REVIEW for *Infection and Immunity*

Bacterial Virulence in the Moonlight: Multitasking Moonlighting Proteins are Important in Bacteria-Host Interactions in Bacterial Infection

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Abstract

Men may not be able to multi-task but it is emerging that proteins can. This capacity of proteins to exhibit more than one function is termed protein moonlighting and, surprisingly, many highly conserved proteins involved in metabolic regulation or the cell stress response have a range of additional biological actions which are involved in bacterial virulence. This review highlights the multiple roles exhibited by a range of bacterial proteins, such as glycolytic and other metabolic enzymes and molecular chaperones, and the role that such moonlighting activity plays in the virulence characteristics of a number of important human pathogens including *Streptococcus pyogenes, Helicobacter pylori* and *Mycobacterium tuberculosis.*

Introduction

Protein hyperspace is the term conjured up by the physicists, Smith and Morovitz (1982), to encompass the theoretical number of proteins that can be generated. The reader may be surprised that the number of 10kDa (100 amino acid residue) proteins that can, in theory, be produced is 20^{100} or, in more normal nomenclature, 10^{130}. This number will clearly be very much larger for proteins with more residues. To put 10^{130} in perspective, it is estimated that the number of stars in the Universe is around 10^{24}. Even if only 1 in 10^{12} of these possible proteins was soluble, and had some functional activity, there would be a multiverse worth of possible proteins in protein hyperspace. Thus with an almost unlimited palette of amino acid sequences and 3.8 billion years to play with (with a doubling time for *E. coli* of 30 minutes, 3.8 billion years allows for $7x10^{13}$ cell divisions) evolution should have had a good chance at utilising protein hyperspace. Up until 1995 it was dogma that the protein product of each gene had only one function. However, in this year Campbell and Scanes reported that certain neuropeptides had immunomodulatory activity as well as their classic neuropeptide function (Campbell and Scanes, 1995). The term that was introduced to describe this ability of a protein, or in this case, peptide, to have more than one biological action was moonlighting - a term which, colloquially, means to have more than one job (the second being done at night). Since this initial finding, a growing range of proteins have been reported to moonlight and in consequence the generic term for such molecules is MOONLIGHTING PROTEINS (Jeffery, 1999,2009). A number of the best studied moonlighting proteins are shown in Table 1. This immediately shows the reader that moonlighting is not a behaviour practised by ‘unusual’ proteins or ‘rare’ proteins but is a phenomenon evolved by a number of highly conserved, often metabolic proteins/enzymes. Some of the moonlighting activities possessed by proteins better known to biological scientists from their university courses are bewildering. Take phosphoglucose isomerase (PGI), a glycolytic enzyme best known for its ability to convert glucose-6-phosphate into fructose-6-phosphate. The mammalian enzyme is now known to function as a neuroleukin (neurotrophic factor) (Faik et al., 1988), an autocrine motility factor (Watanabe et al.,

1996), a differentiation and maturation mediator for myeloid cells (Xu et al., 1996) and an implantation factor (in the ferret) (Schulz and Bahr, 2004).

Fifteen years after the introduction of the moonlighting protein hypothesis, it is clear that one of the major beneficiaries of this evolutionary mechanism, by which individual proteins can have multiple functions, is the bacterium. There are now a significant number of examples of bacterial moonlighting proteins. Many of these proteins appear to play a role in the virulence properties of bacteria and so it is important to recognise this phenomenon in bacteriology. The understanding of protein moonlighting is confused by the fact that not all protein homologues moonlight (Gancedo and Flores 2008) or they may have different moonlighting functions. Readers need to appreciate this fact when reading the moonlighting literature. Major groups of proteins that moonlight in bacterial virulence include: (i) the metabolic enzymes of the glycolytic pathway; (ii) enzymes of other metabolic pathways such as the glyoxylate cycle and (iii) molecular chaperones and protein folding catalysts. Amongst the most commonly identified moonlighting functions of bacterial proteins are adhesion and modulation of leukocyte activity. Given, what appears to be the random nature of bacterial protein moonlighting, the comparatively wide range of moonlighting functions, and the likelihood that investigators have only scratched the surface of this phenomenon, it is difficult to divide up the available literature into a clear narrative. Thus this review will deal with individual groups of moonlighting proteins and delineate the range of their virulence properties.

Readers should be aware that protein moonlighting is also known as gene sharing. This latter title has been introduced by Piatigorsky (Piatigorsky 2007) who discovered the ‘moonlighting’ actions of eye lens proteins in the 1980s. ‘Gene sharing’ is generally used in reference to eukaryotic proteins and will not be employed in this review.

Moonlighting Bacterial Glycolytic Enzymes

Glycolysis exists in all three Kingdoms of life and is assumed to have evolved early in the evolution of life (Fothergill-Gilmore and Michels 1993). In eukaryotes, there are ten enzymes in the glycolytic ‘system’ starting with hexokinase which converts glucose to glucose-6-phosphate and ending with pyruvate kinase which converts

phosphoenolpyruvate into pyruvate which then can be converted into into acetyl CoA for mitochondrial oxidation (Fig 1).

A growing number of enzymes of the glycolytic pathway have been found to act as moonlighting enzymes including aldolase, triose phosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase (GAPD) and enolase. A recent review focused on moonlighting in mammalian glycolytic enzymes reveals the richness of eukaryotic glycolytic moonlighting (Kim and Dang 2006). In addition to moonlighting, there are numerous reports that many bacteria express a number of their glycolytic enzymes on their outer cell surfaces. Currently, Gram-positive organisms such as streptococci and staphylococci appear to express most of the enzymes of the glycolytic pathway on their cell surfaces (Hughes et al 2002; Ling et al, 2004; Encheva et al 2006; Wu et al 2008). As there is no current mechanism for the release of glycolytic enzymes, such reports have been criticised as simply being the result of the binding of enzymes to the bacterial surface as the result of bacterial death and dissolution – with release of enzymes. As will be described, there is incontrovertible evidence that the cell surface GAPD of *Streptococcus pyogenes* is there as part of an as-yet-undescribed system for secreting cytoplasmic enzymes (Boel et al 2005). This hypothesis is supported by studies of the surface location of GAPD in *Lactobacillus plantarum* which reveals that soluble GAPD does not bind to the surface of this organism and that the presence of GAPD on the bacterial surface relates to cell wall permeability (Saad et al 2009). It has to be assumed that the other secreted bacterial cytoplasmic enzymes that exhibit moonlighting activity are also secreted by specific mechanisms which have evolved to aid the bacterium. In this context, it has been reported that glycolytic enzymes associate with the surface of mitochondria in *Arabidopsis thaliana* and that the degree of association is dependent on respiration rate (Graham et al 2007). Such association of glycolytic enzymes with mitochondria has also been reported for yeast (Brandina et al 2006). It is not clear if this is relevant to the situation in bacteria. The moonlighting activity of the bacterial glycolytic enzymes will start with the first enzyme, hexokinase and work down the pathway.

Hexokinase: There are, as yet, no reports that bacterial hexokinases moonlight. However, in mammalian cells, hexokinase binds to the mitochondrial voltage-dependent anion channel (VDAC), a membrane protein controlling metabolite entry into the mitochondrion. The VDAC is also involved in controlling apoptosis and binding of hexokinase to VDAC can inhibit VDAC-induced apoptosis (Shoshan-Barmatz et al 2009). The soluble FimA protein from *E.coli* K1 actually promotes the binding of hexokinase to VDAC1 thus blocking cellular apoptosis (Sukumuran et al 2010), a key requirement for an enteroinvasive bacterium. So here we have an example of two proteins, one bacterial and one from the host, moonlighting and interacting through these moonlighting functions. This could be termed 'systems moonlighting' and a few examples of such behaviour are now entering the literature, as will be described in this review.

Phosphoglucose isomerase (PGI): As described in the Introduction, PGI is a major human/mammalian moonlighting protein with multiple cell signalling actions and with a role in malignancy (Yanagawa et al 2004). It is also an intriguing autoantigen associated with the induction of a rheumatoid arthritis-like condition in mice (Kamradt, Schubert 2005). The assumption is that glycolytic enzymes in eukaryotes would have evolved from those of the prokaryotes or archaeae. However, there is one report that suggests that certain bacterial PGIs may have come as a result of gene transfer from a eukaryotic source (Katz 1996), although this hypothesis has been questioned (Grauvogel et al 2007). Can bacterial PGI proteins also exhibit the moonlighting actions of the mammalian homologues, and in doing so can they induce pathology? The recombinant PGI from *Bacillus stearothermophilus* has been shown to have both autocrine motility factor and neuroleukin activity (Sun et al 1999), suggesting that this bacterium, if it colonised humans could signal via its PGI. However, this result contrasts with that of another group using using recombinant PGI from the bacterium -------, it was found that although the bacterial protein was enzymically active it did not bind the human PGI cellular receptor, gp78/autocrine motility factor (EMF)-receptor (R), and failed to exhibit the cytokine-like actions of the human PGI (Amraei and Nabi, 2002). Clearly more work on this area is required as the bacterial PGIs could have important pathological properties if they have the same cell

signalling activity as the human enzyme. One bacterium, *Xanthomonas oryzae* pv. *oryzae*, which causes bacterial leaf blight in rice, was subjected to transposon mutagenesis to identify avirulent mutants. One such mutant had its PGI gene inactivated. The mechanism of action of this protein in this particular system has not yet been identified (Tsuge et al 2004).

Phosphofructokinase: In *Bacillus subtilis*, phosphofructokinase has been found to interact intracellularly with enzymes involved in RNA processing, possibly as part of a 'RNA degradasome' complex (Comminchau et al 2009). Nothing else is known about the moonlighting activity of this bacterial enzyme.

Aldolase (fructose-1,6-bisphosphate aldolase): In *Streptococcus pneumoniae*, aldolase is a cell surface lectin which is immunogenic in infected children (Ling et al 2004). Analysis of the host target receptor for this aldolase has identified it as a seven-pass transmembrane receptor of the cadherin superfamily, designated Flamingo (Blau et al 2007). Flamingo is an atypical cadherin involved in regulating cell polarity and dendritic and axonal growth. It is unclear if this ability to bind Flamingo is related to the ability this bacterium has to cause long-term neurological sequelae in patients with pneumococcal meningitis (Meli et al 2002). Interestingly, the type IV pili of *Neisseria menigitidis*, another organism causing meningitis, recruits the Par3/Par6/PKCzeta polarity complex that plays a major role in the establishment of eukaryotic cell polarity and the formation of intercellular junctions. It is this interaction that leads to the bacterium entering into brain endothelial cells (Coureuil et al 2009). It has recently been reported that the aldolase enzyme of *Neisseria meningitidis* is present on the bacterial outer membrane. Inactivation of the cytoplasmic aldolase gene had minimal effect on cell growth but had a marked effect on the ability of the bacterium to bind to human cells (Tunio et al 2010). This carries on the tradition of bacterial glycolytic enzymes moonlighting as adhesins. In addition to using aldolase as an adhesin, both bacteria (Reddy et al 2004) and parasites (Starnes et al 2009) can bind to host aldolase – a property that is presumably involved in colonisation and host cell invasion

Triose phosphate isomerase: Little is known about the surface location or moonlighting actions of triose phosphate isomerase (TPI). It is known that altering culture condition for the oral pathogen, *Aggregatibacter actinomycetemcomitans* causes this enzyme to be cell expressed on the bacterial surface (Fletcher et al 2001). In *Staphylococcus aureus,* the TPI functions as an adhesin for binding the fungal pathogen, *Cryptococcus neoformans*. Such binding can result in the killing of the fungus (Ikeda et al, 2007; Furuya and Ikeda 2009). The TPI is believed to bind to the mannan backbone of glucuronoxylomannan which is the major component of the capsule covering this fungus. Definite evidence for the surface localisation of TPI on *S. aureus* has been achieved by use of scanning immunoelectron microscopy (Yamaguchi et al 2010).

GAPD: This is an enzyme with a large and growing range of protein moonlighting functions in both eukaryotes and prokaryotes (Table 2). Some of the moonlighting functions relate to the enzymic activity of this protein and others do not. Glyceraldehyde 3-phosphate dehydrogenase (GAPD) catalyses the conversion of glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate utilising both NAD, which is reduced to NADH and a phosphate group which is transferred to the intermediate compound oxidised by the enzyme. It is this bisphosphoglycerate which is used to generate the first molecule of ATP coming from glycolysis. This enzymic active site proves to be capable of additional effects on cells relevant to the pathogenesis of human infectious disease. In eukaryotic cells, for example, the NAD binding actions of GAPD is thought to be responsible for its function as a DNA binding protein (Demarse et al 2009) and also as a transcriptional regulator (Zheng et al 2003) (see Table 2).

Some of the earliest evidence for protein moonlighting, even before the term was introduced, has come from the study of the GAPD of Group A streptococci. Pancholi and Fischetti first reported that a prominent surface protein, tightly adherent to the surface of *Streptococcus pyogenes* M6 strain, had sequence homology to other GAPD proteins and exhibited GAPD activity, as assayed using glyceraldehyde 3-phosphate and NAD and measuring NADH production. It was shown that this surface protein, termed streptococcal surface GAPDH (SDH) bound to lysozyme, cytoskeletal proteins and fibronectin (Pancholi

and Fischetti 1992). A separate group identified the *Strep. pyogenes* GAPD as a cell surface receptor for plasminogen (Lottenberg et al 1992; Winram and Lottenberg 1996). In an interesting cross-disciplinary interaction, the finding that the signalling gas, nitric oxide (NO) generation in brain cytosolic fractions promoted the ADP-ribosylation of brain GAPD (Zhang and Snyder 1992) led to the identification that SDH is a self ADP ribosylating enzyme whose activity is enhanced by NO (Pancholi and Fischetti 1993). ADP ribosylation of proteins is an enzymic process performed by a range of bacterial toxins (Henkel et al 2010) and it was possible that the cell surface SDH had some form of signalling action with host cells. Indeed, it was found that incubating the human pharyngeal cell line, Detroit 562, with intact streptococci or purified SDH caused specific patterns of protein phosphorylation and that relatively non-specific inhibitors of tyrosine and serine kinases could inhibit the invasion of cells by these bacteria, suggesting that SDH activity was an important signal for cell invasion (Pancholi and Fischetti 1997). These studies also claim to have identified both a 30 and a 32 kDa binding protein (receptors?) for SDH on the Detroit 562 plasma membranes (Pancholi and Fischetti 1997). More detailed examination of the binding of ^{125}I-labelled SDH to isolated Detroit 562 plasma membrane proteins identified a doublet of 30/32 kDa (14-3-3ε proteins) plus additional proteins of 37 (GAPD), 47 (enolase), 55 (uPAR) and 80 kDa (moesin) (Jin et al 2005). uPAR (urokinase receptor/CD87) – a GPI-anchored plasma membrane protein, whose expression is increased in inflammation and with tissue remodelling, and in most human cancers - turned out to be a receptor for SDH. This protein regulates proteolysis at the cell surface by binding urokinase-type plasminogen activator (urokinase) a process that can activate many intracellular signalling pathways and is important in key cellular events such as proliferation, migration and inhibition of cell death. Signalling via uPAR, which has no cytoplasmic domain, requires the participation of co-receptors such as the integrins (Smith and Marshall 2010). Removal of uPAR from the cell surface resulted in a decrease in bacterial binding to Detroit 562 cells (Jin et al 2005).

One seemingly insurmountable problem with deciding if the cell surface location of glycolytic enzymes is an important virulence determinant, is the general inability to

inactivate the genes encoding these proteins. An ingenious solution to this problem has been offered by Pancholi and co-workers (Boel et al 2005). These workers replaced the chromosomal copy of *Strep. pyogenes gapd* with a gene that encoded a functional GAPD protein which contained a 12 residue C-terminal hydrophobic peptide. It was hoped that this hydrophobic segment would prevent egress of the cytoplasmic GAPD. This hypothesis was confirmed by immunocytochemical location studies and the failure of the mutant to show cell surface GAPD activity. So this artificially generated strain is a cell surface enzyme knockout. The GAPD mutant grew normally in culture but was deficient in binding to plasminogen, showed only one third the adherence to Detroit 562 cells and had totally lost its anti-phagocytic activity (Boel et al 2005). The neutrophil is the major phagocyte for bacteria and the complement breakdown product, C5a, is a major signal for attracting and activating neutrophils. It turns out that *Strep. pyogenes* GAPD/SDH inhibits the actions of C5a on neutrophils by binding to this protein and blocking neutrophil chemotaxis and hydrogen peroxide production which contribute to bacterial killing (Terao et al 2006).

Other streptococci also utilise GAPD for various moonlighting functions. The GAPD of the bacterium responsible for causing pneumonia and meningitis, *Strep. pneumoniae*, also acts as a plasminogen binding protein (Bergmann et al 2004a), which is important in the ability of this organism to cross endothelial and epithelial cell barriers (Attali et al 2008). In *Strep. agalactiae,* GAPD is reported to function as a virulence factor with B lymphocyte-modulatory activity (Madureira et al 2007) and the same enzyme from *Strep. oralis* binds to the major fimbriae of *Porphryomonas gingivalis* and appears to be important in the colonisation of the latter organism (Nagata et al 2009). In *Strep. suis* serotype 2 the GAPD enzyme functions as an albumin binding protein (Quessy et al 1999) and also as an adhesin for cell binding (Brassard et al 2004). Lest it be thought that the virulence properties of cell surface GAPD is solely a Gram-positive phenomenon it has been reported that enterohaemorrhagic (EHEC) and enteropathogenic (EPEC) strains of *E. coli* express cell surface GAPD which bind plasminogen and fibrinogen. Non-pathogenic *E. coli* strains do not secrete this enzyme. Host cells interacting with these pathogenic strains show the presence of GAPD on their cell surfaces. An interesting observation is that these bacteria

produce two forms of GAPD differing in their pI with only the more basic form being secreted (Egea et al 2007). The GAPD proteins from both EHEC and EPEC strains possess NAD-ribosylating activity (Aguilera et al 2009). The GAPD enzymes also have adhesive properties in bacteria other than streptococci and staphylococci. Thus *Mycoplasma genitalium* cell surface GAPD is involved in bacterial binding to mucin (Alvarez et al 2003) and cell surface *Lactobacillus plantarum* GAPD binds gastric mucin and also Caco-2 cells - an intestinal epithelial cell line (Kinoshita et al 2008a; Ramiah et al 2008). In addition to binding mucin it has been found that the GAPD of *L. plantarum* binds to the human ABO blood group antigens which are present on intestinal mucin. This binding can be inhibited by NAD suggesting that the enzyme active site is also the site of blood group antigen binding (Kinoshita et al 2008b). Inactivation of the gene encoding the GAPD of *Xanthomonas campestris pv. campestris* was still able to grow on glucose suggesting a role for other pathways in energy production. However, the isogenic mutant had animpairment in *in vivo* virulence and in the ability to synthesise extracellular polysaccharide (Lu et al 2009).

Phosphogylcerate kinase: This is the first of the ATP-generating enzymes of glycolysis. This protein was first found on the surface of *Candida albicans* (Alloush et al 1997) and it was later reported to be on the outer cell wall of *Strep. oralis* (Highes et al 2002; Wilkins et al 2003) and of *Aeromonas salmonicida* (Ebanks et al 2005). This enzyme has been reported to be a plasminogen binding protein in oral streptococci (Kinnby et al 2008). A more surprising finding is that Group B streptococcal surface exposed phosphoglycerate kinase binds to human cellular actin and may be involved in the binding and internalisation of this bacterium into host cells (Burnham et al 2005).

Phosphoglycerate mutase: Another glycolytic enzyme found on the surface of streptococci (Wu et al 2008) and implicated in the binding of plasminogen (Kinnby et al 2008).

Enolase: This is a prototypic moonlighting protein in both prokaryotes and eukaryotes with putative roles in a variety of human diseases (Pancholi 2001). Human cells, such as neurons and endothelial cells and also yeasts and protozoans have cell surface enolases

(Pancholi 2001). Only the bacterial enzyme will be discussed. However, readers should be aware that there is an interesting hypothesis propounded, which claims that neurological movement and psychiatric disorders such as Tourette's syndrome and obsessive-compulsive disorder are caused by antibodies raised against streptococcal cell surface glycolytic enzymes, such as enolase, which then cross-react with the same enzymes on the surface of specific neurons (Dale et al 2006).

A growing range of microbes have been reported to have cell surface enolases which have moonlighting functions (Table 3). As can be seen, these organisms include Gram positive as well as Gram negative (e.g. *Borrelia burgdorferi* – Nowalk et al 2006) bacteria. Most attention has focused on the cell surface enolase of Group A streptococci. Pancholi and Fischetti, who are responsible for much of our understanding of the role of cell surface GAPD in *Strep pyogenes* discussed earlier, are also responsible for the current view of streptococcal cell surface enolase and its role in the virulence of this organism. The plasminogen system of the human is an evolutionary target for both plasminogen activators and receptors (Lahteenmaki et al 2001). Analysis of the plasminogen binding characteristics of cell wall proteins of *Strep. pyogenes* identified enolase as the strongest binder. This cell surface enolase was enzymically active and antibodies to this streptococcal enolase induce opsonisation and enhanced phagocytosis (Pancholi and Fischetti 1998). It turns out that enolase is 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 these bacteria to penetrate a synthetic basement membrane gel (Matrigel™) and so is believed to be important for the invasion of this organism that results in meningitis (Eberhard et al 1999). With this latter organism it was found that soluble recombinant *Strep. pneumoniae* enolase bound to surface of the pneumococci even when associated with plasminogen. Treatment of the cell surface with proteases inhibited such re-association suggesting that it 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 gene (Bergmann et al 2001). In studies of murine enolases it was discovered that enolase

binding to plasminogen was dependent on C-terminal lysyl residues in the enolase which were bound to by lysine binding sites in the plasminogen (Redlitz et al 1995). To test if the pneumococcal enolase also bound plasminogen through C-terminal lysines, both carboxypeptidase treatment and mutation of Lys-433 and Lys-434 was employed. These treatments clearly inhibited plasminogen binding to the enolase (Bergmann et al 2001). A similar analysis of the *Strep. pyogenes* enolase has been made (Derbise et al 2004). In this study, binding of native and mutated enolase to native plasminogen, termed Glu-plasminogen and plasminogen after cleavage by plasmin, termed Lys-plasminogen, has been studied. Deletion or substitution of the lysines in enolase at positions 434-435 resulted in significant decreases in the binding of this glycolytic enzyme to Glu- or Lys-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). A later study also showed that lysines at position 252 and 255 also contribute to plasminogen binding (Cork et al 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 2003b). A similar binding site has been identified in *Bifidobacter* spp enolases (Candela et al 2009).

As has been explained, human cells can also express enolase on their cell surface. Pancholi has suggested that the presence of such cell surface enolase may be important in the invasion of *Strep. pyogenes* into human pharyngeal cells (Detroit 562) (Pancholi et al 2003).

How important is cell surface enolase in the pathogenesis of bacterial infection. As it is not possible to inactivate this enzyme this only leaves the use of strains in which the plasminogen binding sites in enolase have been ablated or the use of immunisation with recombinant bacterial enolase. There are no reports of bacterial strains with modified enolases having lowered virulence, suggesting that experiments with these strains have been attempted, but without a decrease in colonisation or disease severity. However, 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). Interestingly, in this context, it has been reported that the recombinant enolase from the oral bacterium, *Strep. sobrinus*, is an immunosuppressive protein (Viega-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).

It is not only plasminogen that bacterial enolases bind to. The enolase of *Strep. gordonii* has been found to bind to the salivary mucin, Muc7 (Kesimer et al 2009) (Table 3). In addition, it has been recently reported that 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). What do we know about the binding of enolase to the bacterial cell surface. One report reveals 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).

Pyruvate kinase: This enzyme has been found on the surface of neurons and may contribute to the neural sequelae of streptococcal infection (Dale et al 2006). The only report of its presence on the bacterial cell surface is with *Lactococcus lactis* which expresses this protein on its cell surface where is functions to bind yeast mannan (Katakura et al 2010).

Importance of the cell surface location of glycolytic enzymes

Clearly, bacterial glycolytic enzymes exhibit a growing range of moonlighting actions. The common aspect of these variegated moonlighting actions is that they occur when the specific glycolytic enzyme is on the cell surface. This is not to say that bacterial glycolytic enzymes do not also have intracellular moonlighting activity. It is just that such activity has not yet been reported, although a recent yeast two-hybrid analysis does suggest that glycolytic enzymes may participate in novel moonlighting actions within the cytoplasm of

Bacillus subtilis (Commichau et al 2009). From the rapidly expanding number of reports on the cell surface location of bacterial glycolytic enzymes it is a reasonable inference that some bacteria have the whole of the glycolytic pathway on their cell surface (Fig 2). The glucose concentration in the extracellular fluid of the non-diabetic human is 5mM and this can rise much higher in individuals with diabetes. The concentration of glucose within cells is less well known but in mammalian cells it is normally <1mM. Thus there is sufficient glucose in the human extracellular milieu to allow the glycolytic pathway to function on the surface of bacteria that are colonising/infecting *Homo sapiens.* There is some evidence that the glycolytic pathway could have some degree of organisation forming a macromolecular complex in association with mitochondria or the plasma membrane (e.g. Campanella et al 2008). This is of relevance to a recent study which captured two recombinant glycolytic enzymes (hexokinase and glucose-6-phosphate isomerase) on an artificial substratum and showed sequential enzymic activity which was manyfold higher than when the same two enzymes were in solution (Mukai et al 2009). This suggests that the glycolytic pathway, if sequestered on a surface structure, such as that of the outer cell wall of a bacterium, can be significantly more active than the same enzymes free within the cell cytoplasm.

If the glycolytic pathway is functioning on the surfaces of some bacteria what are the consequences for the bacterium and for the host. We have already dealt with what we know about the enzymes as receptors or modifying enzymes (kinases and ADP-ribosylases). In addition to this, could the metabolites produced as the glycolytic pathway proceeds have, themselves, any biological actions? The glycolytic pathway generates two molecules of ATP per cycle of the pathway. Provided there is sufficient ADP in the extracellular milieu this generation of ATP could be used for signalling to cells through the large family of purinergic receptors/purinoceptors of which the P2Y and P2X subtypes recognise ATP (Gever et al 2006). This could be of importance, as evidence is accumulating for a role for the P2X7 purinergic receptor in the release of the potent pro-inflammatory cytokine from monocytes and macrophages (Ward et al 2010). Thus if bacteria generate ATP locally they might promote a pro-inflammatory response – possibly

not the most useful outcome for the bacterium. It is likely that other host cellular pathways are more likely to be induced in response to local ATP synthesis. Alternatively, cell surface ATP synthesis may be utilised by the generating bacterium or nearby bacteria (if in a biofilm) to fuel as yet undefined processes. For example, ATP synthase is found on the surface of neurons and is active in ATP generation. Such extracellular ATP synthesis appears to have an effect on intracellular pH in neurons (Xing et al 2010).

Another possibility is that the substrates that are produced as part of the glycolytic pathway have signalling actions in their own right. Again, such signalling may be to other members of the bacterial species or to other bacterial species or to the host.

Moonlighting actions of other bacterial metabolic enzymes

Cells have a wide range of other carbohydrate metabolic pathways including the tricarboxylic acid cycle, pentose phosphate pathway, glucuronate metabolism, and also pathways of fatty acid synthesis and of other essential components such as amino acids, purines and pyrimidines. 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 this reason the various bacterial metabolic enzymes with moonlighting actions will be simply shown in Table 4. One of the most fascinating moonlighting metabolic enzymes to be described in this section will be the alcohol acetaldehyde dehydrogenase of *Listeria monocytogenes* as it is the first example of moonlighting protein-moonlighting protein interactions. Listeria adhesion protein (LAP) was identified as a key adhesin of this organism (Santiago et al 2006) allowing the bacterium to bind to intestinal epithelial cells. It was then shown that LAP bound to the major molecular chaperone, chaperonin (Cpn)60 or heat shock protein (Hsp)60, on the surface of human host cells (Wampler et al 2004). More detailed analysis revealed that LAP was actually the alcohol acetaldehyde dehydrogenase of *L. monocytogenes* – an enzyme involved in alcohol metabolism. Surprisingly, measurement of the kinetics of binding of LAP to human Hsp60, using surface plasmon resonance, revealed a very high affinity interaction (Kim et al 2006). So here we

have a bacterial moonlighting protein (in a topologically-unusual site) interacting with a human moonlighting protein (also in a topologically unusual site) to create a phenomenon important to human colonisation with a pathogenic bacterium. This is the first example of complex moonlighting-moonlighting interactions but it is unlikely to be the only such complex moonlighting behaviour.

Another bacterial metabolic enzyme with interesting properties is the glutamate dehydrogenase (RocG) of *Bacillus subtilis*, 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).

As will be seen in this review, *Mycobacterium tuberculosis* and other mycobacteria have evolved a number of moonlighting 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 also plays an independent role in controlling cell wall permeability to hydrophobic cytotoxic compounds (Prodobnik 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). Finally, 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 aldolase (Reddy et al 2004).

The rice pathogenic bacterium *Xanthomonas oryzae pv. oryzae* has already been described. This organism 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 posseses a 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)

Bacterial moonlighting proteins which act as adhesins and invasins

Bacteria have a variety of moonlighting proteins which act as adhesins and, depending on the host receptor targeted, such moonlighting adhesins can also aid bacterial invasion of host cells. Enolases from various Gram-positive organisms are reported to bind to human plasminogen or laminin (Antikainen et al., 2007). The enolase of *Streptococcus suis* is enzymically active, and found present on the bacterial surface. The recombinant protein binds to plasminogen and with high affinity (Kd = 21nM). Furthermore, antibodies to this protein inhibit the adhesion and invasion of *Strep. suis* into microvascular endothelial cells (Esgleas et al., 2008). The pentose phosphate pathway enzyme, 6-phosphogluconate dehydrogenase also acts as an adhesin in various pneumococcal strains (Daniely et al 2006). Many bacteria have cell surface molecular chaperones such as Hsp60, Hsp70 or peptidyl prolyl isomerases which act as cellular adhesins. What is so fascinating is the variety of host ligands to which these molecular chaperones bind. These proteins will be discussed in the next section on molecular chaperones.

Fibronectin is a major host component being found at high concentrations in the body fluids, in the extracellular matrix (ECM) and at the interface between cells and the ECM where the fibronectin is bound to cells through specific integrins which act to prepare the fibronectin for its inclusion in the ECM and function as transducers of fibronectin signalling (Henderson et al 2010b). The binding of group A streptococcal GAPD to fibronectin has already been described (Pancholi and Fischetti 1992). *Lactobacillus plantarum* has a cell

surface enolase which binds fibronectin (Castaldo et al., 2009). Fibronectin has a complex domain structure with different parts of the protein binding to different host components including heparin, collagen, gelatin, fibulin etc (Henderson et al 2010b). It turns out that a number of bacterial moonlighting proteins, other than those mentioned above, can also bind to fibronectin. *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; Rosseels et al 2006). Proteins are in the mass range from 30-31kDa and are all able to bind to fibronectin (Wicker and Harboe 1992). 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 was therefore surprising when Patrick Brennan's group reported that the antigen85 complex members each contain a carboxylesterase domain and act as mycolyltransferases, proteins involved in the final stages of the assembly of the complex mycobacterial cell wall (Belisle et al., 1997). Crystal structures of recombinant antigen 85C (Ronning et al., 2000) and 85B (Anderson et al., 2001) from *M. tuberculosis* confirmed that the proteins are members of the α/β-hydrolase family. Of note, the antagonism of the mycolyltransferase activity by 6-azido-6-deoxy-alpha, alpha'-trehalose demonstrated that these proteins are essential and are potential targets for new antimycobacterial drugs (Belisle et al., 1997). Although at least 100 bacterial fibronectin binding proteins have been identified (Henderson et al 2010b) we know very little about the nature of the fibronectin binding sites in these proteins. Analysis of the antigen85B complex member from *Mycobacterium kansasii* identified two fibronectin-binding epitopes, one a 27 residue stretch (84-110) and a second motif of 20 amino acids (211-230). Epitopes were highly conserved in the closely related antigen 85 complexes of other mycobacteria. The 84-110 segment inhibited the binding of fibronectin to the components of the antigen 85 complex of both *M. kansasii* and BCG, but motif 211-230 did not have the same inhibitory effect. Further examination of the 84-110 sequence

using synthetic peptides defined residues 98-108 as the minimum inhibitory motif with six residues (FEWYYQ) being most important for Fn interaction. This Fn-binding motif forms a helix at the surface of the protein and has no homology to other known prokaryotic and eukaryotic Fn-binding features and appears to be unique to the mycobacteria (Naito et al., 1999). 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 Fn (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 at the bacterial surface, binding by an unknown mechanism, where it can bind both fibronectin and laminin (Kinhikar et al., 2006). 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. This is the first glyoxylate cycle enzyme shown to be present on the bacterial cell surface and acting as an adhesin for components of the extracellular matrix.

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* (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). N-terminal sequencing identified these proteins as elongation factor (EF)-Tu and the β-subunit of pyruvate dehydrogenase. Recombinant versions of these proteins were shown to bind fibronectin. Using specific antibodies 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 Fn-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. Substututions 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). This has allowed a molecular model to be constructed of the moonlighting fibronectin binding site in EF-Tu (Fig 3).

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 for fibronectin (Heilman et al 2005). *Staphylococcus epidermidis* Aae (autolysin/adhesin in *S. epidermidis*) is homologous to *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 (Alignet 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). *Staphylococcus saprophyticus* Aas also has haemagglutinating activity and has been tentatively identified with a 160kD *S. saprophyticus* haemagglutinin with fibronectin binding ability that had been previously purified from bacterial strains (Gatermann and Meyer, 1994, Hell et al., 1998).

Moonlighting bacterial molecular chaperones and protein-folding catalysts

Protein moonlighting was first discovered, although not given this title, when the nature of the proteins in the lens of different species was identified. It turns out that a number of well known proteins, including members of the glycolytic pathway can act to transmit light. Amongst the most common of the lens proteins are homologues of the small molecular chaperone family known as the crystallins (Piatigorsky 2007). It is rapidly emerging that molecular chaperones and the associated protein-folding catalysts from bacteria are major classes of moonlighting proteins (Henderson and Pockley 2005; Henderson et al 2008; Pockley et al 2010). The literature on the role of bacterial molecular chaperones and protein folding catalysts was reviewed in this journal in 2006 (Henderson et al 2006) and only relevant aspects of the literature published after this will be described in detail.

A fascinating aspect of the moonlighting biology of the molecular chaperones of prokaryotes and eukaryotes is the fact that these proteins can function both as agonists for receptors and as receptors themselves. These largely include the 60 and 70 kDa heat shock proteins. A number of reports suggest that the bacterial Hsp70 protein, DnaK, is a cell surface protein in a growing number of bacteria and functions as a receptor for plasminogen. This was first revealed with the cell surface proteome of *Listeria monocytogenes* which contains a number of proteins already discussed: GAPD, enolase, EF-Tu and including DnaK (Schaumberg et al 2004). DnaK was first reported to be a plasmingen binding protein in *Neisseria meningitidis* (Knaust et al 2007). This was followed by the report that cell surface DnaK in *M. tuberculosis* bound to plasminogen (Xolalpa et al 2007). A recent report has established that the Hsp70 protein, DnaK of *Bifidobacterium*

animalis subsp.lactis is a cell surface receptor for plasminogen whose expression on the surface is upregulated in the presence of bile salts. (Candela et al 2010).

As has been described (Henderson et al 2006), a number of host cell surface molecular chaperones can act as receptors for both bacteria and viruses. These include Hsp70 and Hsp90 which are part of the receptor for lipopolysaccharide (LPS) (Triantafilou and Triantafilou 2002) and BiP which can bind to various viruses (Honda et al 2009). Trophoblast giant (TG) cells are key points of entry for pathogens capable of inducing abortion. Evidence exists that these TG cells bind to bacteria through a cell surface Hsp70 protein (or proteins) that recognise tetratricopeptide repeats on proteins on the bacterial surface (Watanabe et al 2009).

Bacterial molecular chaperones stimulate mammalian monocytes: The most common moonlighting activity of bacterial molecular chaperones is their ability to activate (or inhibit) monocyte cytokine synthesis. This was first shown in 1983 when Friedland and co-workers reported that the chaperonin (Cpn) 60.2 (Hsp65) protein of *M. tuberculosis* stimulated monocytes to secrete pro-inflammatory cytokines (Friedland et al 1993). This was taken to being equivalent to the classical activation state of macrophages which is induced by LPS or interferon-γ (IFN-γ) (Martinez et al 2009). It also resulted in the literature on this aspect of biology, particularly the monocyte signalling actions of human molecular chaperones being heavily criticised as being due to contamination of the molecular chaperones with bacterial contaminants, principally LPS (Tsan and Gao 2009). Much of this controversy could have been avoided if workers in the field had read the paper by Peetermans et al (1994) which revealed that unlike LPS and IFN-γ, the *M. tuberculosis* Cpn60.2 protein failed to upregulate MHC II or Fc receptor expression in monocytes and also failed to stimulate these cells to generate oxygen-derived free radicals. These are all classic alterations in cell behaviour seen in the classically-activated macrophage (Martinez et al 2009). Thus this Cpn60 protein stimulated what is now termed alternative macrophage activation (Martinez et al 2009) and therefore this particular Cpn60 protein was clearly not contaminated by LPS. Indeed, a complete analysis of the literature on molecular chaperone activity has revealed that bacterial contaminants play no part in the

activity of these proteins (Henderson et al 2010a,c). A recent study of *Francisella tularensis* has revealed that the LPS of this organism is a very weak pro-inflammatory signal and that the Cpn60 protein is a more active monocyte/endothelial cell activator and that the LPS and Cpn60 of this organism actually synergise to activate macrophages or vascular endothelial cells (Noah et al 2010).

The Cpn60 proteins of a growing number of bacteria have been examined and a bewildering variety of activities have been recorded (Table 5). Only a few of the reports in Table 5 will be described in detail and the next section will deal with the molecular chaperones of *M. tuberculosis.*

The bacteria whose molecular chaperones, largely Cpn60, have been studied in some detail are: *Helicobacter pylori*, *Chlamydia pneumoniae* and *M. tuberculosis.*

Helicobacter pylori: The Cpn60 protein of *H. pylori* is a major antigen in patients with gastroduodenal disease with potential diagnostic significance (Macchia et al 1993; Yunoki et al 2000). This immunogenicity may be enhanced by the finding that Cpn60 is a cell surface protein in *H. pylori* (Yamaguchi et al 1996). Curiously, a monoclonal antibody to *H. pylori* Cpn60 is reported to inhibit the growth of the bacterium, suggesting that the surface localisation of this protein is involved in growth control (Yamaguchi et al 1997). What is most intriguing is the reports that the *H. pylori* Cpn60 protein may be involved in the process of gastric carcinoma formation (e.g. Lin et al 2010). There is some confusion in the literature about the mechanism of activation of monocytes and epithelial cells by recombinant or purified *H. pylori* Cpn60 with most reports suggesting that the Cpn60 protein works by binding to toll-like receptor (TLR) 2 (Takenaka et al 2004; Zhao et al 2007). In contrast, using a non-recombinant, purified *H. pylori* Cpn60 protein, it was claimed that activation of murine macrophage cytokine synthesis did not require TLR2/4 or myeloid differentiation factor (MyD) 88 (Gobert et al 2004). This may be because the purified Cpn60 is post-translationally modified compared to recombinant protein or may have something to do with the oligomeric state of the protein as this has been proposed to influence cell signalling activity (Lin et al 2009). In addition to acting as a cell signal, cell surface *H. pylori* Cpn60 also functions as an adhesin for binding to human epithelial cells

(Kamiya et al 1998). The other molecular chaperone which acts as a *H. pylori* moonlighting chaperone is peptidyl prolyl isomerase (PPI). The PPI is immunogenic in patients with gastric ulceration (Atanassov et al 2002). Interestingly, this PPI, which is a secreted protein, induced gastric epithelial cell apoptosis in a TLR4-dependent manner (Basak et al 2005). 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).

Chlamydia pneumoniae: This obligate intracellular bacterium causes around 10% of community-acquired pneumonia (CAP) cases and 5% of cases of bronchitis (Burillo and Bouza 2010). Controversially, it is also implicated as a causative factor in the pathology of atherosclerosis (Watson and Alp 2008). It is this latter hypothesis that has focused on the moonlighting actions of chlamydial molecular chaperones. The first report on the Cpn60 of this bacterium was from Peter Libby's group which revealed the presence of *C. pneumoniae* Cpn60 in atherosclerotic plaques and the ability of this protein to stimulate monocyte pro-inflammatory cytokine and metalloproteinase synthesis (Kol et al 1998). There is also a significant body of work on the immune responses to *C. pneumoniae* Cpn60 (e.g. Mayr et al 1999) which will be largely ignored here as it is nothing to do with protein moonlighting. Later studies revealed that *C. pneumoniae* recombinant Cpn60 stimulated murine monocytes and human microvascular endothelial cells through a conventional TLR4/MD-2/Myd88-dependent pathway (Bulut et al 2002). Activity was heat labile and blocked by antibodies to *C. pneumoniae* Cpn60 thus controlling for LPS contamination. Recombinant *C. pneumoniae* Cpn60 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 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). *In vivo* administration of purified chlamydial HSP60 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 was a more potent neutrophil attractant than was endotoxin or the CpG oligonucleotide 1668 (DaCosta et al 2004). There are few studies of the *in vivo* actions of bacterial molecular chaperones. However one group has administered recombinant *C. pneumoniae* Cpn60 by the intra-tracheal route and has shown that in wild type mice this results in local accumulation of inflammatory cells and up-regulation of cytokine levels (Bulut et al 2009)

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). Unexpectedly, *C. pneumoniae* Cpn60.1 also inhibits the generation of the potent bioactive gas, nitric oxide (NO) from human coronary artery vascular endothelial cells. If this were to happen *in vivo* it would result in endothelial dysfunction (Chen et al 2009).

Genome sequencing is continuing to reveal that a growing proportion of microorganisms have multiple genes encoding chaperonin 60 proteins (Lund et al 2009). In this context it is established that the Chlamydia normally have three genes encoding Cpn60 proteins (Karunakaran et al 2003). All of our information on the chaperonin 60 proteins of the Chlamydia comes from the study of the Cpn60.1/GroEL1 protein. Evidence now exists for the hypothesis that the Cpn60.1 protein of *C. pneumoniae* is a cell surface protein with adhesive properties which facilitates infection of cells with Chlamydia. In contrast the Cpn60.2 and Cpn60.3 proteins are not adhesive (Wuppermann et al 2008).

Mycobacterium tuberculosis: The literature on the moonlighting molecular chaperones of *M. tuberculosis* has recently been reviewed (Henderson et al 2010c) and will only be briefly described. This bacterium has two Cpn60 proteins and the cytokine-inducing, but not monocyte-activating ability of the Cpn60.2 protein has been described. The Cpn60.2 protein is essential, while the gene encoding the Cpn60.1 protein can be dispensed with (Hu et al 2008). While the Δcpn60.1 isogenic mutant responds as the wild type organism to various stressors, and grows *in vitro* and *in vivo* at the same rate as the wild type

bacterium, it fails to induce the classic granulomatous response in the lungs of infected mice. Complementation of the mutant returns its granuloma-inducing activity (Hu et al 2008). This suggests that the *M. tuberculosis* Cpn60.1 protein is involved in the generation of the cells that generate granulomas. Direct confirmation of this has come from testing the Δcpn60.1 isogenic mutant in a human granuloma assay. The mutant produced only 10% of the multinucleate giant cells from whole human blood compared with the wild type or complemented virulent *M. tuberculosis* strain H37Rv (Cehovin et al 2010). When these data are taken into account, together with the fact that the *M. tuberculosis* Cpn60.1 protein is a potent inhibitor of osteoclastogenesis (osteoclasts being one of the only two naturally-occurring multinucleate cells in the mammal) (Winrow et al 2008), the hypothesis generated is that the Cpn60.1 protein of *M. tuberculosis* is able to modulate the cell signalling pathways in macrophages to induce giant cell formation and inhibit osteoclast formation. As it is not known how these two cell populations differ in their differentiation the *M. tuberculosis* Cpn60.1 protein provides a molecular probe to identify the different signalling pathways involved in these two, apparently similar, cell lineages.

It is clear from what has been written above and an earlier description of the activity of the *M. tuberculosis* Cpn60.2 protein that these proteins are unusual modulators of myeloid cell activity and are certainly classifiable as alternative macrophage activators (Henderson and Henderson 2009). While both the Cpn60.1 and Cpn60.2 proteins have been shown to stimulate monocytes to synthesise certain cytokines (Friedland et al 1993; Lewthwaite et al 2001) it has also been shown that the Cpn60.1 protein of *M. tuberculosis* can inhibit the normal cytokine-inducing action that mycobacterial purified protein derivative (PPD) has on macrophages. Thus recombinant *M. tuberculosis* Cpn60.1 inhibits PPD-induced expression of macrophage IL-12P40 by a mechanism involving induction of cell surface TLR2 and the binding of Cpn60.1 to this receptor protein. This results in the down-regulation of nuclear *c-rel* and, in consequence, blocks IL-12P40 transcription (Khan et al 2008).

As has been stated previously in this review, protein homologues may show distinct moonlighting patterns of activity. The two Cpn60 proteins of *M. tuberculosis* have >60% sequence identity. In our current BIOINFORMATIC world this generates the hypothesis that

these proteins are near identical in both structure and function. However, it is well known that single amino acid mutations can lead to significant changes in protein behaviour. For example, one residue difference in haemoglobin generates sickle cell disease through aggregation of haemoglobin. Thus it is perhaps not so surprising that these two Cpn60 proteins have different biological activities. For example, these proteins do not compete for binding to the human monocyte cell surface suggesting that they bind to different receptors (Cehovin et al 2010). The Cpn60.2 protein has no influence on bone breakdown or on osteoclastogenesis while the Cpn60.1 protein inhibits both processes (Winrow et al 2008). A recent series of experiments to determine the role of Cpn60.2 in *M. tuberculosis*:macrophage interactions has revealed the potential importance of this molecular chaperone in the infectious process in tuberculosis. This was first suggested by the finding that Cpn60.2 exists upon the surface of *M. tuberculosis* in significant amount – as does DnaK, the Hsp70 protein of this organism (Hickey et al 2009). The potential importance of the surface location of this protein was shown by experiments that revealed that recombinant *M. tuberculosis* Cpn60.2 inhibits the binding of *M. tuberculosis* to macrophages, as do antibodies to Cpn60.2 (Hickey et al 2009). It has been demonstrated that a large sialylated glycoprotein, CD43, is important in the uptake of *M. tuberculosis* into macrophages (Fratazzi et al 2000). This is the key step in the infection of the host with *M. tuberculosis*. It turns out that *M. tuberculosis* Cpn60.2 is a ligand for CD43 important in the adherence and uptake of this bacterium into macrophages (Hickey et al 2010).

There is a great deal more to learn about the Cpn60 proteins of the mycobacteria. For example, Henderson's group have been unable to show that the Cpn60.1 protein has any molecular chaperone activity (Hu et al 2008). Surprisingly, Mande and co-workers have presented evidence that neither *cpn60* gene can complement an *E. coli* groEL mutant (Kumar et al 2009). It is assumed that some form of technical artefact is to blame for the failure to show folding with the Cpn60.2 protein. Other moonlighting activities reported for the Cpn60.1 protein of the mycobacteria include a role in biofilm formation (Ohja et al 2005). Thus inactivation of the *cpn60.1* gene in *Mycobacterium smegmatis* results in a mutant that grows normally in planktonic culture but which fails to form biofilms at liquid-

air interfaces. The explanation is that Cpn60.1 interacts with KsaA, a protein involved in the type II fatty acid synthase which generates cell surface mycolic acids. To generate biofilms, the bacterium must produce elevated levels of short-chain fatty acids and failure to do so results in altered cell surface properties and an inability to form a biofilm. The same gene inactivation in *M. tuberculosis* does not result in impairment of biofilm formation (Hu et al 2008). Finally, it has been reported that the *M. tuberculosis* Cpn60.1 protein binds to DNA and can be protective of this macromolecule (Basu et al 2009).

In addition to the Cpn60 proteins there is now good evidence for moonlighting of the DnaK/Hsp70 protein of *M. tuberculosis*.

The characteristic granuloma formation found in mycobacterial infections is increasingly thought to be controlled by CC and CXC chemokines (Mendez-Samperio 2008) and therefore understanding how mycobacteria induce expression of these cytokines in important. One component of *M. tuberculosis* which stimulates chemokine synthesis and activates human myeloid and lymphoid cells is the DnaK/Hsp70 protein. The activity of the *M. tuberculosis* Hsp70 protein was first identified with naive primate CD8-enriched lymphocytes which secrete the CC chemokines CCL3, CCL4 and CCL5 (Lehner et al 2000). Human Hsp70 proteins (there are at least 12 Hsp70 genes in *Homo sapiens*) bind to myeloid cells through TLR2/TLR4 (e.g. Vabulas et al 2002) and so it was presumed that the DnaK of *M. tuberculosis* would also bind to this receptor. However, analysis of the binding of *M. tuberculosis* DnaK to myeloid cells identified CD40, a member of the tumour necrosis receptor superfamily, as the key signalling receptor for this ligand. Binding of DnaK via CD40 resulted in monocytes synthesising the CC chemokines described above and the DnaK also induces the maturation of dendritic cells (Wang et al 2001). One study of the human Hsp70 protein has identified that is does bind to CD40. However the binding site in the human Hsp70 protein is within the N-terminal ATP-binding domain which, as will be explained, differs from the binding site in *M. tuberculosis* DnaK, suggesting that the moonlighting activity of these two Hsp70 proteins evolved independently (Becker et al 2002). Now, in all these studies, there is the criticism that the activity of the recombinant protein is due to LPS contamination. Lehner's group, who have conducted these studies,

have rigorously controlled for LPS contamination using five separate controls and have conclusively demonstrated that contamination with this ubiquitous bacterial component is not responsible for the biological actions of *M.tuberculosis* DnaK (Wang et al 2001; Henderson et al 2010a). Using truncation mutagenesis and overlapping synthetic peptides, the binding site in DnaK for CD40 was shown to be within the C-terminus which distinguishes it from the human Hsp70 protein (Wang et al 2002). It later turned out that *M. tuberculosis* DnaK also binds to the HIV co-receptor CCR5 (Whittall et al 2006; Floto et al 2006). This is an interesting finding given that there is synergy between infection with HIV and *M. tuberculosis* (Becker and Wood 2010). Now, as HIV and *M. tuberculosis* DnaK (which is present on the bacterial surface – Hickey et al 2009) both bind to CCR5, can the Hsp70 block HIV binding? Surprisingly, the answer is yes suggesting that this mycobacterial protein may have some therapeutic potential (Babaahmady et al 2007).

Conclusions

It is clear that protein moonlighting contributes to bacterial virulence in a range of important pathogens, including *M. tuberculosis, H. pylori, C. pneumonia, S. aureus, L. monocytogenes* and group A streptococci. Major proteins involved in key metabolic processes, and essential molecular chaperones, essential for dealing with the bacterium's response to stress, also have unexpected functions which contribute to bacterial virulence. Identifying moonlighting activity is clearly not an easy process with most of the currently identified bacterial moonlighting proteins being discovered by accident. The problem of identifying the 'moonlight-ome' for any particular organism is obvious. How does one identify protein moonlighting? A first stage would be to try to understand how moonlighting has evolved and to examine the structural and sequence features of moonlighting proteins. In particular, it would be informative to examine the regions of the proteins' surfaces that are involved in alternative functions and, where possible, to compare orthologous proteins with the same primary function which do and do not exhibit moonlighting behaviour. As more and more data on moonlighting proteins become available, it will become possible to develop statistical, data mining and machine learning approaches to examining these proteins and making predictions.

Acknowledgements

BH acknowledges support from the British Heart Foundation.

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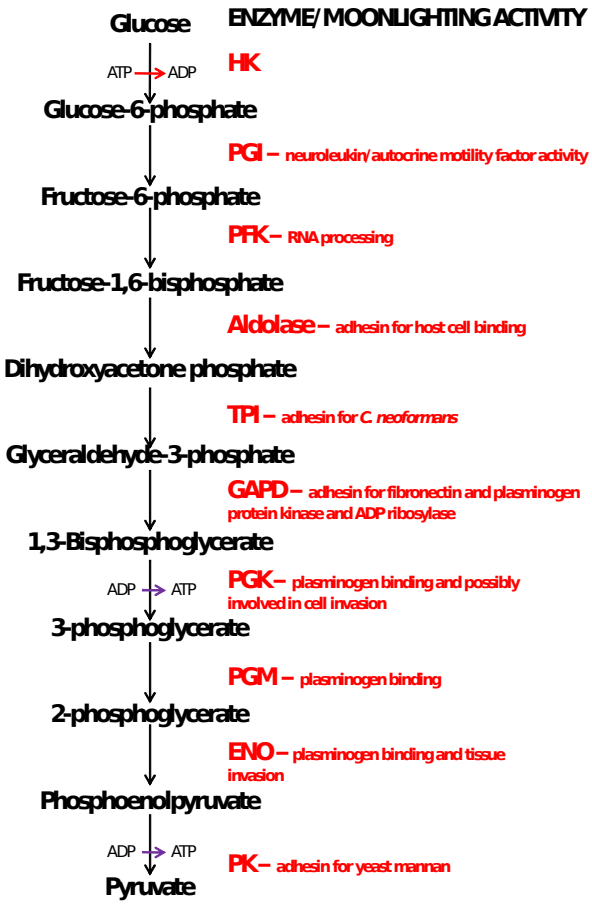
Legends

Figure 1. The glycolytic pathway and moonlighting actions of glycolytic proteins



HK- hexokinase; PGI – phosphoglucose isomerase; PFK – phosphofructokinase; TPI – triose phosphate isomerase; GAPD – glyceraldehydes-3-phosphate dehydrogenase; PGK – phaosphoglycerate kinase; PGM – phosphoglycerate mutase; ENO – enolase; PK- pyruvate kinase.

Figure 2. The glycolytic pathway on the cell surface of bacteria and the possible consequences of having this metabolic pathway functioning in this topological site. The generation of metabolic intermediates and of ATP at the bacterial surface may provide signals to host cell receptors such as the purinoceptor P2X4, whose crystal structure is shown.

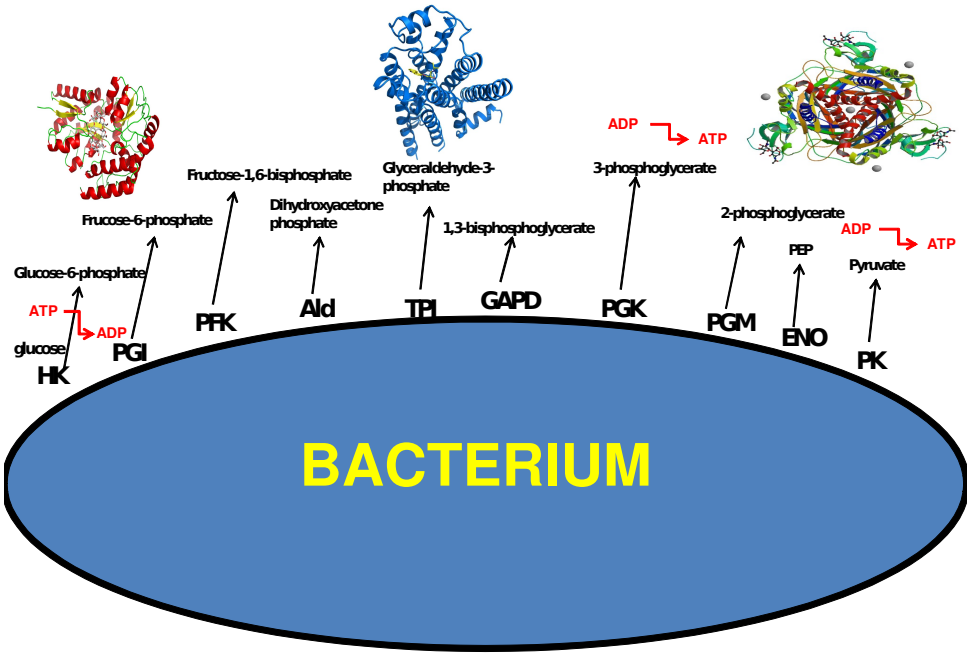


Figure 3. A molecular model of the residues in M. pneumoniae EF-Tu responsible for binding to fibronectin, The M. pneumoniae EF-Tu_{Mp} amino acid sequence was modeled on the structure of EF-Tu from *T. thermophilus* (PDB code 2c78). Crystallographic and biochemical studies suggest that EF-Tu is organized into three domains: 1 (green), 2 purple), and 3 (blue). The regions 193 to 204 and 343 to 357, which are included within Fn binding regions 1 and 2, respectively, are shown in orange and yellow. Residues in these regions that differ from those in *M*. *genitalium* and are believed to participate in Fn binding are shown in ball-and-stick representation. (Reproduced from Balasubramanian et al 2009, with permission).

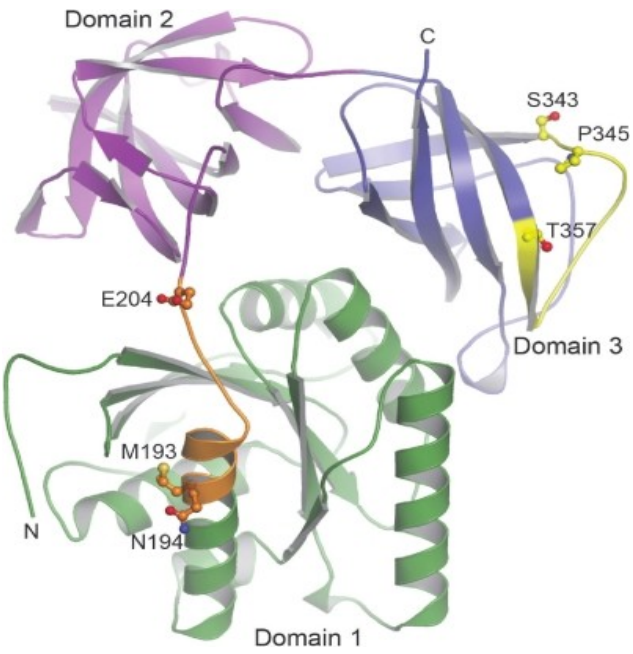


Table 1. Some Moonlighting Proteins in Eukaryotes

Protein	Source	Original Function	Moonlighting Functions
Aldehyde dehydrogenase	cow	alcohol metabolism	lens protein
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

See Piatigorsky 2007 for references

Table 2. Moonlighting Functions of Glyceraldehyde 3-phosphate Dehydrogenase from Eukaryotes and Prokaryotes

Source	Moonlighting Function	Reference
Gecko	lens protein and UV filter	Jimenez-Asensio et al (1995)
Pea chloroplast	DNA glycosylase	Wang et al 1999
Human	control of apoptosis	Saunders et al (1999)
Human	inhibition of caspase 3 and control of apoptosis	Jang et al (2009)
Human	transcriptional regulator	Zheng et al (2003)
Human	binding to telomeric DNA	Sundararaj et al (2004)
Human	interaction with viral RNA	e.g. Sikora et al (2009)
Human	nuclear accumulation	Yego and Mohr (2010)
Chlamydomonas reinhardtii	thio-disulphide exchange	Erales et al (2009)
Xenopus	nuclear membrane assembly	Nakagawa et al (2003)
S. aureus	transferrin binding protein	Modun et al (2000)
Streptococci etc	plasminogen binding protein	Boel et al (2005)
Streptococci etc	ADP ribosylase	Pancholi and Fsichetti (1997)
Streptococci etc	fibronectin binding protein	Pancholi and Fischetti (1992)

Table 3. Bacteria with cell surface moonlighting enolase

Bacterium	Moonlighting function of enolase	Reference
Aeromonas hydrophila	plasminogen binding	Sha et al (2009)
Bacillus anthracis	plasminogen binding	Agarwal et al (2008)
Bifidobacter spp	plasminogen binding	Candela et al (2009)
Borrelia burgdorferi	?	Nowalk et al 2006
Lactobacillus jenseni	inhibitor of neisseria binding	Spurbeck & Arvidson (2010)
Lactobacillus plantarum	fibronectin binding protein/adhesin	Castaldo et al (2009)
Mycoplasma fermentans	plasminogen binding	Yavlovich et al (2007)
Neisseria meningitidis	plasminogen binding	Knaust et al (2007)
Paenibacillus larvae	?	Antunez et al 2010
Staph. aureus	laminin binding protein	Carneiro et al (2004)
Strep. gordonii	MUC7 binding	Kesimer et al (2009)
Strep. mutans	salivary mucin MG2 and plasminogen	Ge et al (2004)
Strep. pneumoniae	plasminogen binding	Kolberg et al (2006)
Strep. suis	fibronectin binding protein/adhesin	Esgleas et al (2008)
Trichomomas vaginalis	plasminogen binding	Mundodi et al (2008)

Table 4. Moonlighting Actions of Bacterial Metabolic Proteins

Bacterium	Metabolic Protein	Moonlighting Activity	Reference
Bacillus stearothermophilus	PGI	similar moonlighting actions to human PGI	Sun et al (1999)
Xanthomonas oryzae pv. *oryzae*	PGI	involved in virulence	Tsuge et al (2004)
Bacillus subtilis	Phosphofructokinase	interactions with RNA processing enzymes	Comminchau et al (2009)
Neisseria meningitidis	Aldolase	adhesin	Tunio et al (2010)
Streptococcus pneumoniae	Aldolase	adhesion for atypical cadherin, Flamingo	Blau et al (2007)
Staphylococcus aureus	TPI	adhesion to fungal mannans	Furuya and Ikeda (2009)
Staphylococcus aureus	GAPD	transferrin receptor	Modun et al (2000)
Streptococcus pyogenes	GAPD	fibronectin binding protein	Pancholi and Fischetti (1992)
Streptococcus pyogenes	GAP	plasminogen binding protein	Lottenber et al (1992)
Streptococcus pyogenes	GAPD	cell signalling kinase/ADP ribosylase	Pancholi and Fischetti (1993,1997)
Streptococcus pyogenes	GAPD	neutrophil evasion protein	Terao et al (2006)
Streptococcus pneumoniae	GAPD	plasminogen binding protein	Bergmann et al (2004a)
Streptococcus oralis	GAPD	binds major fimbriae of P. gingivalis	Nagata et al (2009)
Streptococcus agalactiae	GAPD	immunomodulator	Madureira et al (2007)
EHEC and EPEC	GAPD	bind plasminogen and fibrinogen	Egea et al (2007)
EHEC and EPEC	GAPD	NAD-ribosylating activity	Aguilera et al (2009)
Xanthomonas campestris	GAPD	role in extracellular polysaccharide synthesis	Lu et al (2009)
Mycoplasma pneumoniae	GAPD	adhesin for mucin	Alvarez et al (2003)
Lactobacillus plantarum	GAPD	binds mucus and Caco-2 cells	Kinoshita et al (2008a); Ramiah et al (2008)
Lactobacillus plantarum	GAPD	human ABO blood group	Kinoshita et al (2008b)
Oral streptococci	Phosphoglycerate kinase	plasminogen binding protein	Kinnby et al (2008)
Group B streptococci	Phosphoglycerate kinase	actin binding protein	Burnham et al (2005)
Oral streptococci	Phosphoglycerate mutase	plasminogen binding protein	Kinnby et al (2008)
For Enolase see Table 3			
Lactococcus lactis	Pyruvate kinase	binds to yeast mannan	Katakura et al (2010)
Listeria monocytogenes	Alcohol acetaldehyde dehydrogenase	Binding to human Hsp60	Kim et al (2006)
Bacillus subtilis	Glutamate dehydrogenase	transcription factor binding activity	Gunka et al (2010)
Mycobacterium tuberculosis	Glutamate racemase	DNA gyrase	Sengupta et al (2008)
Mycobacterium tuberculosis	c-AMP phosphodiesterase	controls cell wall permeability	Podobnik et al (2009)
Mycobacterium tuberculosis	Aconitase	iron-dependent RNA-binding activity	Banerjee et al (2007)
Mycobacterium avium	Superoxide dismutase	adhesin binding mucus-associated proteins	Reddy et al (2004)
Mycobacterium tuberculosis	Superoxide dismutase	adhesin binding host GAPD	Reddy and Suleman (2004)
Mycobacterium tuberculosis	Malate synthase	laminin/fibronectin binding protein	Kinhikar et al (2006)
Mycobacterium tuberculosis	Mycosyl transferases	fibronectin binding proteins	Wicker /Harboe (1992); Rosseels et al (2006)
Xanthomonas oryzae pv. *oryzae*	chorismate mutase	involved in virulence	Degrassi et al (2010)
Mycoplasma pneumoniae	β-subunit of pyruvate dehydrogenase	fibronectin binding protein	Dallo et al (2002)

Table 5. Selected Bacterial Molecular Chaperones and their Signalling/Functional Actions

Bacterium	Protein	Signalling Activity	Reference
M. tuberculosis	Cpn60.2	activates monocyte cytokine synthesis	Friedland et al 1993
M. tuberculosis	Cpn60.2	as above but fails to activate macrophages	Peetermans et al 1994
M. tuberculosis	Cpn60.2	cell surface protein involved in macrophage binding	Hickey et al 2009
M. tuberculosis	Cpn60.2	cell surface protein binding macrophage CD43	Hickey et al 2010
M. tuberculosis	Cpn60.1	activates monocyte cytokine synthesis	Lewthwaite et al 2001
M. tuberculosis	Cpn60.1	inhibits osteoclast formation	Winrow et al 2008
M. tuberculosis	Cpn60.1	stimulates multinucleat giant cell formation	Cehovin et al 2010
M. tuberculosis	Cpn60.1	inhibits murine experimental asthma	Riffo-Vasquez et al 2004
M. tuberculosis	Cpn60.1	stimulates formation of granulomas	Hu et al 2008
M. tuberculosis	Cpn60.1	inhibits PPD-induced IL-12 synthesis	Khan et al 2008
M. tuberculosis	Cpn60.1	DNA binding protein	Basu et al 2009
M. tuberculosis	DnaK	stimulates CD8 lymphocyte chemokine production	Lehner et al 2000
M. tuberculosis	DnaK	stimulates monocyte chemokine synthesis and dendritic cell maturation by binding CD40	Wang et al 2001
M. tuberculosis	DnaK	binds to HIV co-receptor CCR5	Floto et al 2006
M. tuberculosis	DnaK	competes with HIV for binding to CCR5	Babaahmady et al 2007
M. smegmatis	Cpn60.1	essential for biofilm formation	Ohja et al 2005
M. leprae	Cpn60.2	inhibits murine experimental asthma	Rha et al 2002
M. tuberculosis	DnaK	plasminogen binding protein	Xolala et al 2007
H. pylori	Cpn60	surface location controls bacterial growth	Yamaguchi et al 1997
H. pylori	Cpn60	stimulates monocyte cytokine synthesis by TLR2	Takenaka et al 2004
H. pylori	Cpn60	stimulates epithelial cell cytokine synthesis via TLR2	Zhao et al 2007
H. pylori	Cpn60	stimulates macrophage cytokine synthesis – no TLR Involvement	Gobert et al 2004
H. pylori	Cpn60	adhesin for human epithelial cells	Kamiya et al 1998
H. pylori	PPI	apoptosis of gastric epithelial cells	Basak et al 2005
H. pylori	PPI	activates monocyte IL-6 synthesis	Pathak et al 2006
C. pneumoniae	Cpn60	activates monocyte cytokine synthesis	Kol et al 1998
C. pneumoniae	Cpn60	oxidation of LDL	Kalayoglu et al 2000
C. pneumoniae	Cpn60	stimulation of vascular smooth muscle cell proliferation	Sasu et al 2001
C. pneumoniae	Cpn60	activates human vascular endothelial cells	Bulut et al 2002
C. pneumoniae	Cpn60	murine dendritic cell maturation	Costa et al 2002
C. pneumoniae	Cpn60	in vivo promotion of neutrophil accumulation	DaCosta et al 2004
C. pneumoniae	Cpn60	human monocyte-derived dendrtitic cell maturation	Ausiello et al 2006
C. pneumoniae	Cpn60	cell adhesin	Wuppermann et al 2008
C. pneumoniae	Cpn60	inhibition of vascular endothelial cell NO synthesis	Chen et al 2009
C. pneumoniae	Cpn60	local administration induces lung inflammation	Bulut et al 2009
Francisella tularensis	Cpn60	more active than LPS and synergises with LPS	Noah et al 2010
Aggregatibacter actinomycetemcomitans	Cpn60	stimulation of bone resorption	Kirby et al (1995)
E. coli	Cpn60	stimulation of osteoclastogenesis	Reddi et al 1998
E. coli	Cpn60	stimulation of monocyte cytokine synthesis	Tabona et al 1998
Enterobacter aerogenes	Cpn60	insect neurotoxin	Yoshida et al 2001