INFECTION AND IMMUNITY, Sept. 2011, p. 3476–3491 Vol. 79, No. 9
0019-9567/11/$12.00 doi:10.1128/IAI.00179-11

MINIREVIEW

Bacterial Virulence in the Moonlight: Multitasking Bacterial Moonlighting Proteins Are Virulence Determinants in Infectious Disease▽

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Men may not be able to multitask, 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 *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Helicobacter pylori*, and *Mycobacterium tuberculosis*.

Protein hyperspace is the term conjured up by the physicists Smith and Morovitz (166) to encompass the theoretical number of proteins that it is possible to generate. For example, the number of 10-kDa (100-amino-acid residue) proteins that can, in theory, be produced is 20^{100} or 10^{130}. 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^{30} of these potential proteins was soluble and had some functional activity, there would still be an unimaginable number of proteins. Thus, with an almost unlimited palette of amino acid sequences and 3.8 billion years to play with (with a doubling time for *Escherichia coli* of 30 min, 3.8 billion years allows for 6.7×10^{13} cell divisions), evolution should have had a good chance at utilizing 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 (34). 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 (with 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 (88, 89). Surprisingly, many of the moonlighting proteins in eukaryotes and prokaryotes are highly conserved proteins, often metabolic proteins/enzymes or molecular chaperones. 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) (55), an autocrine motility factor (AMF) (185), a differentiation and maturation mediator for myeloid cells (196), and an implantation factor (in the ferret) (161). There is now clear evidence that PGI is an important modulator of tumor progression and a target for cancer therapy (56). As will be appreciated from reading this review, the term protein moonlighting can encompass two phenomena. The first is the presence of a second (third, fourth, etc) "biochemical" site on the protein which allows additional biological activities to be displayed. However, these additional activities may occur only when the moonlighting protein is in a different location from that which it normally occupies. Many bacteria secrete glycolytic enzymes which associate with the cell surface and moonlight. Thus, in addition to biochemical moonlighting, it might be said that there is also "geographical" moonlighting, and both may be required to allow any one protein to exhibit true moonlighting activity.

Fifteen years after the introduction of the moonlighting protein hypothesis, it is clear that one of the major beneficiaries of this evolutionary mechanism 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 recognize this phenomenon in bacteriology. The understanding of protein moonlighting is complicated by the fact that not all protein homologues moonlight (62), 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 the following: (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. Among the most commonly identified moonlighting vir-

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▽ Published ahead of print on 6 June 2011.

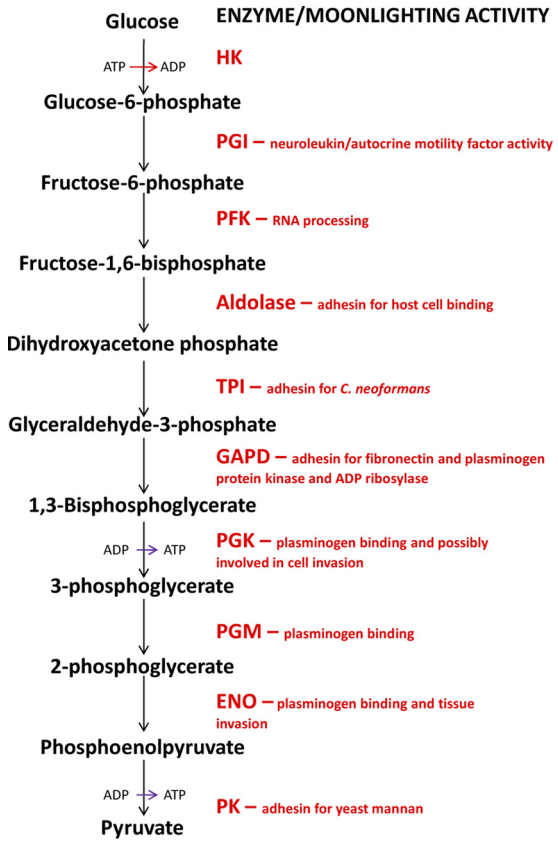

FIG. 1. The glycolytic pathway and moonlighting actions of bacterial glycolytic proteins. HK, hexokinase; PGI, phosphoglucose isomerase; PFK, phosphofructokinase; TPI, triose phosphate isomerase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; PGM, phosphoglycerate mutase; ENO, enolase; PK, pyruvate kinase.

ulence functions of bacterial proteins are adhesion and modulation of leukocyte activity.

MOONLIGHTING BACTERIAL GLYCOLYTIC ENZYMES

Glycolysis exists in all three kingdoms of life and is assumed to have evolved early in the evolution of life (58). In eukaryotes, there are 10 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 acetyl-coenzyme A (CoA) for mitochondrial oxidation (Fig. 1).

A growing number of enzymes of the glycolytic pathway have been found to act as moonlighting proteins, including aldolase, triose phosphate isomerase (TPI), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and enolase. A recent review focused on moonlighting in mammalian glycolytic enzymes reveals the richness of eukaryotic glycolytic moonlighting (99). In addition to moonlighting, there are numerous reports that many bacteria have a number of their glycolytic enzymes associated with the outer aspect of the bacterial cell wall. Currently, Gram-positive organisms such as streptococci and staphylococci appear to have most of the enzymes of the glycolytic pathway on their cell surfaces (53, 86, 115, 192). As there is no currently known mechanism for the selective release of glycolytic enzymes, such reports have been criticized as simply being the result of the binding of enzymes to the bacterial surface as the result of the death of bacteria and the release of such enzymes. However, as will be described, there is incontrovertible evidence that the cell surface GAPDH of *Streptococcus pyogenes* is there as part of an as-yet-undescribed system for secreting cytoplasmic enzymes (26). This hypothesis is supported by studies of the surface location of GAPDH in *Lactobacillus plantarum*, which reveals that soluble GAPDH does not bind to the surface of the intact organism (157). It has to be assumed that the other bacterial cytoplasmic enzymes that are found on the cell surface and 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 the plant's respiration rate (67). Such association of glycolytic enzymes with mitochondria has also been reported for *Saccharomyces cerevisiae* (27). It is not clear if this is relevant to glycolytic enzyme binding in bacteria. The only evidence that cell wall-associated glycolytic enzymes play any role in bacterial physiology is the report that in the presence of low glucose levels, *L. plantarum* has an elevated level of cell wall-associated GAPDH (157). The moonlighting actions of bacterial glycolytic enzymes is summarized in Table 1, and this section will deal with only three of the glycolytic enzymes in any detail.

PGI. As described in the introduction, phosphoglucose isomerase (PGI) is a major human/mammalian moonlighting protein with multiple cell signaling actions and with a role in malignancy in its guise as autocrine motility factor (200). It is also an intriguing autoantigen associated with the induction of a rheumatoid arthritis-like condition in mice (93). The obvious assumption is that glycolytic enzymes in eukaryotes would have evolved from those of the prokaryotes or archaea. However, there is one report that suggests that certain bacterial PGIs may have arisen as a result of gene transfer from a eukaryotic source (96) although this hypothesis has been questioned (68). 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 (169), suggesting that this bacterium, if it colonized humans, could signal via its PGI. However, this result contrasts with that of another group using recombinant PGI from the same bacterium, where it was found that although the bacterial protein was enzymatically active, it did not bind the human PGI cellular receptor, gp78/autocrine motility factor receptor (AMFR), and failed to exhibit the cytokine-like actions of the human PGI (5). The major difference in these studies was that Sun et al. (169) cloned the *pgi* gene and expressed the enzyme and showed that it could crystallize (and therefore was properly folded). In contrast, Amraei and Nabi (5) used a commercial PGI preparation which may have undergone structural alterations to the moonlighting site. Clearly, more work on this area is required as the bacterial PGIs could have important pathological properties if they have the same cell signaling activity as the human enzyme. One

TABLE 1. Moonlighting actions of bacterial glycolytic enzymes

Glycolytic enzyme	Moonlighting function[a]	Reference(s)
Hexokinase	In eukaryotes binds to mitochondrial VDAC receptor	164
	E. coli K1 FimA promotes binding of VDAC1 to mitochondria blocking cellular apoptosis	168
PGI	Described in text	
Phosphofructokinase	Part of a RNA degradosome complex	41
Aldolase	In *S. pneumoniae*, a cell surface lectin which binds host cadherin receptor termed flamingo	25, 115
	In *N. meningitidis* aldolase is a cell surface protein involved in cell adherence	178
	M. avium binds to aldolase from the host	151
Triose phosphate isomerase	*S. aureus* enzyme acts as adhesin for the fungus *C. neoformans*	61, 87, 199
GAPDH	Described in text	
Phosphoglycerate kinase	Plasminogen binding protein in oral streptococci	102
	In group B streptococci cell surface phosphoglycerate kinase binds to cellular actin and may be involved in cell binding and internalization	32
Phosphoglycerate mutase	Streptococcal cell surface plasminogen binding protein	102, 192
Enolase	Described in text	
Pyruvate kinase	*L. lactis* cell surface protein that binds to yeast mannan	95

[a] *C. neoformans*, *Cryptococcus neoformans*; *L. lactis*, *Lactococcus lactis*.

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 (177).

GAPDH. Some of the earliest evidence for protein moonlighting, even before the term was introduced, has come from the study of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 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 GAPDH proteins and exhibited GAPDH enzyme activity. It was shown that this surface protein, termed streptococcal surface GAPDH (SDH) bound to lysozyme, cytoskeletal proteins, and fibronectin (Fn) (137). A separate group identified the *S. pyogenes* GAPDH as a cell surface receptor for plasminogen (116, 190). In an interesting cross-disciplinary interaction, the finding that generation of the signaling gas nitric oxide (NO) in brain cytosolic fractions promoted the ADP-ribosylation of brain GAPDH (204) led to the identification of SDH as a self-ADP-ribosylating enzyme whose activity is enhanced by NO (138). ADP-ribosylation of proteins is an enzymatic process performed by a range of bacterial toxins (80), and it was possible that the cell surface SDH had some form of signaling 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 nonspecific 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 (139). These studies also claim to have identified both a 30- and a 32-kDa binding protein (receptor?) for SDH on the Detroit 562 plasma membranes (139). More detailed examination of the binding of ^{125}I-labeled SDH to isolated Detroit 562 plasma membrane proteins identified a doublet of 30/32 kDa (identified as 14-3-3ε proteins, which are important in eukaryotic cell signaling) plus additional proteins of 37 (GAPDH), 47 (enolase), 55 (urokinase-type plasminogen activator receptor [uPAR]), and 80 (moesin) kDa (90). uPAR (CD87), a glycosylphosphatidylinositol (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 signaling pathways and is important in key cellular events such as proliferation, migration, and inhibition of cell death. Signaling via uPAR, which has no cytoplasmic domain, requires the participation of coreceptors such as the integrins (165). Removal of uPAR from the cell surface resulted in a decrease in bacterial binding to Detroit 562 cells (90).

One seemingly insurmountable problem to 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 provided by Boël and coworkers (26). These workers replaced the chromosomal copy of *S. pyogenes gapdh* with a gene that encoded a functional protein but which contained a 12-residue C-terminal hydrophobic peptide. It was hoped that this hydrophobic segment would prevent egress of the cytoplasmic GAPDH. This hypothesis was confirmed by immunocytochemical localization studies and the failure of the mutant to show cell surface GAPDH activity. So this artificially generated strain is a cell surface enzyme knockout (a "topological mutant"). The GAPDH 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 antiphagocytic activity (26). 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 *S. pyogenes* GAPDH/SDH inhibits the actions of C5a on neutrophils by binding to this protein and blocking neutrophil chemotaxis and hydrogen peroxide production, both of which contribute to bacterial killing (173).

Other streptococci also utilize GAPDH for various moonlighting functions. The GAPDH of the bacterium responsible for causing pneumonia and meningitis, *Streptococcus pneumoniae*, also acts as a plasminogen binding protein (24), which is important in the ability of this organism to cross endothelial and epithelial cell barriers (11). In *Streptococcus agalactiae*, GAPDH is reported to function as a virulence factor with B

lymphocyte-modulatory activity (120), and the same enzyme from *Streptococcus oralis* binds to the major fimbriae of *Porphyromonas gingivalis* and appears to be important in the colonization of the latter organism (130). In *Streptococcus suis* serotype 2, the GAPDH enzyme functions as an albumin binding protein (147) and also as an adhesin for cell binding (28). Lest it be thought that the virulence properties of cell surface GAPDH are solely Gram-positive phenomena, it has been reported that enterohemorrhagic (EHEC) and enteropathogenic (EPEC) strains of *E. coli* express cell surface GAPDH proteins which bind plasminogen and fibrinogen. Nonpathogenic *E. coli* strains do not express this enzyme. Host cells interacting with these pathogenic strains show the presence of GAPDH on their cell surfaces. An interesting observation is that these bacteria produce two forms of GAPDH, differing in their pI, with only the more basic form being secreted (52). The GAPDH proteins from both EHEC and EPEC strains possess NAD-ribosylating activity (2). The GAPDH enzymes also have adhesive properties in bacteria other than streptococci and staphylococci. Thus, *Mycoplasma genitalium* cell surface GAPDH is involved in bacterial binding to mucin (4), and cell surface *L. plantarum* GAPDH binds gastric mucin and also Caco-2 cells, an intestinal epithelial cell line (103, 148). In addition to binding mucin, it has been found that the GAPDH 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 (104). An isogenic mutant generated by inactivation of the gene encoding the GAPDH 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 impairment in *in vivo* virulence and in the ability to synthesize extracellular polysaccharide (117).

Surprisingly, with the relatively large literature on bacterial cell surface GAPDH, there are only two reports of direct evidence for the role of this cell surface protein in bacterial virulence. This is due to the inability to inactivate the gene encoding this protein, and it is surprising that the cell surface GAPD knockout has not been tested *in vivo*. Thus, the finding that nonpathogenic strains of *E. coli* have no cell surface GAPDH is indirect evidence for a role in bacterial virulence. However, direct evidence for a role in virulence has come from the study of the GAPDH of *S. agalactiae*, a protein which has a biological activity not reported for other bacterial homologues, namely, its ability to activate B lymphocytes. To ascertain if this protein had a role in the virulence/colonization of this bacterium, a strain was generated which overexpressed GAPDH. When this GAPDH-overexpressing strain was used to infect mice that are not susceptible to *S. agalactiae* infection, there was a significant increase in bacterial colonization of the livers of mice. This was shown not to be due to differences in growth kinetics of the wild-type and overexpressing strains *in vivo* (120).

Enolase. Enolase is a prototypic moonlighting protein in both prokaryotes and eukaryotes, with putative roles in a variety of human diseases (136). Human cells, such as neurons and endothelial cells, and also yeasts and protozoa have cell surface enolases (136). Only the bacterial enzyme will be discussed. However, readers should be aware that there is an interesting hypothesis 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 human brain neurons (45).

TABLE 2. Bacteria with cell surface moonlighting enolases and moonlighting functions

Bacterium[a]	Moonlighting function of enolase	Reference
A. hydrophila	Plasminogen binding	163
B. anthracis	Plasminogen binding	1
Bifidobacterium spp.	Plasminogen binding	35
B. burgdorferi	Unknown	134
L. jensenii	Inhibitor of *Neisseria* binding	167
L. plantarum	Fibronectin binding protein/adhesin	37
M. fermentans	Plasminogen binding	201
N. meningitidis	Plasminogen binding	106
P. larvae	Unknown	9
S. aureus	Laminin binding protein	36
S. gordonii	MUC7 binding	97
S. mutans	Salivary mucin MG2 and plasminogen binding	64
S. pneumoniae	Plasminogen binding	108
S. suis	Fibronectin binding protein/adhesin	54
T. vaginalis	Plasminogen binding	128

[a] *B. anthracis*, *Bacillus anthracis*; *M. fermentans*, *Mycoplasma fermentans*; *S. mutans*, *Streptococcus mutans*; *T. vaginalis*, *Trichomonas vaginalis*.

A growing range of microbes have been reported to have cell surface enolases which have moonlighting functions (Table 2). As can be seen, these organisms include Gram-positive as well as Gram-negative (e.g., *Borrelia burgdorferi* [134]) 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 GAPDH in *S. 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 (110). Analysis of the plasminogen-binding characteristics of cell wall proteins of *S. pyogenes* identified enolase as the strongest binder. This cell surface enolase was enzymatically active, and antibodies to it induced opsonization and enhanced phagocytosis (140). It turns out that enolase is present on the surface of most streptococci (140) including cells of *S. pneumoniae* (22). 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 (51). It was found that soluble recombinant *S. pneumoniae* enolase bound to the surfaces of the pneumococci even when it was associated with plasminogen. Treatment of the cell surface with proteases inhibited such reassociation, suggesting that binding was due to protein-protein/peptide interactions (22). Inactivation of the enolase gene in *S. pneumoniae* resulted in nonviable cells, showing the essential nature of this gene (22). In studies of mammalian enolases, it was discovered that enolase binding to plasminogen was dependent on C-terminal lysyl residues

in the enolase which bound to lysine binding sites in the plasminogen (152). To test if the pneumococcal enolase also bound plasminogen through C-terminal lysines, both carboxypeptidase treatment and mutation of Lys-433 and Lys-434 were employed. These treatments clearly inhibited plasminogen binding to the enolase, but these studies were done under reducing conditions (22). However, additional analysis of binding, under native conditions, revealed that it was an internal nonapeptide, consisting of residues 248 to 256 (FYDKERKVY), that was responsible for the binding of enolase to plasminogen (23). A similar analysis of the *S. pyogenes* enolase has been made (49). In initial studies, binding of native and mutated enolase to native plasminogen, termed Glu-plasminogen, and to plasminogen after cleavage by plasmin, termed Lys-plasminogen, was investigated. Deletion or substitution of the lysines in enolase at positions 434 and 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 to penetrate a synthetic extracellular matrix (49). A later study showed that lysines at position 252 and 255 also contribute to plasminogen binding (42). A similar binding site has been identified in *Bifidobacterium* spp. enolases (35).

How important is cell surface enolase in the pathogenesis of bacterial infection. As it is not possible to inactivate this enzyme, this leaves only the use of strains in which the plasminogen binding sites in enolase have been ablated/mutated or the use of immunization with recombinant bacterial enolase. There is one small study of the former. Mutation of the *S. pneumoniae* enolase at the C-terminal residues or of these residues plus the internal site (residues 248 to 256) revealed that bacteria producing enolase missing the internal plasminogen binding site were significantly less virulent in a nasal inoculation model in the mouse (23). The recombinant enolase of the Gram-negative organism *Aeromonas hydrophila* (which also binds to plasminogen) has been used to immunize mice, and this markedly decreased the pathology consequent upon infection with this bacterium (163). Thus, it can be concluded that cell surface enolase does contribute to bacterial virulence. Interestingly, in this context, it has been reported that the recombinant enolase from the oral bacterium *Streptococcus sobrinus* is an immunosuppressive protein (180) which can be used, if administered orally, to protect against dental caries in the rat (50). 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 (9).

It is not only plasminogen that bacterial enolases bind to. The enolase of *Streptococcus gordonii* has been found to bind to the salivary mucin, Muc7 (97) (Table 2). In addition, it has been recently reported that the cell surface enolase of the vaginal Gram-positive commensal organism *Lactobacillus jensenii* is a potent inhibitor of the adherence of the Gram-negative *Neisseria gonorrhoeae* to epithelial cells (167). What do we know about the binding of enolase to the bacterial cell surface? One report reveals that enolase (and GAPDH) associates with the surface lipoteichoic acids of *Lactobacillus crispatus* at pH 5 but dissociates at alkaline pH (7).

IMPORTANCE OF THE CELL SURFACE LOCATION OF BACTERIAL GLYCOLYTIC ENZYMES

The moonlighting activity of bacterial glycolytic enzymes is related to their surface location. 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 the Gram-positive organism *Bacillus subtilis* (41). 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 surfaces. Is this significant? In sperm, the glycolytic enzymes form an almost solid-state arrangement associated with a structure near the sperm surface called the fibrous sheath (127). In some tumor cells, or in cells where mitochondria have been knocked out, it is now recognized that such cells consume oxygen at the cell surface via a process called trans-plasma membrane electron transport (tPMET) (81). So could these bacterial cell surface glycolytic enzymes be functional? The glucose concentration in the extracellular fluid of the nondiabetic human is 5 mM, 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 <1 mM although some reports suggest that levels of >1 mM may occur (174). Thus, there is sufficient glucose in the human extracellular milieu to allow the glycolytic pathway to function on the surface of bacteria that are colonizing/infecting *Homo sapiens.* There is some evidence that the glycolytic pathway could have some degree of organization forming a macromolecular complex in association with mitochondria or the plasma membrane (33). This is of relevance to a recent study which captured two recombinant glycolytic enzymes (hexokinase and glucose-6-phosphate isomerase [PGI]) on an artificial substratum and showed sequential enzymatic activity which was manyfold higher than when the same two enzymes were in solution (127). This suggests that the glycolytic pathway, if sequestered on a surface structure such as that of the outer cell wall of a bacterium (or in sperm), could 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 by the glycolytic pathway have 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 signaling to cells through the large family of purinergic receptors/purinoceptors of which the P2Y and P2X subtypes recognize ATP (65). This could be of importance as evidence is accumulating for a role for the

P2X7 purinergic receptor in the release of a potent proinflammatory cytokine from monocytes and macrophages (184). Thus, if bacteria generate ATP locally, they might promote a proinflammatory response—possibly not the most useful outcome for the bacterium. It is certain that other host cellular pathways are more likely to be induced in response to local ATP synthesis. Alternatively, cell surface ATP synthesis may be utilized 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 (194). Another possibility is that the substrates that are produced as part of the glycolytic pathway have signaling actions in their own right. Again, such signaling 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 metabolic pathways, and such pathways involve an extremely large number of individual enzymes whose moonlighting functions are largely unknown. The literature highlights only individual enzymes in individual organisms, and it is difficult to say much about the relevance of such moonlighting activity in a generic sense. The various bacterial metabolic enzymes, including glycolytic enzymes, with moonlighting actions are shown in Table 3, and only a brief description of the nonglycolytic enzymes will be provided. One of the most fascinating moonlighting metabolic enzymes is the alcohol acetaldehyde dehydrogenase of *Listeria monocytogenes* as it is one of the first examples of moonlighting protein-moonlighting protein interactions. Listeria adhesion protein (LAP) was identified as a key adhesin of this organism (158), allowing the bacterium to bind to intestinal epithelial cells. It was then shown that LAP bound to the molecular chaperone chaperonin 60 (Cpn60) or heat shock protein 60 (Hsp60) on the surface of human host cells (181). Then LAP was identified as the alcohol acetaldehyde dehydrogenase, an enzyme involved in alcohol metabolism, of *L. monocytogenes*. Surprisingly, measurement of the kinetics of binding of LAP to human Hsp60, using surface plasmon resonance, revealed a very high-affinity interaction (100). 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 colonization with a pathogenic bacterium.

Another bacterial metabolic enzyme with interesting properties is the glutamate dehydrogenase (RocG) of *B. 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 retain only the binding to the transcription factor (69).

As will be seen later in this review, *M. tuberculosis* and other mycobacteria have evolved a number of moonlighting proteins. Among 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*, Murl 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 (162). *M. tuberculosis* has only one cyclic AMP (cAMP) phosphodiesterase which also plays an independent role in controlling cell wall permeability to hydrophobic cytotoxic compounds (145). 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 tricarboxylic acid (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 posttranscriptional regulation of the expression of proteins involved in iron homeostasis (16). 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 GAPDH and aldolase (150).

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 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 (48).

BACTERIAL MOLECULAR CHAPERONES AND PROTEIN-FOLDING CATALYSTS AS VIRULENCE DETERMINANTS

It is rapidly emerging that molecular chaperones and the associated protein-folding catalysts from bacteria are major classes of moonlighting proteins (39, 73, 76, 144). The literature on the role of bacterial molecular chaperones and protein folding catalysts was reviewed in this journal in 2006 (74), 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 functional receptors. These largely include the 60-, 70-, and 90-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 *L. monocytogenes* which contains a number of proteins already discussed: GAPDH, enolase, elongation factor (EF)-Tu, and including DnaK (160). DnaK was first reported to be a plasminogen binding protein in *Neisseria meningitidis* (106). This was followed by the report that cell surface DnaK in *M. tuberculosis* bound to plasminogen (195). A recent

TABLE 3. Moonlighting actions of bacterial metabolic proteins[a]

Bacterium[b]	Metabolic protein	Moonlighting activity	Reference(s)
B. stearothermophilus	PGI	Similar moonlighting actions to human PGI	169
X. oryzae pv. oryzae	PGI	Involved in virulence	177
	Chorismate mutase	Involved in virulence	48
B. subtilis	Phosphofructokinase	Interactions with RNA processing enzymes	41
	Glutamate dehydrogenase	Transcription factor binding activity	69
N. meningitidis	Aldolase	Adhesin	178
S. pneumoniae	Aldolase	Adhesion for atypical cadherin, Flamingo	25
	GAPDH	Plasminogen binding protein	24
S. aureus	TPI	Adhesion to fungal mannans	61
	GAPDH	Transferrin receptor	126
S. pyogenes	GAPDH	Fibronectin binding protein	137
	GAPDH	Plasminogen binding protein	116
	GAPDH	Cell signaling kinase/ADP ribosylase	138, 139
	GAPDH	Neutrophil evasion protein	173
S. oralis	GAPDH	Binds major fimbriae of *Porphyromonas gingivalis*	130
S. agalactiae	GAPDH	Immunomodulator	120
EHEC and EPEC	GAPDH	Bind plasminogen and fibrinogen	52
	GAPDH	NAD-ribosylating activity	2
X. campestris	GAPDH	Role in extracellular polysaccharide synthesis	117
M. pneumoniae	GAPDH	Adhesin for mucin	4
	β-Subunit of pyruvate dehydrogenase	Fibronectin binding protein	46
L. plantarum	GAPDH	Binds mucus and Caco-2 cells	103, 148
	GAPDH	Binds human ABO blood group antigens	104
Oral streptococci	Phosphoglycerate kinase	Plasminogen binding protein	102
	Phosphoglycerate mutase	Plasminogen binding protein	102
Group B streptococci	Phosphoglycerate kinase	Actin binding protein	32
L. lactis	Pyruvate kinase	Binds to yeast mannan	95
L. monocytogenes	Alcohol acetaldehyde dehydrogenase	Binds to human Hsp60	100
M. tuberculosis	Glutamate racemase	DNA gyrase	162
	cAMP phosphodiesterase	Controls cell wall permeability	145
	Aconitase	Iron-dependent RNA-binding activity	16
	Superoxide dismutase	Adhesin binding host GAPD	150
	Malate synthase	Laminin/fibronectin binding protein	101
	Mycolyl transferases	Fibronectin binding proteins	156, 188
M. avium	Superoxide dismutase	Adhesin binding mucus-associated proteins	151

[a] For enolase, see Table 2.
[b] *L. lactis*, *Lactococcus lactis*.

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 (35).

As has been described previously (74), 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) (175), and BiP, which can bind to various viruses (84). 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 recognizes tetratricopeptide repeats on proteins on the bacterial surface (186). The two 90-kDa heat shock proteins of human cells, Hsp90 and gp96, are increasingly seen to play important roles in bacterial virulence and may open up new therapeutic avenues. The role of these proteins was first discovered when it was found that gp96 on the outer surface of

human brain microvascular endothelial cells (Ec-gp96) bound to the outer membrane protein A of *E. coli* K1 (146). Invasion is crucially dependent on intracellular signaling via the transcription factor signal transducer and activator of transcription 3 (Stat3) (122), and the levels of Ec-gp96 are controlled by nitric oxide/cGMP signaling (125). gp96 is an endoplasmic reticulum (ER) Hsp90 family member, and evidence has recently been found for its role in the uptake of bacterial toxins. *Clostridium difficile* toxin A is the main cause of the antibiotic-associated colitis caused by this organism. Cell surface gp96 on human colonocytes is the plasma membrane receptor for this toxin, and downregulation of the surface expression of gp96 inhibits cellular intoxication (129). Cholera toxin is transported from the cell surface to the ER by retrograde transport, and then the catalytic A1 subunit dissociates and, by an ill-defined mechanism, enters the cytosol. It is now reported that the cytosolic molecular chaperone Hsp90 controls this passage of the toxin in an ATP-dependent manner. The action of Hsp90 on toxin transport can be blocked by geldanamycin, an inhibitor of the ATPase active site in this protein, suggesting that such Hsp90 inhibitors could have therapeutic use in treating cholera (172).

Bacterial molecular chaperones modulate mononuclear phagocyte function. The most common moonlighting activity of bacterial molecular chaperones is their ability to activate (or inhibit) mononuclear phagocyte cytokine synthesis. This was first shown in 1993 when Friedland and coworkers reported that the chaperonin 60.2 (Cpn60.2, also called Hsp65) protein of *M. tuberculosis* stimulated monocytes to secrete proinflammatory cytokines (60). It was tacitly assumed that cytokine synthesis was equivalent to the classical activation state which monocytes enter when they are stimulated with LPS and/or gamma interferon (IFN-γ) (121). Unfortunately, this finding has resulted in the literature on this aspect of cellular microbiology, particularly the monocyte signaling actions of human molecular chaperones, being heavily criticized as being due to contamination of the molecular chaperones with bacterial contaminants, principally LPS (176). Much of this controversy could have been avoided if workers in the field had read the paper by Peetermans and coworkers (143), which revealed that, unlike LPS and IFN-γ, the *M. tuberculosis* Cpn60.2 protein failed to upregulate major histocompatibility complex (MHC) class II antigen or Fc receptor expression in monocytes and also failed to stimulate these cells to generate oxygen-derived free radicals. These are the cellular alterations characteristic of the classically activated macrophage (121). Thus, this mycobacterial Cpn60 protein stimulated what is now termed alternative macrophage activation (121), and therefore this particular Cpn60 protein was clearly not contaminated with 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 (77, 79). A recent study of *Francisella tularensis* has revealed that the LPS of this organism is a very weak proinflammatory signal and that the Cpn60 protein is a more active monocyte/endothelial cell activator. Moreover, the LPS and Cpn60 of this organism actually synergize to activate macrophages or vascular endothelial cells (133).

The Cpn60 proteins of a growing number of bacteria have been examined, and a bewildering variety of biological activities have been identified (Table 4). The bacteria whose molecular chaperones, largely Cpn60, have been studied in any detail are *Helicobacter pylori*, *Chlamydia pneumoniae*, and *M. tuberculosis*, and the literature on these proteins will be dealt with in turn.

***Helicobacter pylori*.** The Cpn60 protein of *H. pylori* is a major antigen in patients with gastroduodenal disease with potential diagnostic significance (119, 203). This immunogenicity may be enhanced by the finding that Cpn60 is a cell surface protein in *H. pylori* (197). Curiously, a monoclonal antibody to *H. pylori* Cpn60 is reported to inhibit the growth of the bacterium, suggesting that the surface localization of this protein is involved in growth control (198). What is most intriguing are the reports that the *H. pylori* Cpn60 protein may be involved in the process of gastric carcinoma formation (see, e.g., reference 113). 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 2 (TLR2) (171, 205). In contrast, using a nonrecombinant, purified *H. pylori* Cpn60 protein, it was claimed that activation of murine macrophage cytokine synthesis did not require TLR2/TLR4 or myeloid differentiation factor-88 (MyD88) (66). This may be because the purified Cpn60 is posttranslationally modified, which does not happen with the *E. coli* recombinant protein, or it may have something to do with the oligomeric state of the protein as this has been reported to influence cell signaling activity (114). In addition to acting as a cell signal, cell surface *H. pylori* Cpn60 also functions as an adhesin for binding to human epithelial cells (92). The other molecular chaperone which acts as an *H. pylori* moonlighting protein is peptidyl prolyl isomerase (PPI). The PPI is immunogenic in patients with gastric ulceration (10). Interestingly, this PPI, which is a secreted protein, induced gastric epithelial cell apoptosis in a TLR4-dependent manner (17). In addition to gastric epithelial cell destruction, the gastropathy associated with *H. pylori* infection involves an inflammatory response, with overexpression of cytokines, particularly interleukin-6 (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 attenuated IL-6-inducing activity (141).

***Chlamydia pneumoniae*.** *C. pneumoniae* is an obligate intracellular bacterium that causes around 10% of community-acquired pneumonia cases and 5% of cases of bronchitis (31). Controversially, it is also implicated as a causative factor in the pathology of atherosclerosis (187). It is the latter hypothesis that has focused on the moonlighting actions of chlamydial molecular chaperones. The first report on the Cpn60 protein of this bacterium was from Kol et al., which revealed the presence of *C. pneumoniae* Cpn60 in atherosclerotic plaques and the ability of this protein to stimulate monocyte proinflammatory cytokine and metalloproteinase synthesis (107). There is also a significant body of work on the immune responses to *C. pneumoniae* Cpn60 (see, e.g., reference 123), 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

TABLE 4. Selected bacterial molecular chaperones and their signaling/functional actions

Bacterium[a]	Protein	Signaling activity	Reference
M. tuberculosis	Cpn60.2	Activates monocyte cytokine synthesis	60
	Cpn60.2	As above but fails to activate macrophages	143
	Cpn60.2	Cell surface protein involved in macrophage binding	82
	Cpn60.2	Cell surface protein binding macrophage CD43	83
	Cpn60.1	Activates monocyte cytokine synthesis	112
	Cpn60.1	Inhibits osteoclast formation	191
	Cpn60.1	Stimulates multinucleate giant cell formation	38
	Cpn60.1	Inhibits murine exptl asthma	154
	Cpn60.1	Stimulates formation of granulomas	85
	Cpn60.1	Inhibits PPD-induced IL-12 synthesis	98
	Cpn60.1	DNA binding protein	18
	DnaK	Stimulates CD8 lymphocyte chemokine production	111
	DnaK	Stimulates monocyte chemokine synthesis and dendritic cell maturation by binding CD40	182
	DnaK	Binds to HIV coreceptor CCR5	57
	DnaK	Competes with HIV for binding to CCR5	13
	DnaK	Plasminogen binding protein	195
M. smegmatis	Cpn60.1	Essential for biofilm formation	135
M. leprae	Cpn60.2	Inhibits murine exptl asthma	153
H. pylori	Cpn60	Surface location controls bacterial growth	198
	Cpn60	Stimulates monocyte cytokine synthesis by TLR2	171
	Cpn60	Stimulates epithelial cell cytokine synthesis via TLR2	205
	Cpn60	Stimulates macrophage cytokine synthesis; no TLR involvement	66
	Cpn60	Adhesin for human epithelial cells	92
	PPI	Induces apoptosis of gastric epithelial cells	17
	PPI	Activates monocyte IL-6 synthesis	141
C. pneumoniae	Cpn60	Activates monocyte cytokine synthesis	107
	Cpn60	Oxidation of LDL	91
	Cpn60	Stimulation of vascular smooth muscle cell proliferation	159
	Cpn60	Activates human vascular endothelial cells	29
	Cpn60	Murine dendritic cell maturation	43
	Cpn60	*In vivo* promotion of neutrophil accumulation	44
	Cpn60	Human monocyte-derived dendritic cell maturation	12
	Cpn60	Cell adhesin	193
	Cpn60	Inhibition of vascular endothelial cell NO synthesis	49
	Cpn60	Local administration induces lung inflammation	29
F. tularensis	Cpn60	More active monocyte activator than LPS and synergizes with LPS	133
A. actinomycetemcomitans	Cpn60	Stimulation of bone resorption	105
E. coli	Cpn60	Stimulation of osteoclastogenesis	149
	Cpn60	Stimulation of monocyte cytokine synthesis	170
E. aerogenes	Cpn60	Insect neurotoxin	202

[a] *M. leprae*, *Mycobacterium leprae*; *A. actinomycetemcomitans*, *Aggregatibacter actinomycetemcomitans*; *E. aerogenes*, *Enterobacter aerogenes*.

conventional TLR4/MD-2/Myd88-dependent pathway (30). 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 TLR2/TLR4-dependent manner (43). 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 (12).

There are few studies of the *in vivo* actions of bacterial molecular chaperones. *In vivo* administration of purified chlamydial Cpn60 to the peritoneal 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 (44). Intratracheal administration of recombinant *C. pneumoniae* Cpn60 in wild-type mice resulted in local accumulation of inflammatory cells and upregulation of cytokine levels (30).

In addition to stimulating cellular cytokine synthesis, it has been reported that *C. pneumoniae* Cpn60, but not Cpn10, is capable of inducing the oxidation of low-density lipoprotein (LDL) (91). It also promotes the proliferation of human vascular smooth muscle cells by a mechanism dependent on TLR4 binding and the activation of p44/42 mitogen-activated protein (MAP) kinase (159). Unexpectedly, *C. pneumoniae* Cpn60 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 (40).

Genome sequencing is continuing to reveal that a growing proportion of microorganisms have multiple genes encoding chaperonin 60 proteins (118). In this context it is established that the *Chlamydiae* normally have three genes encoding Cpn60 proteins (94). All of our information on the chaperonin 60 proteins of the *Chlamydiae* 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 host cells. In contrast, the Cpn60.2 and Cpn60.3 proteins are not adhesive (193).

***M. tuberculosis*.** The literature on the moonlighting molecular chaperones of *M. tuberculosis* has recently been reviewed (79) and will be only 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 (85). 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 (85). This suggests that the *M. tuberculosis* Cpn60.1 protein is involved in the production of the cells that generate granulomas. Direct confirmation of this has come from testing the Δ*cpn60.1* isogenic mutant in a human blood granuloma assay. The mutant produced only 10% of the multinucleate giant cells from whole human blood that was induced by the wild type or complemented virulent *M. tuberculosis* strain H37Rv (38). 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) (191), the hypothesis generated is that the Cpn60.1 protein of *M. tuberculosis* is able to modulate the cell signaling 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 maturation into multinucleate cells, the *M. tuberculosis* Cpn60.1 protein provides a molecular probe to identify the different signaling pathways involved in these two, apparently similar, cell lineages.

It is clear from what has been written above and from 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 (75). While both the Cpn60.1 and Cpn60.2 proteins have been shown to stimulate monocytes to synthesize certain cytokines (60, 112), it has also been shown that the Cpn60.1 protein of *M. tuberculosis* can inhibit the proinflammatory activity of mycobacterial purified protein derivative (PPD) 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 downregulation of nuclear *c-rel* and, in consequence, blocks IL-12p40 transcription (98).

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 nearly identical in both structure and function. However, it is well known that single amino acid mutations can lead to significant changes in protein behavior. For example, one residue difference in hemoglobin generates sickle cell disease through aggregation of hemoglobin. 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 (38). The Cpn60.2 protein has no influence on bone breakdown or on osteoclastogenesis while the Cpn60.1 protein inhibits both processes (191). A recent series of experiments to determine the role of Cpn60.2 in *M. tuberculosis* cell-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* cells in significant amounts, as does DnaK, the Hsp70 protein of this organism (82). 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 (82). It has been demonstrated that a large sialylated glycoprotein, CD43, is important in the uptake of *M. tuberculosis* into macrophages (59). 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 and is important in the adherence and uptake of this bacterium into macrophages (83).

There is a great deal more to learn about the Cpn60 proteins of the mycobacteria. For example, Hu et al. have been unable to show that the Cpn60.1 protein has any molecular chaperone activity (85). Surprisingly, Kumar et al. have presented evidence that neither *cpn60* gene can complement an *E. coli* groEL mutant (109). It is assumed that some form of technical artifact 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 (135). 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 (85). Finally, it has been reported that the *M. tuberculosis* Cpn60.1 protein binds to single-stranded DNA and can be protective of this macromolecule (18).

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 (124), and therefore understanding how

mycobacteria induce expression of these cytokines is 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 (111). Human Hsp70 proteins (there are at least 12 Hsp70 genes in *Homo sapiens*) bind to myeloid cells through TLR2/TLR4 (e.g., 179), 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 tumor necrosis receptor superfamily, as the key signaling receptor for this ligand. Binding of DnaK via CD40 resulted in monocytes synthesizing the CC chemokines described above, and the DnaK also induces the maturation of dendritic cells (182). One study of the human Hsp70 protein has determined 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 (19). 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 (77, 182). 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 (183). It later turned out that *M. tuberculosis* DnaK also binds to the HIV coreceptor CCR5 (57, 189). This is an interesting finding, given that there is synergy between infection with HIV and *M. tuberculosis* (20). Now, as HIV and *M. tuberculosis* DnaK (which is present on the bacterial surface [82]) both bind to CCR5, can DnaK block HIV binding? Surprisingly, the answer is yes, suggesting that this mycobacterial protein may have some therapeutic potential (13).

BACTERIAL MOONLIGHTING PROTEINS WHICH ACT AS ADHESINS AND INVASINS

Bacterial adhesion is essential for the infectious process, and many bacteria also invade cells to colonize and cause disease. Moonlighting proteins from bacteria are involved in both adhesion and invasion. Enolases from various Gram-positive organisms are reported to bind to human plasminogen or laminin (8). The enolase of *Streptococcus suis* is enzymatically active and found present on the bacterial surface. The recombinant protein binds to plasminogen and with high affinity (dissociation constant [K_d] of 21 nM). Furthermore, antibodies to this protein inhibit the adhesion and invasion of *S. suis* into microvascular endothelial cells (54). The pentose phosphate pathway enzyme, 6-phosphogluconate dehydrogenase, also acts as an adhesin in various pneumococcal strains (47). 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.

Fibronectin is a major host component, being found at high concentrations in 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 which function as transducers of fibronectin signaling (78). The binding of group A streptococcal GAPDH to fibronectin has already been described (137). *Lactobacillus plantarum* has a cell surface enolase which binds fibronectin (37). Fibronectin has a complex domain structure, with different parts of the protein binding to different host components including heparin, collagen, gelatin, fibulin, etc. (78). It turns out that a number of bacterial moonlighting proteins, other than those mentioned above, can also bind to fibronectin. *M. 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 that are located at different loci in the genome and show significant nucleotide and amino acid sequence homology and marked immune cross-reactivity (56, 188). Proteins are in the mass range of from 30 to 31 kDa and are all able to bind to fibronectin (188). The site of interaction of the antigen 85 complex proteins has been reported variously as the gelatin binding domain for the *Mycobacterium bovis* protein (142) and the heparin and cell-wall binding regions for the *Mycobacterium kansasii* protein (132). It was therefore surprising when Belisle et al. reported that the antigen 85 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 (21). Crystal structures of recombinant antigen 85C (155) and 85B (6) 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 (21). Although at least 100 bacterial fibronectin binding proteins have been identified (78) we know very little about the nature of the fibronectin binding sites in these proteins. Analysis of the antigen 85B complex member from *M. kansasii* identified two fibronectin-binding epitopes, one a 27-residue stretch (residues 84 to 110) and a second motif of 20 amino acids (residues 211 to 230). Epitopes were highly conserved in the closely related antigen 85 complexes of other mycobacteria. The segment of residues 84 to 110 inhibited the binding of fibronectin to the components of the antigen 85 complex of both *M. kansasii* and *M. bovis* BCG, but motif at residues 211 to 230 did not have the same inhibitory effect. Further examination of the sequence of residues 84 to 110 using synthetic peptides defined residues 98 to 108 as the minimum inhibitory motif, with six residues (FEWYYQ) being most important for fibronectin interaction. This fibronectin binding motif 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 (131). It is also argued that a large region of conserved surface residues among antigen 85A, -B, and -C proteins is a probable site for the interaction of these proteins with fibronectin (155). 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 (101). The binding site in 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.

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 45 kDa, were identified in *Mycoplasma pneumoniae*, and N-terminal sequencing identified these proteins as elongation factor (EF)-Tu and the β-subunit of pyruvate dehydrogenase (46). 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-subunits and one β-subunit which transform pyruvate into acetyl-CoA for mitochondrial oxidation (46). 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. pneumoniae*, and both antibodies could inhibit the binding of *M. pneumoniae* 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 (14). 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 allowed the identification of the moonlighting site in *M. pneumoniae* EF-Tu for binding to fibronectin. 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 to 358 in this *M. pneumoniae* 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 (15).

Autolysins are important peptidoglycan-degrading enzymes. A number of the autolysins of the staphylococci have been shown to function also as fibronectin binding proteins. These include Aaa (autolysin/adhesion of *Staphylococcus aureus*) which binds fibronectin with high affinity (K_d of 30 nM) and which is involved in bacterial adherence to fibronectin (71). *Staphylococcus epidermidis* Aae (autolysin/adhesin in *S. epidermidis*) is homologous to *S. aureus* Aaa and binds to the 29-kDa heparin-binding module of fibronectin (70). Two other staphylococcal autolysins also function as fibronectin binding proteins. These are large (155 kDa) homologous proteins, *Staphylococcus caprae* Atlc (autolysin caprae) (3) and *Staphylococcus saprophyticus* Aas (72), 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 (repeat regions 1 to 3 [R1-R3]) that is sandwiched between two enzymatic domains and has no recognizable similarity to other proteins. The repeat region is responsible for binding to fibronectin, but exactly what binds is still unclear. Using far-Western blotting, only recombinant R1-R3 and R3 alone bind fibronectin. In contrast, using enzyme-linked immunosorbent assay (ELISA) or surface plasmon resonance methods, all recombinant domain constructs bind fibronectin (3). The binding site for fibronectin in the *S. saprophyticus* autolysin has been localized as lying between the two enzymatic domains, within residues 714 to 1202, and inactivation of the gene was shown to result in loss of fibronectin binding (72). *S. saprophyticus* Aas also has hemagglutinating activity and has been tentatively identified with a 160-kDa *S. saprophyticus* hemagglutinin with fibronectin binding ability that had been previously purified from bacterial strains (63, 72).

CONCLUSIONS

It is clear that protein moonlighting contributes to bacterial virulence in a range of important pathogens, including *M. tuberculosis*, *H. pylori*, *C. pneumoniae*, *S. aureus*, *L. monocytogenes*, *Mycoplasma genitalium*, 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 functions which do and do not exhibit moonlighting behavior. 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.

ACKNOWLEDGMENT

B.H. acknowledges support from the British Heart Foundation.

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Editor: H. L. Andrews-Polymenis