaction. However, the exact role of GAPD binding to plasminogen in bacterial virulence is still unclear. Thus, Winram and Lottenberg (1998) identified a C-terminal lysly residue as responsible for high affinity plasmin binding by *Strep. pyogenes* GAPD. The gene encoding this mutated protein was used to replace the wild type *gapd* gene. However, this site-directed isogenic mutant bound as much plasminogen as the wild type organism. This finding does not square with the study in which the *Strep pyogenes* GAPD was mutated such that it could not be secreted. In this study (Boel et al, 2005) there was less plasminogen binding by the mutant.

A number of Gram-positive bacteria including: group B streptococci (Seifert et al, 2003), *Strep. suis* (Jobin et al, 2004), *Strep. pneumoniae* (Bergmann et al, 2004), *Lactobacillus crispatus* (Hurmalainen et al, 2007; Antikainen et al, 2007) and *Bacillus anthracis* (Matta et al, 2010) have cell surface GAPDs which bind plasminogen. In the latter organism, immunisation of mice with the bacterial GAPD protein conferred significant protection from infection with the bacterium (Matta et al, 2010). To date there is only one report of GAPD as a cell surface plasminogen binding protein in a Gram-negative bacterium. Enterohaemorrhagic and enteropathogenci strains of *E. coli* secrete GAPD which binds to plasminogen (Egea et al, 2007). Non-pathogenic strains of this bacterium do not secrete GAPD. Curiously, analysis of the *E. coli* GAPD has revealed two different electrophoretic variants with only the more basic being secreted. Indeed, GAPD binding to plasminogen is not simply confined to bacteria as there are reports that the multicellular parasites *Onchocerca volvulus* (Erttmann et al, 2005) and *Schistosoma bovis* (Ramajo-Hernández et al, 2007) also use this enzyme to bind plasminogen.

Finally, an early report of GAPD surface binding in *Strep. pyogenes* found binding to fibronectin (Pancholi and Fischetti, 1992). The protist, *Trichomonas vaginalis* has also been reported to have a surface associated GAPD which binds to fibronectin (Lama et al, 2009).

12.5.2.2. Enolase

Enolase is the other major cell surface moonlighting glycolytic enzyme of prokaryotes and eukaryotes. However, before turning to this protein, what is known of the moonlighting actions of the two glycolytic enzymes, phosphoglycerate kinase and phosphoglycerate mutase, that lie between GAPD and enolase will be briefly described. Phosphoglycerate kinase has been found on the cell wall of *Candida albicans* (Alloush et al, 1997) and the Gram-negative organism *Aeromonas salmonicida* (Ebanks et al, 2005) as well as in Gram positive organisms. It is involved in the binding of plasminogen by oral streptococci (Kinnby et al, 2008). In group B streptococci, phosphoglycerate kinase is a cell surface enzyme which appears to be involved in inhibiting binding of bacteria to epithelial cells (Burnham et al, 2005). The role of this protein is still inexplicable. Phosphoglycerate mutase is also found on the surface of oral streptococci (Kinnby et al, 2008) and by Wu et al (2008). In *M. tuberculosis* this enzyme has been reported to be involved in resistance of the bacterium to oxidative stress (Chaturvedi et al, 2010). In the yeast, *Candida albicans*, phosphoglycerate mutase is

found on the cell surface where it acts as: (i) a plasminogen binding protein (Crowe et al, 2003) and a complement binding protein, specifically for factor H and factor H-like (FHL-1) protein (Polterman et al, 2007). Binding of factor H and FHL-1 is a key event in the pathogenesis of streptococcal infection (Oliver et al, 2008) and thus phosphoglycerate mutase may contribute to the virulence of these bacteria.

Returning to the subject of this section, enolase, it is established that this protein has multiple moonlighting actions in both eukaryotes and prokaryotes (Pancholi, 2001). For example, enolase in involved in tumour biogenesis and is now seen as a therapeutic target (Capello et al, 2011). Encystation is a survival strategy used by many parasites. Enolase has recently been shown to be involved in the encystation mechanism of *Entamoeba invadens* (Segovia-Gamboa et al, 2010.) A growing number of bacteria and other microbes are reported to express moonlighting enolases on their cell surfaces. Currently, most examples of bacteria with cell surface enolase are Gram positive, but there are also Gram negative (e.g. *Borrelia burgdorferi* (Nowalk et al, 2006)) in this group. Attention has largely focused on the cell surface enolases of Group A streptococci. Pancholi and Fischetti, whose work on *Strep. pyogenes* cell surface GAPD was discussed, have also been responsible for much of the work on streptococcal cell surface enolase and its role in bacterial virulence. The complex plasminogen activation system of the vertebrate is essential for survival and is also used by tumour cells to invade tissues (Dano et al, 2005). This capacity of the plasminogen activation system to catalyse tissue invasion has meant that it is an evolutionary target for bacterial plasminogen activators and receptors (Lähteenmäki et al, 2001, 2005). Analysis of the plasminogen binding characteristics of cell wall proteins of *Strep. pyogenes* identified enolase as the strongest binder, with this cell surface enolase being enzymically active, and antibodies to it induced opsonisation and enhanced phagocytosis (Pancholi and Fischetti, 1998). Enolase is now recognised to be present on the surface of most streptococci (Pancholi and Fischetti, 1998) including *Strep pneumoniae* (Bergmann et al, 2001). Plasminogen binding to the surface of pneumococci enables the bacteria to penetrate a synthetic basement membrane gel (Matrigel™). It is this process that is believed to be important for the invasion of this organism and the consequence of invasion - meningitis (Eberhard et al, 1999). In a separate study it was shown that soluble recombinant *Strep. pneumoniae* enolase bound to the surface of the pneumococci even when associated with

plasminogen. Treatment of the cell surface with proteases inhibited such re-association suggesting that binding was due to protein-protein/peptide interactions (Bergmann et al, 2001). Inactivation of the enolase gene in *Strep. pneumoniae* resulted in non-viable cells, showing the essential nature of this protein, presumably for its role in glycolysis (Bergmann et al, 2001). In mammalian enolases binding to plasminogen is dependent on C-terminal lysyl residues in the enolase which binds to lysine binding sites in the plasminogen (Redlitz et al, 1995). To test if pneumococcal enolase also bound plasminogen through these C-terminal lysines, the enolase was treated with carboxypeptidase or was mutated at Lys-433 and Lys-434 resulting in inhibition of enolase binding to plasminogen (Bergmann et al, 2001). In *Strep. pyogenes*, binding of native and C-terminal mutated enolase to native plasminogen, termed Glu-plasminogen and plasminogen after cleavage by plasmin, termed Lys-plasminogen, was investigated. Deletion or substitution of the lysines in enolase at positions 434-435 resulted in significant decreases in the binding of this glycolytic enzyme to both forms of plasminogen. Moreover, the bacteria encoding the mutated enolase demonstrated a significant decrease in the ability to acquire plasminogen from human plasma and penetrate a synthetic extracellular matrix (Derbise et al, 2004). The lysines at position 252 and 255 also contribute to plasminogen binding (Cork et ak, 2009). This supports earlier studies of the plasminogen binding sites of the enolase of *Strep. pneumoniae* which had identified residues 248-256 in enolase (FYDKERKVYD) as an additional internal plasminogen-binding motif (Bergmann et al, 2003). A similar binding site has been identified in *Bifidobacteria* spp enolases (Candela et al, 2009).

The contribution of enolase-plasminogen binding to bacterial virulence is still unclear in the absence of gene inactivation studies. The surface enolase of *Streptococcus suis* binds to plasminogen with high affinity (Kd = 21nM) and antibodies to this protein inhibit the adhesion and invasion of the organism into microvascular endothelial cells (Esgleas et al, 2008). The recombinant enolase of the Gram-negative organism, *Aeromonas hydrophila*, (which also binds to plasminogen) has been used to immunise mice and this markedly decreased the pathology consequent upon infection with this bacterium (Sha et al, 2009). These data confirm that enolase binding is a virulence attribute for these bacteria.

It is not only plasminogen that bacterial enolases bind to. The enolase of *Strep. gordonii* binds to the salivary mucin, Muc7 (Kesimer et al, 2009). The *Lactobacillus plantarum* enolase binds to fibronectin

(Castaldo et al, 2009). In addition, the cell surface enolase of the vaginal commensal organism, *Lactobacillus jensenii*, is a potent inhibitor of the adherence of *Neisseria gonorrhoeae* to epithelial cells (Spurbeck and Arvidson, 2010).

Other functions have been ascribed to the bacterial cell surface enolase. For example the enolase of *Strep. sobrinus* is an immunosuppressive protein (Veiga-Malta et al, 2004) which can be used, if administered orally, to protect against dental caries in the rat (Dinis et al, 2009). In contrast, with *Paenibacillus larvae,* the Gram-positive causative agent of American Foulbrood (AFB), which affects the larvae of the honeybee, *Apis mellifera*, the enolase is a secreted highly immunogenic protein which is thought to play a role in the virulence of this bacterium (Antunez et al, 2010).

Surprisingly, little is known about the binding of enolase to the bacterial surface. It has been shown that enolase (and GAPD) associate with the surface lipoteichoic acids of *Lactobacillus crispatus* at pH 5 but dissociate at alkaline pH (Antikainen et al, 2007).

Of the two remaining enzymes of glycolysis, pyruvate kinase is a cell surface enzyme in *Lactococcus lactis* which can bind the hyper-mannosylated yeast invertase (Katakura et al, 2010). Thus far there are no reports of cell surface lactate dehydrogenase on bacteria.

12.5.3. Other Bacterial Moonlighting Adhesins

Moonlighting adhesins other than molecular chaperones and glycolytic enzymes exist. However, one of the problems in bringing together the literature on these proteins is attempting to find some common thread to the discussion. Fortunately, the most abundant of the moonlighting bacterial adhesins are proteins that bind to the complex host adhesive glycoprotein, fibronectin. This protein is found in high concentration in the fluids of the body and in the extracellular matrix (ECM) and plays a major role in linking cells to the ECM through specific integrins, which can function as transducers of changes in the matrix (Henderson et al, 2011). Fibronectin has a complex domain structure with different parts of the protein binding to different host components including heparin, collagen, gelatin, fibulin, DNA and so on (Henderson et al, 2011). The binding of *Strep. pyogenes* GAPD (Pancholi and Fischetti, 1992) and the *Lactobacillus plantarum* cell surface enolase (Castaldo et al, 2009) to fibronectin has already been discussed. Another Gram-negative moonlighting fibronectin binding protein is the C5a peptidase of group B streptococci. This protein was identified as cleaving and inactivating the complement component and anaphylatoxin, C5a. It also binds fibronectin and

inactivation of the gene encoding this protein results in bacteria having 50% less fibronectin binding capacity than the wild type organism (Beckmann et al, 2002).

Mycobacterium tuberculosis secretes three protein homologues, termed the antigen 85 complex, consisting of proteins 85A, 85B and 85C. These are the products of three different genes located at different loci in the genome and showing significant nucleotide and amino acid sequence identity and marked immune cross-reactivity (Wicker and Harboe 1992). Proteins are in the mass range from 30-31kDa and are all able to bind to fibronectin (Abou-Zeid, 1988; Wicker and Harboe, 1992). In this guise these proteins are also termed fibronectin binding proteins (FBPs)1-3. The site of interaction of the antigen85 complex proteins has been reported variously as the gelatin binding domain for the *M. bovis* protein (Peake et al, 1993), and the heparin and cell-wall binding regions for the *M. kansasii* protein (Naito et al, 2000). It turns out that in addition to fibronectin binding, the antigen 85 complex proteins contain a carboxylesterase domain and act as mycolyltransferases, which are proteins involved in the final stages of the assembly of the complex mycobacterial cell wall (Belisle et al, 1997). All three proteins appear to have the same, or similar, enzymic roles in terms of transferring mycoloyl residues (Puech et al, 2002). This clearly defined these molecules as moonlighting proteins. The fibronectin-binding motif in the antigen 85 complex proteins forms a helix at the surface of the protein and has no homology to other known prokaryotic and eukaryotic fibronectin binding features and appears to be unique to the mycobacteria (Naito et al, 1998). It is also argued that a large region of conserved surface residues among antigen85 proteins A, B and C is a probable site for the interaction of these proteins with fibronectin (Ronning et al, 2000). Another mycobacterial fibronectin binding protein brings us back to the role of metabolic enzymes in protein moonlighting. The malate synthase of *M.tuberculosis*, a cytoplasmic protein involved in the glyoxylate pathway, a cytoplasmic metabolic pathway, has also been found to occur on the bacterial surface, associating by an unknown mechanism, where it can bind both fibronectin and laminin. The binding site in the malate synthase for fibronectin lies in a C-terminal region of the protein that is unique to *M. tuberculosis,* but it is not known to which domain in fibronectin it binds (Kinhikar et al, 2006). This is the first glyoxylate cycle enzyme shown to be present on the bacterial cell surface.

The mycoplasmas are cell-wall-less organisms that have evolved from a Gram-positive ancestor, and are probably the smallest living form capable of autonomous growth. Using fibronectin affinity

chromatography two fibronectin binding proteins, of 30 and 45kDa were identified in *Mycoplasma pneumonia* and N-terminal sequencing identified these proteins as elongation factor (EF)-Tu and the β-subunit of pyruvate dehydrogenase (Dallo et al, 2002). Elongation factor (EF)-Tu is normally assumed to be a cytoplasmic protein responsible for critical steps in protein synthesis. Pyruvate dehydrogenase is an enzyme complex formed of two α and one β-subunit which transform pyruvate into acetyl CoA for mitochondrial oxidation (Dallo et al, 2002). Recombinant versions of these proteins were shown to bind fibronectin. Antibody labelling revealed that both of these proteins were present on the surface of *M. pneumonia* and both antibodies could inhibit the binding of *M. pneumonia* to fibronectin. Subsequent studies revealed that a 179 residue region in the C-terminus of EF-Tu is responsible for fibronectin binding. Using C-terminal constructs and truncation mutants, two distinct sites with different fibronectin-binding efficiencies were identified. Immunogold electron microscopy, using antibodies raised against recombinant constructs, demonstrated the surface accessibility of the EF-Tu carboxyl region and fractionation of mycoplasma confirmed the association of EF-Tu with the mycoplasma outer membrane (Balasubramanian et al, 2008). As has been stated, the rules governing protein moonlighting are not understood. This may explain why the EF-Tu protein of *Mycoplasma genitalium* does not bind to fibronectin even though it shares 96% identity with the *M. pneumoniae* protein. This has enabled the moonlighting site in *M. pneumoniae* EF-Tu for binding to fibronectin to be identified. Substitutions of amino acids: serine 343, proline 345, and threonine 357 markedly reduced the Fn binding of the *M. pneumoniae* EF-Tu. Moreover, synthetic peptides corresponding to residues 340-358 in this *M. pneumonae* EF-Tu protein were able to block the binding of recombinant EF-Tu to fibronectin and also the binding of *M. pneumoniae* to this protein (Balasubramanian et al, 2009).

Autolysins are important peptidoglycan-degrading enzymes. A number of the autolysins of the staphylococci have been shown to also function as fibronectin binding proteins. These include Aaa (autolysin/adhesion of *S. aureus*) which binds fibronectin with high affinity (Kd = 30nM) and which is involved in bacterial adherence to fibronectin (Heilmann et al, 2005). *Staphylococcus epidermidis* Aae (autolysin/adhesin in *S. epidermidis*) is a homologue of *S. aureus* Aaa and binds to the 29kDa heparin-binding module of fibronectin (Heilmann et al, 2003). Two other staphylococcal autolysins also function as fibronectin binding proteins. These are large (155kDa) homologous proteins – *S.*

caprae Atlc (autolysin caprae) (Allignet et al, 2001) and *S. saprophyticus* Aas (Hell et al, 1998) which, interestingly, have no obvious cell wall anchor motif. AtlC is the only fibronectin binding protein so far identified in *S. caprae* and it is a bifunctional enzyme that contains a repeat region (R1-R3), with no recognisable similarity to other proteins, sandwiched between two enzymic domains. The repeat region is responsible for binding to fibronectin, but exactly what binds is still unclear. Using far-western blots, only recombinant R1-R3 and R3 alone bind fibronectin. In contrast, using ELISA or surface plasmon resonance methods, all recombinant domain constructs bind fibronectin (Allignet et al, 2001). The binding site for fibronectin in the *S. saprophyticus* autolysin has been localised as lying between the two enzymic domains, within residues 714-1202, and inactivation of the gene was shown to result in loss of fibronectin binding (Hell et al, 1998). The AtlA autolysin of *S. mutans* is also a fibronectin binding protein and inactivation of the gene encoding this protein decreases the virulence of this organism in a rat model of infective endocarditis (Jung et al, 2009). Inactivation of the autolysin gene encoding the IspC protein of *Listeria monocytogenes* (found on the cell surface), had, contrary to expectation, no effect on rates of cell growth or cell separation but had a major influence on *in vivo* virulence of the bacterium (Wang and Lin, 2008). This appeared to be, at least in part, due to a decreased ability to bind to target cells.

While not likely to be a moonlighting protein, the CagL protein of *H. pylori* is a small cell surface protein (of 26kDa) with an RGD motif, which mimics the activity of host fibronectin and can modulate host cell signalling (Tegtmeyer et al, 2010).

A number of other bacterial proteins moonlight as adhesins. Again, these are examples of cell surface metabolic enzymes functioning as adhesins. The pentose phosphate pathway enzyme, 6-phosphogluconate dehydrogenase (6PGD) is a cell surface located enzyme which also acts as an adhesin in various pneumococcal strains. Binding of *Strep. pneumoniae* to lung epithelial cells was inhibited by recombinant 6PGD and immunisation of mice with this protein gave significant protection against infection with *Strep. pneumoniae* (Daniely et al, 2006). A similar story was found with the 6PGD of *Streptococcus suis* with the immunisation of mice with this protein able to provide 80% protection against a lethal dose of the bacterium (Tan et al, 2008). These results clearly show the virulence phenotype that can be induced by a moonlighting protein.

The protozoan, *Trichomonas vaginalis*, which causes the sexually trasmittted disease Trichomonosis, binds to the vaginal epithelium. The hydrogenosomal enzyme pyruvate:ferredoxin oxidoreductase is an iron-induced cell surface protein which acts as an epithelial cell adhesin in this organism (Moreno-Brito et al, 2005).

Perhaps the most intriguing moonlighting adhesin is the alcohol acetaldehyde dehydrogenase of *Listeria monocytogenes*. Listeria adhesion protein (LAP) was identified as a key cell-binding adhesin of this organism (Santiago et al, 2006) allowing the bacterium to bind to intestinal epithelial cells. The surprising finding was then made that the host cell receptor for LPS was human (Cpn)60 or heat shock protein (Hsp)60 (Wampler et al, 2004). Surprise followed surprise when LAP was identified as the alcohol acetaldehyde dehydrogenase of *L. monocytogenes* – yet another metabolic enzyme involved in moonlighting. Measurement of the kinetics of the interaction of LAP with human Cpn60, using surface plasmon resonance, revealed a Kd value in the low nanomolar range, which is a respectable binding affinity (Kim et al, 2006). So, like the example of PGI as AMF binding to a ubiquitin ligase here we have another example of a moonlighting protein (alcohol acetaldehyde dehydrogenase) binding, with high affinity, to another moonlighting protein (human Cpn60) to allow a bacterium to colonise its host and cause disease. How may other such examples will Nature provide us with? Further analysis of LAP/alcohol acetaldehyde dehydrogenase in non-pathogenic strains of *Listeria* have found that while these strains produce this enzyme there is very little of it on the bacterial surface and so only pathogenic strains bind to target cells via LAP/Hsp60 binding (Jagadeesan et al, 2010). As human Cpn60 (Hsp60) is a stress protein, the role of cell stress in *Listeria* infection has been examined. Thus exposure of CaCo-2 cells used for infection assays to various stressors increased intracellular Hsp60 levels and enhanced the adhesion, but not invasion of *L. monocytogenes.* Knock-down of Hsp60 with inhibitory RNA reduced the adhesion and translocation of wild-type *L. monocytogenes* but a *lap* mutant showed unchanged adhesion. Overexpression of Hsp60 enhanced wild type adhesion and cellular translocation but there was no change in the *lap* mutant. Of importance, infection with *L. monocytogenes* increased plasma membrane expression of Hsp60. Thus there is a dynamic response between these two moonlighting proteins to enhance *L. monocytogenes* infection (Burkholder and Bhunia, 2010).

As final examples of 'moonlighting adhesivity' serum opacity factor, a streptococcal virulence factor which binds to high density lipoproteins and disrupts them forming large lipid vesicles (Courtney and Pownall, 2010) also binds the host ECM protein, fibulin (Courtney et al, 2009). The superoxide dismutase of *M. tuberculosis* has also been reported to function as an adhesin, binding to a number of host moonlighting proteins such as GAPD and cyclophilin A (Reddy and Suleman, 2004) – again potentially a moonlighting-moonlighting interaction.

12.5.4. Other Moonlighting Actions of Bacterial Proteins

Cells have a wide range of other metabolic pathways, and such pathways involve an extremely large number of individual enzymes, most of which we know nothing about in relation to their moonlighting functions. The literature only highlights individual enzymes in individual organisms and it is difficult to say much about the relevance of such moonlighting activity in a generic sense. For example, *Bacillus subtilis* encodes a glutamate dehydrogenase (RocG) which, in addition to deaminating glutamate to form α-ketoglutarate, also binds to the transcription factor GltC which functions to regulate glutamate production from α-ketoglutarate and so links these two metabolic pathways. Mutants of RocG have been isolated which have lost their dehydrogenase activity and only retain the binding to the transcription factor (Gunka et al, 2010).

Mycobacterium tuberculosis, and other mycobacteria, have evolved moonlighting actions in various of their proteins. Amongst these is the enzyme glutamate racemase (MurI) which generates d-glutamate, a key component of the peptidoglycan of the bacterial cell wall. In mycobacteria, including *M. tuberculosis,* MurI also functions as a DNA gyrase. This DNA gyrase activity is not related to the racemase function and overexpression of MurI *in vivo* results in the bacterium being more resistant to ciprofloxacin, an antibiotic targeting DNA gyrases, thus showing that this protein is important in DNA function in the intact organism (Sengupta et al, 2008). *Mycobacterium tuberculosis* has only one cAMP phosphodiesterase which has recently been shown to play an independent role in controlling cell wall permeability to hydrophobic cytotoxic compounds (Podobnik et al, 2009). Such influence on cell wall functioning is likely to contribute to the survival and virulence of this bacterium. The aconitase of *M. tuberculosis,* as well as being a TCA cycle enzyme, also functions as an iron-responsive protein (IRP). Such proteins interact with iron-responsive elements (IREs) present at untranslated regions of mRNAs and such binding controls the post-transcriptional regulation of the

expression of proteins involved in iron homeostasis (Banerjee et al, 2007). The rice pathogenic bacterium *Xanthomonas oryzae pv. oryzae* has a moonlighting chorismate mutase, which is an important enzyme in the shikimate pathway responsible for aromatic amino acid synthesis. Bacteria have two forms of chorismate mutases termed AroQ and AroH, and some pathogenic bacteria are reported to possess a subgroup of these enzymes which have been named AroQ(gamma). Now *X. oryzae pv. oryzae* XKK.12 possesses an AroQ(gamma), and inactivation of the gene coding for this enzyme leads to an isogenic mutant which is hypervirulent, implying an important moonlighting role for this protein in bacterium:rice interactions (Degrassi et al, 2010).

12.6. Identification of the Moonlighting Sites in Bacterial Moonlighting Proteins

An obvious requirement, if we are to understand protein moonlighting, is to identify the moonlighting site, or sites, in moonlighting proteins to determine how they evolved and how they relate to the initially discovered 'active site' of the protein in question. Unfortunately, only a few moonlighting sites have been conclusively identified in bacterial proteins. These are shown in Table 12.5 and mapped onto structures in Fig. 12.3. As one would expect, all the moonlighting peptides clearly map to the surface of the proteins, with the partial exception of Hsp70. In this case the moonlighting peptide is at the interface of the homo-dimer raising the possibility that Hsp70 adopts its moonlighting role when disassociated into monomers although the residues identified by Wang et al (2005) as the most important are exposed in the dimer. It will only be with the accumulation of more and defined moonlighting sites that this information can be used to ascertain the relationship between moonlighting sequence, structure and function and between the moonlighting sites(s) and the active site which provided the said protein with its original (activity) name. Having mapped moonlighting regions to protein structures, it should be possible to compare the shape and chemical nature of the region with the normal protein. For example, as described above, Ef-Tu from Mycoplasma pneumoniae binds to fibronectin. One could then compare the structural properties of the fibronectin-binding region of Ef-Tu with the protein surfaces that normally bind to fibronectin.

12.7. Conclusions

This review has described a relatively large number of defined moonlighting proteins with actions which are clearly associated with bacterial virulence (Fig 12.4). These include proteins such as GAPD, enolase and chaperonin 60 whose moonlighting actions are utilised by a wide range of

bacteria and other microorganisms to interact with their hosts. These proteins also have additional functions such as DNA binding or enhancement of biofilm formation. In some cases, although the moonlighting protein seems to be a chaperone or enzyme, it has actually evolved away from this activity and appears to have purely the moonlighting function. A key question is how pervasive is protein moonlighting in bacteria and in bacteria-host interactions. Clearly, other bacterial proteins exist which seem to be moonlighting proteins but, for which, definitive evidence is not yet available. For example, *Strep. pyogenes* encodes a DNase (SpnA) which is cell surface-associated. Inactivation of the gene encoding SpnA produces a less virulent strain, suggesting that this surface DNase is contributing to virulence (Hasegawa et al, 2010). However, the mechanism by which this protein induces a virulence phenotype is not known. Thus, it is likely that we are just at the beginning of identifying the full panoply of moonlighting bacterial proteins that are contributing to bacterial virulence and definite searches should begin to be made to identify more. The bacterium employing the greatest number of moonlighting proteins is *M. tuberculosis* which, currently, has twelve moonlighting proteins in its armamentarium. It is also interesting as to how many bacteria (and other microbes) have been identified that use either GAPD, enolase or Cpn60 as moonlighting virulence proteins.

Another way in which surface-expressed moonlighting proteins can contribute to pathology is by antibodies to such bacterial proteins cross-reacting with the homologues of the host. Perhaps the most surprising manifestation of this is the proposal that antibody cross-reactivity between glycloytic enymes of group A streptococci and human neuronal surface glycolytic enzymes is responsible for conditions such as Tourette's syndrome and obsessive-compulsive disorder (Dale et al, 2006). Other conditions, such as psoriasis, are also believed to be driven by similar immunological corss-reactivity (McFadden et al, 2009).

Clearly, this review has focused on the role that bacterial moonlighting proteins play in bacterial/host interactions and in the process of bacterial virulence. This is largely because the moonlighting proteins discussed have been discovered by chance and their role in virulence has been the result of additional experimentation. In addition to playing a role in virulence the whole moonlighting phenomenon, which is likely to be much more pervasive than the few examples shown in Table 12.4, will impinge on our weltanschauung of bacteria and their interaction with their hosts. There are many

reasons for this. If every, or a large proportion of, proteins moonlight then this implies a much more complex cellular system with many more interactions than there would be if each protein had only a single function (see Sriram et al, 2010). One of the problems of that has arisen in biology in recent years has resulted from genome sequencing. This has revealed that complex organisms like *Homo sapiens* have unexpectedly small numbers of genes (open reading frames(ORFs)) in the their genomes. Currently, the human genome is estimated to contain only 20-25,000 genes. Despite the fact that it is estimated that on average each gene has 3 splice variants, this seems a very low number of working protein components to generate the complexity of a human being. Initial estimates of the human genome were in the 10^5 range. Moonlighting, which may increase the number of functions each protein has by 2-20 times, would, potentially, provide a system with sufficient number of protein interactors to generate what might be thought of as 'optimal complexity'. This increased complexity of protein-protein interactions would have to be matched by an increased complexity in the control of gene transcription, RNA synthesis/manipulation and protein translation. If the production of each moonlighting protein has to be tailored to match differences in each moonlighting activity, then this implies a much more complex cellular network of information control than is currently envisaged.

Moonlighting also has major implications for evolutionary theory which currently envisages only one 'site' which is susceptible to the results of mutation, with most mutations being assumed to be neutral. However, if proteins actually have a much larger proportion of their sequences devoted to biological activity, then a greater proportion of the mutations are likely not to be neutral. This could have major consequences for human genetics, and already there is evidence that this is the case (Sriram et al, 2005). It would be convenient to utilise bacteria to study the evolution and the genetics and biological consequences of protein moonlighting.

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Table 12.1. Eukaryotic Proteins with Protein Moonlighting Actions

Protein	Source	Original Function	Moonlighting Functions
Aldehyde dehydrogenase	cow	alcohol metabolism	lens protein
Thymidine phosphorylase	human	DNA metabolism	platelet-derived endothelial cell growth factor
Fumarate hydratase	human	TCA cycle	tumour suppressor
Gelsolin	human	structural protein	controlling apoptosis
Glycogen synthase kinase 3β	rat	sugar metabolism	role in embryonic development
Lactate dehydrogenase	human	glycolysis	protein translation factor
Lactate dehydrogenase	rat	glycolysis	DNA maintenance
Citrate synthase	tetrahymena	TCA cycle	Structural filament- forming protein
Hexokinase	human	glycolysis	controlling apoptosis
Thioredoxin	multiple	redox enzyme	multiple moonlighting functions
Xanthine oxidoreductase	mouse	oxidase	structural role in milk secretion
Cytochrome C	many	electron transport chain	controlling apoptosis
Phosphoglycerate kinase	human	glycolysis	controlling angiogenesis
Quinone oxidoreductase	guinea pig	electron transport chain	lens protein
Succinate dehydrogenase	human	TCA cycle	tumour suppressor gene
Aconitase	yeast	TCA cycle	DNA maintenance
Enolase	yeast	glycolysis	molecular chaperone
Isocitrate dehydrogenase	yeast	TCA cycle	RNA metabolism
STAT3	rat	signalling protein	electron transport chain
Chaperonin 10	human	molecular chaperone	immunosuppressant
Chaperonin 60	human	molecular chaperone	immunomodulator

References can be found in Piatigorsky 2007 or in this text.

Table 12.2. Prokaryotic Molecular Chaperones*

Chaperonin (Hsp)10

Thioredoxin family

Glutaredoxin

Trigger factor

Peroxiredoxins

Small heat shock proteins

Peptidylprolyl isomerases

DnaJ/Hsp40

GrpE

Protein disulphide isomerases

Chaperonin (Hsp)60

DnaK/Hsp70

HtpG/Hsp90

ClpA/Hsp100 family

Spy

*Names highlighted are the molecular chaperones and protein-folding catalysts from bacteria currently known to moonlight. In Eukaryotes most of the homologues of these Prokaryotic proteins have moonlighting actions.

Table 12.3. The Bacteria Currently Known to Employ Moonlighting Proteins

Bacterium	Moonlighting Proteins
Aeromonas hydrophila	enolase
Aeromonas salmonicida	phosphoglycerate kinase
Aggregatibacter actinomycetemcomitans	Cpn60
Bacillus anthracis	GAPD
Bacillus stearothermophilus	PGI
Bacillus subtilis	glutamate dehydrogenase
Bartonella bacilliformis	Cpn60
Bifidobacterium animalis	Hsp70
Bifidobacteria spp *subsp. lactis*	enolase
Borrelia burgdorferi	Cpn60
Brucella abortis	Cpn60
Candida albicans	phosphoglycerate kinase, phosphoglycerate mutase
Chlamydia pneumoniae	Cpn60.1
Chlamydia trachomatis	Cpn60.2, PPI
Clostridium difficile	Cpn60
Coxiella burnetti	Hsp70
Entamoeba invadens	enolase
Enterobacter aerogenes	Cpn60
Enteropathogenic *E. coli*	Hsp70, GAPD
Enterohaemorrhagic *E. coli*	GAPD
Francisella tularensis	Cpn60
Haemophilus ducreyi	Cpn60
Haemophilus influenza	Hsp70
Helicobacter pylori	Hsp20, Hsp60, Hsp70, PPI, HtrA
Histoplasma capsulatum	Cpn60, PPI
Lactobacillus crispatus	GAPD, enolase
Lactobacillus jensenii	enolase
Lactobacillus johnsonii	Cpn60
Lactococcus lactis	Cpn60, Hsp70, pyruvate kinase
Lactobacillus plantarum	GAPD, enolase
Legionella dumoffi	DnaK (DjlA)
Legionella pneumophila	Cpn60, Hsp70, PPI
Leishmania donovani	Hexokinase
Listeria monocytogenes	GAPD, InlB, alcohol acetaldehyde dehydrogenase , IspC
Mycobacterium avium	Cpn60, Hsp70
Mycobacterium bovis BCG	Cpn60.1
Mycobacterium leprae	Cpn60.2
Mycobacterium smegmatis	Cpn60.1
Mycobacterium tuberculosis	Cpn10, Cpn60.1, Cpn60.2, Hsp70, GAPD, glutamate racemase, mycosyltransferases, malate synthase, phosphodiesterase, phosphoglycerate mutase, superoxide dismutase, aconitase
Mycobacterium ulcerans	small heat shock protein (18kDa)
Mycoplasma pneumoniae	Ef-TU, β-subunit of pyruvate dehydrogenase
Mycoplasma suis	GAPD
Neisseria gonorrhoeae	PPI
Neisseria meningitiditis	peroxiredoxin,Hsp70, GAPD, fructose-bisphosphate aldolase
Paenibacillus larvae	enolase
Phycomyces blakesleeanus	GAPD
Plesiomonas shigelloides	Cpn60
Rhizobium leguminosarum	Cpn60.1
Rickettsia prowazekii	PPI
Salmonella enterica serovar Typhimurium	Cpn60
Schistosoma bovis	GAPD
Staphylococcus aureus	GAPD, triose phosphate isomerase, Aaa
Staphylococcus epidermidis	GAPD, Aae,
Streptococcus agalactiae	Cpn60, Hsp70, GAPD, phosphoglycerate kinase, enolase, C5a peptidase
Streptococcus caprae	Atlc
Streptococcus equisimilis	GAPD, enolase
Streptococcus gordoni	enolase
Streptococci oral	phosphoglycerate mutase
Streptococcus mutans	AtlA
Streptococcus pneumoniae	PPI, GAPD, fructose-bisphosphate aldolase, enolase, 6-phosphogluconate dehydrogenase
Streptococcus pyogenes	GAPD, enolase, serum opacity factor, SpnA
Streptococcus sobrinus	enolase
Streptococcus saprophyticus	Aas
Streptococcus suis	enolase, 6-phosphogluconate dehydrogenase

Toxoplasma gondii	fructose-bisphosphate aldolase
Trichomoas vaginalis	GAPD, pyruvate:ferredoxin oxidoreductase
Trypanosoma brucei	GAPD
Xanthomonas oryzae pv. oryzae	chorismate mutase
Xenorhabdus nematophila	Cpn60

In red- fungus
In blue - protozoan

Table 12.4. Microbial Molecular Chaperones and Protein-Folding Catalysts Present on the Cell Surface with the Identified Host Receptor Proteins Where Known

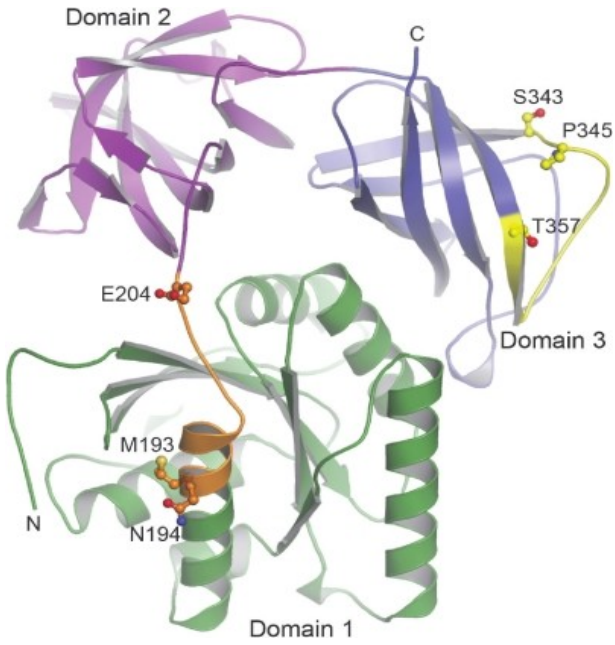
Bacterium	Molecular chaperone	Host receptor	Reference
A. actinomycetemcomitans	Cpn60	?	Goulhen et al, 1998
Bifidobacterium animalis subsp. lactis	Hsp70	plasminogen	Candela et al, 2010
Borrelia burgdorferi	Cpn60	Glycosphingolipid	Kaneda et al, 1997
Brucella abortis	Cpn60	?	Watarai et al, 2003
Chlamydia pneumoniae	Cpn60.1	?	Wuppermann et al, 2008
Chlamydia trachomatis	PPI	?	Lundermose et al, 1993
Clostridium difficile	Cpn60	?	Hennequin et al, 2001
Coxiella burnetti	Hsp70	?	Macellaro et al ,1998
Enteropathogenic *E. coli*	Hsp70	Sulphogalacosylceramide	de Jesus et al, 2005
Haemophilus ducreyi	Cpn60	?	Frisk et al, 1998
Haemophilus ducreyi	Cpn60	glycosphingolipids	Pantzar et al, 2006
Haemophilus influenza	Hsp70	?	Hartman and Lingwood, 199
Helicobacter pylori	Hsp20	?	Du and Ho, 2003
Helicobacter pylori	Cpn60	?	Yamaguchi et al, 1996,1997
Helicobacter pylori	Cpn60	Lactoferrin	Amini et al, 1996
Helicobacter pylori	Hsp70	Sulphatides	Huesca et al, 1998
Histoplasma capsulatum	Cpn60	CD11/CD18	Long et al, 2003
Histoplasma capsulatum	PPI	VLA5	Gomez et al, 2008
Lactobacillus johnsonii	Cpn60	Mucin	Bergonzelli et al, 2006
Lactococcus lactis	Cpn60	Yeast invertase	Katakura et al, 2010
Lactococcus lactis	Hsp70	Yeast invertase	Katakura et al, 2010
Legionella pneumophila	Cpn60	?	Garduno et al, 1998a,b
Legionella pneumophila	Hsp70	?	Hoffman and Garduno, 1999
Legionella pneumophila	PPI	?	Helbig et al 2001,2003
Legionella pneumophila	PPI	Various collagens	Wagner et al, 2007
Mycobacterium avium	Cpn60	$\alpha_v\beta_3$	Hayashi et al, 1997
Mycobacterium avium	Hsp70	?	Ratnakar et al, 1996
Mycobacterium bovis BCG	Cpn60.1	DC-SIGN	Carroll et al, 2010
Mycobacterium leprae	Cpn60.2	?	Esaguy and Aguas, 1997
Mycobacterium tuberculosis	Cpn60.2	CD43	Hickey et al, 2010
Mycobacterium tuberculosis	Hsp70	CD40	Wang et al, 2001
Mycobacterium tuberculosis	Hsp70	CCR5	Floto et al, 2006
Mycobacterium smegmatis	Cpn60	?	Esaguy and Aguas, 1997
Neisseria gonorrhoeae	PPI	?	Leuzzi et al, 2005
Neisseria meningitiditis	peroxiredoxin	plasminogen	Knaust et al, 2007
Neisseria meningitidis	Hsp70	Plasminogen	Knaust et al, 2007
Plesiomonas shigelloides	Cpn60	?	Tsugawa et al, 2007
Rickettsia prowazekii	PPI	?	Emelyanov and Loukianov, 2004
Salmonella enterica serovar Typhimurium	Cpn60	Mucus	Ensgraber and Loos, 1992
Streptococcus agalactiae	Cpn60	?	Hughes et al, 2002
Streptococcus agalactiae	Hsp70	?	Hughes et al, 2002
Streptococcus pneumoniae	PPI	?	Hermans et al, 2006
Streptococcus suis	Cpn10	?	Wu et al, 2008
Streptococcus suis	Cpn60	?	Wu et al, 2008
Streptococcus suis	GrpE (Hsp90)	?	Wu et al, 2008

Table 12.5. Moonlighting Sites in Bacterial Moonlighting Proteins

Bacterium	Moonlighting Protein	Moonlighting Sequence	PDB	Structure Species	ID
M. tuberculosis	Hsp70	**QPSV**QIQVYQGER**E**IAAHNK[1]	3qnj	*E.coli*	12/20
M. tuberculosis	Antigen 85 complex	FEWYYQ[2]	1sfr	*M. tuberculosis*	5/6
M. tuberculosis	Malate synthase	CGAQQPNGYTEPILHRRRREFKARAAEKPAPSDRAGDD[3]	1n8i	*M. tuberculosis*	26/27*
M. pneumoniae	Ef-Tu	GSISLPENTEMVLPGDNTS[4]	2y0u	*T. thermophilus*	10/19
Xen. nematophila	Cpn60	QIRQQIEES[5]	3e76	*E.coli*	8/9

PDB indicates PDB codes for a structure of the protein, or an orthologue from a related species as indicated under 'Structure Species'. ID indicates the number of residues in the structure identical to the 'Moonlighting Sequence' and the length of the matched stretch.

[1]Wang et al (2005) *J immunol* 174: 3306-3316. [residues highlighted in bold are most important for biological activity]
[2]Naito et al (1998) *J Biol Chem* 273:2905-2909.
[3]Kinhikar et al (2006) *Mol Microbiol* 60:999-1013.
[4]Balasubramanian et al (2009) *Infect Immun* 77:3533-3541.
[5]Joshi et al (2008) *J Biol Chem* 283:28287-28296.
*The PDB structure is truncated after the first 27 residues (i.e. KPAPSDRAGDD missing)



The structure shows the two fibronectin binding sites in the Ef-Tu of *Mycoplasma pneumoniae*, in particular, showing the residues 342-357 (S343, P345, T357) which represent the higher affinity binding site for fibronectin. This site is surface exposed (From Balasubramanian *et al* 2009 with permission).

Figure 12.1. A cartoon illustration of the positions of the moonlighting sites in proteins

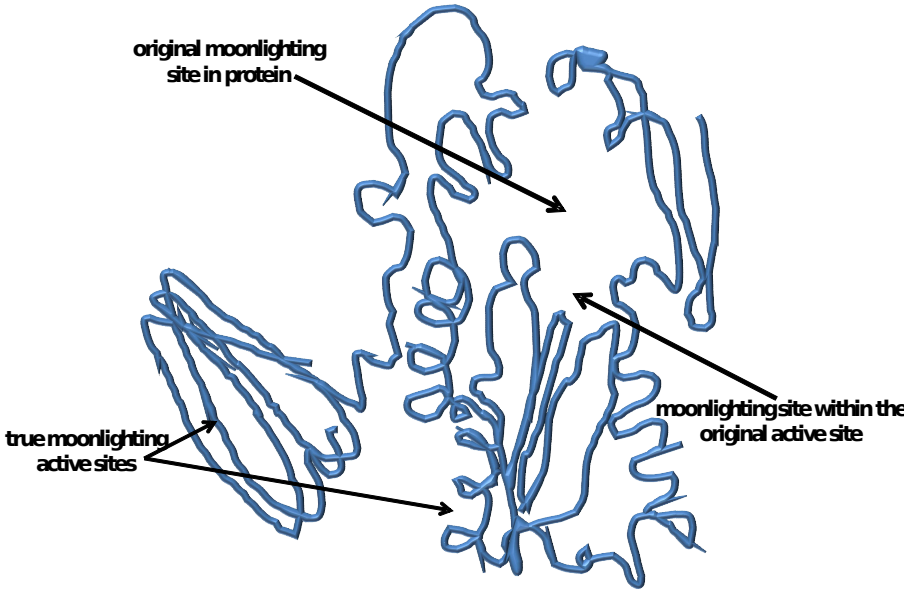


Figure 12.2. Interactions of *Mycobacterium tuberculosis* Chaperonin 60.1 with Myeloid Cells. The Cpn60.1 protein can stimulate monocyte/macrophage cytokine synthesis bt it is not known if this protein can cause classical activation of macrophages. However, the Cpn60.1 protein is able to inhibit the development of osteoclasts from osteoclast precursor cells (both in vitro and in vivo) while also being able to promote the formation of multinucleate giant cells from their precursors. This means that the M. tuberculosis Cpn60.1 protein is a molecular probe to dissect the differences between osteoclasts and multinucleate giant cells.

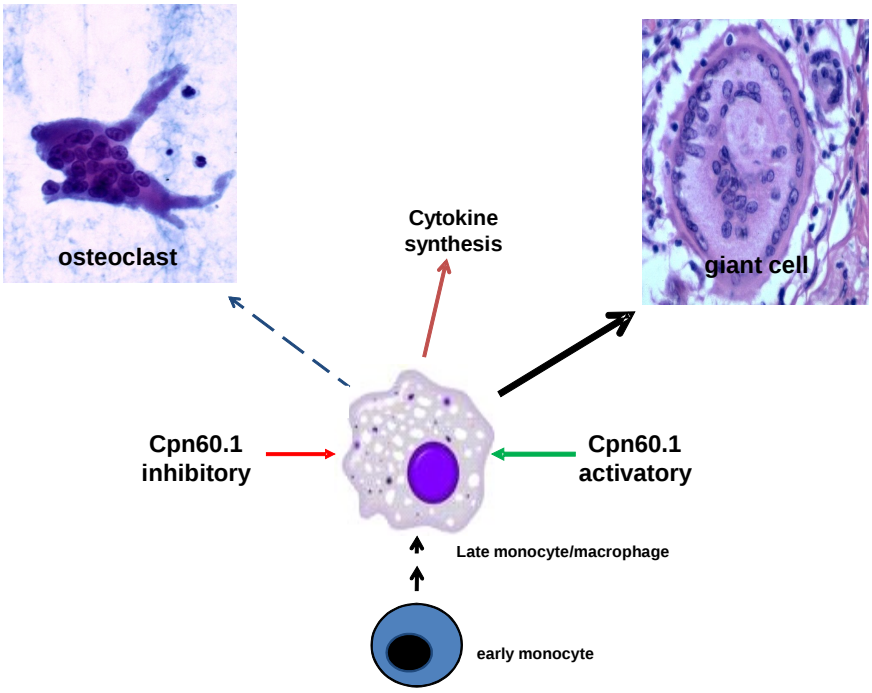


Figure 12.4. Schematic Diagram of the Role of Moonlighting Proteins in Bacterial Virulence. On the slide is shown a number of the reported moonlighting protein in bacteria and the roles that they play, particularly in bacteria-host interactions including induction of cell signalling, adhesion to ECM and cells and degradation of matrices. [6PGD – 6-phosphogluconate dehydrogenase; AAH – alcohol acetaldehyde dehydrogense; PGI – phosphoglucose isomerase; TPI – triose phosphate isomerase]

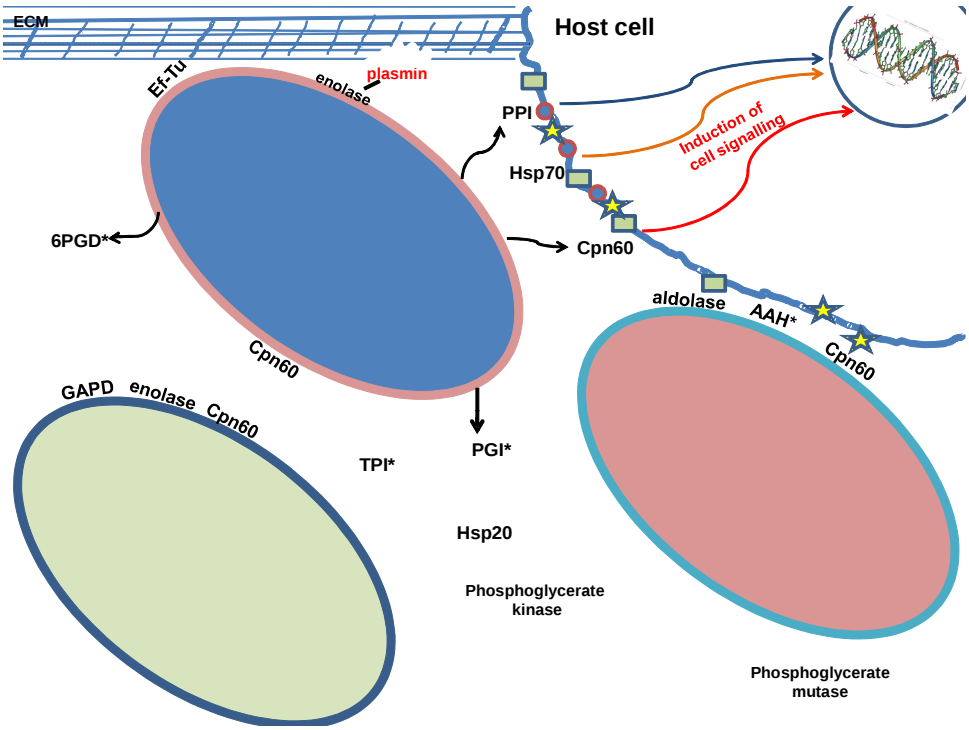
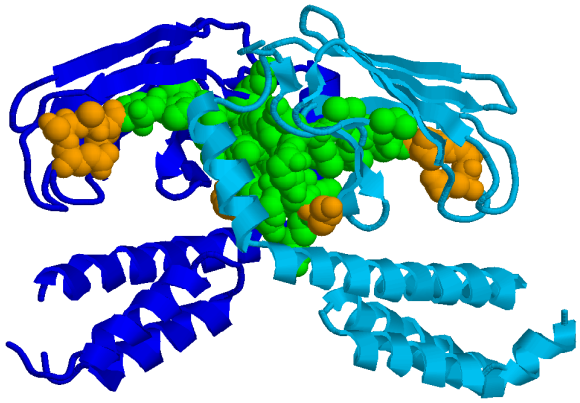
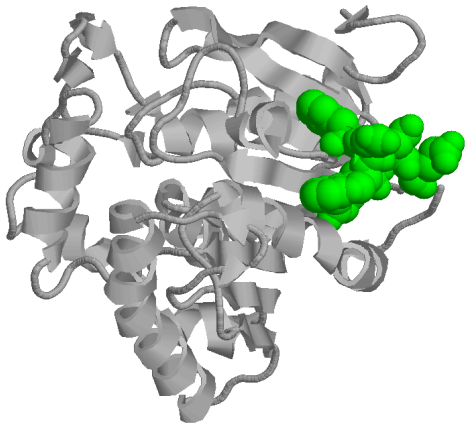


Figure 12.3. Known Bacterial Moonlighting Sites Mapped to Protein Structures. Known moonlighting sequences as shown in Table 12.5 were mapped to protein structures from the same, or related, species to identify the location of the moonlighting site in the structure. Moonlighting sequences are highlighted in green spacefilling with the most important residues in Hsp70 shown in orange. A: Hsp70, B: Antigen 85 Complex, C: Malate Synthase, D: Ef-Tu, E: Cpn60

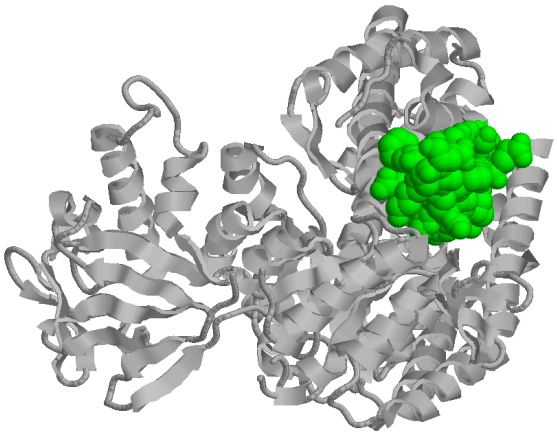
A.



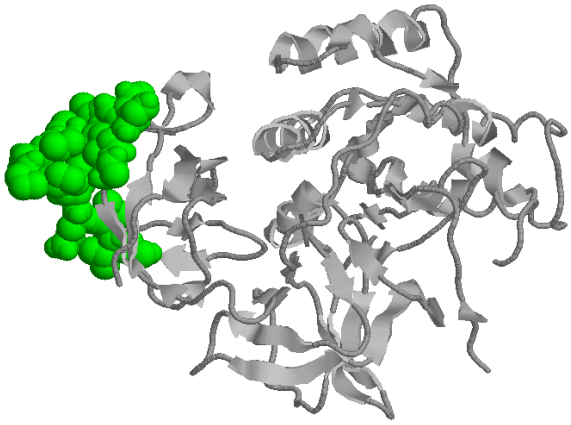
B.



C.



D.



E.