Running title: Effectors of mycoplasmal virulence

Virulence Effectors of Pathogenic Mycoplasmas

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Abstract

Members of the genus *Mycoplasma* and related organisms impose a substantial burden of infectious diseases on humans and animals, but the last comprehensive review of mycoplasmal pathogenicity was published 20 years ago. Post-genomic analyses have now begun to support the discovery and detailed molecular biological characterization of a number of specific mycoplasmal virulence factors. This review covers three categories of defined mycoplasmal virulence effectors: 1) specific macromolecules including the superantigen MAM, the ADP-ribosylating CARDS toxin, sialidase, cytotoxic nucleases, cell-activating diacylated lipopeptides, and phosphocholine-containing glycoglycerolipids; 2) the small molecule effectors hydrogen peroxide, hydrogen sulfide, and ammonia; and 3) several putative mycoplasmal orthologs of virulence effectors documented in other bacteria. Understanding such effectors and their mechanisms of action at the molecular level connects the biology of the bacteria to direct effects on the host and host responses they elicit, and is expected to translate into new interventions for human and veterinary mycoplasmosis.

Keywords: mycoplasma, virulence effectors, pathogenesis

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Introduction

Members of the genus *Mycoplasma* and related wall-less organisms are among the smallest free-living eubacteria and have genomes thought to encode little more than the minimal information essential for independent cellular life (Brown *et al*., 2010). Despite their consequent anatomic and metabolic simplicity, mycoplasmas nevertheless impose a substantial burden of infectious diseases on humans and animals. The respiratory and urogenital tracts are most commonly affected but conjunctivitis, arthritis, mastitis and numerous other chronic inflammatory manifestations result from mycoplasmosis. The intrinsic resistance of mycoplasmas to all cell wall-targeting antibiotics plus sulfonamides, trimethoprim, rifampicin, and polymixins, as well as their emerging resistance to macrolides and quinolones (Bébéar and Kempf, 2005), combined with limited success in developing effective vaccines especially for common conditions such as primary atypical pneumonia (Waites and Talkington, 2004) highlight the importance of understanding individual mycoplasmal virulence factors as potential alternative targets of specific therapeutic interventions (Butt *et al*., 2012).

When the last comprehensive review of mycoplasmal pathogenicity was published 20 years ago, although much was known about the biology of the bacteria and host responses they elicit, the identity of any specific mycoplasmal virulence effectors and their mechanisms of action at the molecular level remained "largely elusive" (Razin *et al*., 1998). Written at the dawn of the microbial genomics era, that review was necessarily limited in general to non-specific factors affecting colonization, evasion of the host immune system, and pro-inflammatory outcomes of infection that lead to disease. Since then, the complete annotated genomes of about 70 species of mycoplasma have been published, and

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sophisticated epigenetic (Lluch-Senar *et al*., 2013), transcriptomic (Madsen *et al*., 2008; Vivancos *et al*., 2010; Mazin *et al*., 2014; Siqueira *et al*., 2014), proteomic (Balasubramanian *et al*., 2000; Catrein & Herrmann, 2011; Párraga-Niño *et al*., 2012; Leal Zimmer *et al*., 2018; Paes *et al*., 2018) and metabolomic analyses (Maier *et al*., 2013; Vanyushkina *et al*., 2014; Lluch-Senar *et al*., 2015; Ferrarini *et al*., 2016; Masukagami *et al*., 2018) are now accelerating the detailed characterization of pathogenic mycoplasmas. This new dawn of post-genomic mycoplasmology is an occasion to summarize the current state of knowledge about exactly how mycoplasmas cause diseases, and what remains to be discovered through new molecular approaches.

This review focuses on three categories of defined mycoplasmal virulence effectors: 1) specific protein and lipid macromolecules; 2) small molecule effectors; and 3) putative mycoplasmal orthologs of virulence effectors documented in other bacteria. Pre-requisites for virulence of some mycoplasmas that may factor in pathogenicity but fall outside the scope of this review because they are not yet linked to specific effectors or diseases include: transmission or colonization factors, or related mechanisms (e.g., adhesins or biofilms); persistence factors (e.g., CRISPR-Cas systems); *in vitro* competition for host cellular nutrients (e.g., arginine acquisition); host defense evasion factors (e.g., capsules, biofilms, anti-oxidants of host-generated ROS, immunoglobulin-binding proteins, immunoglobulin-specific proteases, or variable surface antigen systems); host immune dysregulation by heterogeneous factors that collectively include monocyte, lymphocyte or TLR agonists/antagonists (e.g., "lipid-associated membrane protein [LAMP] antigen" fractions); endogenous DNA-modifying enzymes (e.g., methylases, endonucleases or recombinases); and endogenous metabolic attenuation factors (e.g., iron-sulfur cluster

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biosynthesis proteins or dihydrolipoamide dehydrogenase). Entrypoints to the extensive primary literature on those aspects of mycoplasma-host interactions have been provided elsewhere (Brown *et al*., 2010; May *et al*., 2014).

Specific Effector Proteins and Lipids

The superantigen *Mycoplasma arthritidis* mitogen (MAM). Findlay *et al*. (1939) confirmed a causal relationship of certain strains of mycoplasma, subsequently named *Mycoplasma arthritidis*, with acute or chronic relapsing polyarthritis in rats. The mitogenicity of live or cell-free extracts of *M. arthritidis* for cultured rodent lymphocytes was established (Cole *et al*., 1975, 1977; Naot *et al*., 1977), and Cole *et al*. (1981) found that the transforming factor was secreted into cell-free *M. arthritidis* culture supernatant (MAS). The specific effector identified through fractionation of MAS was a soluble protein that became known as *M. arthritidis* mitogen, or MAM (Atkin *et al*., 1986). The *M. arthritidis* gene *mam* (Marth_orf036; Dybvig *et al*., 2008) encodes a 238 a.a. basic (pI 10.1) protein including a 25 a.a. signal peptide that is cleaved from the mature MAM (Cole *et al*., 1996). Two functional domains, predicted through competitive lymphocyte proliferation inhibition assays using a series of overlapping synthetic MAM peptides, were similar to those of microbial superantigens, and to the "β" motif responsible for T cell activation by lectins such as concanavalin A, respectively. This finding supported the large body of detailed evidence that MAM is an MHC class II β chain and T cell receptor (TCR) Vβ chain haplotype-restricted superantigen that interacts directly with murine or human T cells without any processing by antigen presenting cells (APC). MAM can also induce polyclonal

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plasma cell proliferation and antibody secretion by bridging T_H cells to resting B lymphocytes (Cole and Atkin, 1991; Cole *et al*., 1993, 1997), and can induce cytokine and nitric oxide secretion by APC possibly through dimerization of MHC class II molecules by binding to the α chain of one molecule and to the α or β chain of a second (Bernatchez *et al*., 1997; Ribeiro-Dias *et al*., 2003; Shio *et al*., 2008). Crystallographic analyses of MAM monomers and dimers, complexed with TCR and MHC class II molecules either unloaded or loaded with a peptide antigen, showed that MAM adopts a conformation entirely different than that of the "pyrogenic toxin" superantigens from *Staphylococcus* and *Streptococcus*, and also that in the complex MAM directly interacts with the TCR α chain (Zhao *et al*., 2004; Wang *et al*., 2007; Liu *et al*., 2010). For example, the crystal structure of MAM in ternary complex with a murine single-chain T-cell receptor and a human MHCII receptor bound to a synthetic hemagglutinin peptide was depicted by Wang *et al*. (2007). In addition, Mu *et al*. (2005, 2011) used TLR blocking antibodies and TLR2 or TLR4 deficient mice to show that MAM interacts directly with certain TLRs, possibly by cross-linking them with MHC class II molecules. The interaction is potentiated by co-expression of the MHC class II cell-surface receptor HLA-DR (Shio *et al*., 2014). The $TLR2^+/TLR4^+$ mouse T_H cytokine response to MAM is anti-inflammatory, dominated by IL-4, IL-6 and IL-10, but in $TLR4^-$ mice a pro-inflammatory IFNγ, TNFα and IL-12 response dominates. TLR4-blocking antibodies suppressed IL-17, but $TLR2^-$ mice exhibited enhanced production of IL-17 in response to MAM, showing that interactions between MAM and TLRs can directly modulate T_H type 1, type 2 or IL-17/Th17 responses in disease (Mu *et al*., 2011, 2014). No ortholog of *mam* has been recognized in any other species of bacteria, although a candidate paralog, Marth_orf729, occurs in *M. arthritidis*. Neither of these proteins is essential for growth of *M.*

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arthritidis (Dybvig *et al*., 2008), and MAM overexpressing or knockout strains grew at the same rate as wildtype *M. arthritidis* (Luo *et al*., 2008). MAM was surprisingly reported also to exhibit potent DNase activity (Diedershagen *et al*., 2007), but this could not be confirmed using MAM overexpressing or knockout strains of *M. arthritidis* (Luo *et al*., 2008).

Due to their capacity to induce profound immune dysregulation, superantigens have been proposed to play important roles in a number of infectious or autoimmune diseases including arthritis. Although MHC class II haplotype with respect to sensitivity to MAM predicts the severity of reactions of rats or mice to experimental inoculation with *M. arthritidis*, no specific natural disease or syndrome has been attributable solely to MAM (Cole, 1988; Cole and Atkin, 1991; Cole *et al*., 2000). Both virulent and avirulent strains of *M. arthritidis* produce MAM. Conversely, MAM knockout strains of *M. arthritidis* colonized and persisted in the joints of experimentally infected mice as well as controls did (Luo *et al*., 2008). Intra-articular injection of MAM caused rat synovitis that resolved spontaneously in a few days with no residual deficits, but had no effects on the joints of mice (Cannon *et al*., 1988; Sustackova *et al*., 1995). Systemic administration of MAM did exacerbate the severity of collagen-induced autoimmune arthritis in mice, and also induced the onset of arthritis in mice previously exposed to a sub-arthritogenic dose of collagen (Cole and Griffiths, 1993). Splenic lymphocytes recovered from mice injected with MAM become anergic *in vitro*, and such mice were less susceptible to experimental atopic dermatitis and rejected skin allografts more slowly than controls (Cole and Atkin, 1991; Cole *et al*., 1993). MAM is not toxic (Cole, 1988; Cole *et al*., 2000), but a MAM overexpressing strain of *M. arthritidis* was lethal to mice having a defect in the complement C5 protein (Luo *et al*., 2008). Antibodies crossreactive with MAM antigen occur in sera of

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healthy humans as well as patients affected by rheumatoid arthritis or systemic lupus erythematosus (Sawitzke *et al*., 2000; da Rocha Sobrinho *et al*., 2011). Despite the lack of direct evidence for it, the misconception that MAM itself can induce spontaneous chronic arthritis in rodents has been regrettably perpetuated in some recent papers (Zhao *et al*., 2004; Liu *et al*., 2010).

The community-acquired respiratory distress syndrome (CARDS) toxin. Exploration of *M. pneumoniae* binding to alternative host ligands led to the discovery of a hypothetical protein (MPN372) that bound human surfactant protein A (Kannan *et al*., 2005) and annexin A2 (Somarajan *et al*., 2014). Further characterization of this protein indicated that it belongs to the superfamily of toxins that includes pertussis toxin, diphtheria toxin and cholera toxin (Becker *et al*., 2015), and functional analysis indicated that it mediates ADP-ribosylation of at least five host proteins (Kannan *et al*., 2014). MPN372 was renamed community-acquired respiratory distress syndrome (CARDS) toxin, and appears to be unique to *M. pneumoniae* among mycoplasmas (Kannan and Baseman, 2006). Convalescent sera from *M. pneumoniae*-infected patients reacted strongly to purified CARDS toxin, indicating that it is synthesized during infection and contains immunogenic epitopes. Treatment of mammalian cells and tracheal rings with CARDS toxin resulted in vacuolization and cell death, and exposure of mice and baboons to purified toxin resulted in pulmonary lesions reminiscent of *M. pneumoniae* disease (Hardy *et al*., 2009; Kannan and Baseman, 2006). CARDS toxin was associated with airway hyperresponsiveness characterized by eosinophil and lymphocyte infiltration in exposed animals, and marked increases in IL-1α, 1β, 6, 12, 17, TNF-α and IFN-γ (Hardy *et al*., 2009; Medina *et al*., 2012).

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Interestingly, detection of CARDS toxin was deemed the most sensitive way to diagnose persistent *M. pneumoniae* infection in patients with refractory asthma (Peters *et al*., 2011). CARDS toxin production was found to be significantly variable between strains, and strains producing the highest levels of toxin were associated with more severe clinical presentation as measured by colonization, replication, persistence, and lung histopathology in a murine model (Techasaensiri *et al*., 2010). Taken together, these findings present the possibility that the airway hyperresponsiveness associated with high levels of CARDS toxin contributes to reactive airway disease, and explains the successful use of this molecule as a diagnostic antigen in refractory asthma cases.

CARDS toxin is not constitutively expressed *in vitro*. Its mRNA is most abundant during log-phase growth, and infection of mammalian cells leads to enhanced expression versus broth-grown cultures. In addition, quantitation of CARDS toxin in the lung tissue of experimentally infected animals demonstrated further increases in expression, indicating that CARDS toxin production is carefully regulated by *M. pneumoniae* (Kannan *et al*., 2010). Though purified toxin elicits lung pathology, CARDS toxin produced by *M. pneumoniae* during both *in vitro* and *in vivo* infection remains exclusively associated with the bacterial cells, with a small percentage exposed on the surface and the majority remaining intracellular (Kannan and Baseman, 2006; Kannan *et al*., 2010). Despite the lack of secretion, CARDS toxin is likely internalized by host cells using clathrin-mediated endocytosis. Recombinant CARDS toxin is readily internalized via this mechanism, although cell-associated toxin uptake exploiting the same mechanism has yet to be described (Krishnan *et al*., 2013). Following internalization, vacuole formation associated with the host endosomal GTPase Rab9 occurs (Johnson *et al*., 2011), and CARDS toxin

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stimulates the NLRP3 inflammasome complex by catalyzing the ADP-ribosylation of NLRP3 (Bose *et al*., 2014). Manipulation of host Rab GTPases including Rab9 has been implicated in pathogen-mediated uptake and intracellular survival for other bacteria and enveloped viruses (Smith *et al*., 2007; Murray *et al*., 2005). Given the observation of intracellular *M. pneumoniae* during infection and the association of CARDS toxin with increased bacterial persistence, it is plausible that toxin-mediated endocytosis and vacuolization represents a strategy unique among mycoplasmas to promote host cell invasion and intracellular survival.

Sialidase. Carbohydrate cleavage from complex substrates is associated with virulence in many pathogens. Formerly thought to be absent or rare among mycoplasmas, sialidase (neuraminidase) is presently the best-characterized mycoplasmal effector of virulence in the category of glycosidases. The surface-anchored exo-α-sialidase expressed by the avian pathogen *Mycoplasma synoviae* cleaves terminal α-2,6-linked sialic acid from IgG and other serum glycoproteins, and can remove α-2,3-linked sialic acid from tracheal mucus to promote colonization and persistence in the avian trachea [Berčič *et al*., 2011]. Quantitative measurements of sialidase activity among strains of *M. synoviae* indicated that the level of activity varies significantly among strains and correlates significantly with strain virulence (May *et al*., 2007). Activity level is also proportional to the avidity of *M. synoviae* binding to sialylated host erythrocyte surface receptors, suggesting a functional balance between the two activities (May and Brown, 2011).

Because the proportions of α-2,3- and α-2,6-linked sialo-conjugates expressed at various anatomical sites differ among hosts, glycosidic linkage specificities may be an

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important determinant of host range and anatomical niche tropisms. For the avian pathogen *M. gallisepticum* the preferred receptors are α-2,6-linked sialo-conjugates [Glasgow and Hill, 1980], and its sialidase, a true homolog of that expressed by *M. synoviae* (May and Brown, 2009), degrades α-2,6-linkages more efficiently than α-2,3-linked substrates (Sethi and Müller, 1972). Disruption of the sialidase gene in *M. gallisepticum* resulted in an attenuated phenotype as measured by bacterial recovery rates, tracheal lesion scores, and tracheal thickness measurements in experimentally infected Leghorn chickens, although virulence could not be completely restored by genetic complementation of sialidase activity (May *et al*., 2012).

Comparative genome sequencing of the hypervirulent *Mycoplasma alligatoris* revealed the presence of two orthologous sialidase genes, one surface-associated and the other cytosolic, whereas closely-related *Mycoplasma crocodyli* lacked these genes, a key difference between these otherwise similar genomes (Brown *et al*., 2011). This is meaningful because *M. crocodyli* tends to cause disease with a classical course for mycoplasmosis, whereas *M. alligatoris* has a degree of lethal virulence that is unprecedented in the genus (Brown *et al*., 2004, 2011). Infection of alligator fibroblasts with *M. alligatoris* induces apoptosis, and the induction of cell death can be blocked by the addition of the sialidase competitive inhibitor 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid (Hunt and Brown, 2005, 2007). The sialidases of *M. alligatoris* were specific for terminal α-2,3-linked sialic acids; cleavage of α-2,6-linked residues from galactose was not observed (Shama SM, Brown DR, unpublished).

Strains of *Mycoplasma canis*, *Mycoplasma cynos* and *Mycoplasma molare* express an alternative form of sialidase that is secreted into culture supernatant fluid (May and

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Brown, 2009). The secreted sialidase of *M. canis* cleaved α-2,3-linked sialic acid rapidly from fetuin, and α-2,6-linked sialic acid from transferrin at a slower rate (D. L. Michaels *et al*., submitted). Sialidase (either the enzymatic activity, putative genes, or both) has also been reported for strains of *Mycoplasma anseris*, *Mycoplasma cloacale*, *Mycoplasma corogypsi*, *Mycoplasma meleagridis*, *Mycoplasma neurolyticum* and *Mycoplasma pullorum*, but contribution of the activity to virulence of those species is unexplored (Berčič *et al*., 2008, 2011). A retrospective survey indicated that sialidase is not a virulence factor in *M. pneumoniae* mycoplasmosis (May and Brown, 2011).

Cytotoxic nucleases. Cytotoxic nucleases have been described for *Mycoplasma gallisepticum* (MGA_0676), *Mycoplasma genitalium* (MG_186), *Mycoplasma penetrans* (P40), *Mycoplasma hyorhinis* and *Mycoplasma hyopneumoniae* (Mhp379). Though not encoded by homologous genes, in each case nuclease activity was membrane-associated, relied on divalent cations (Ca2+, Mg2+, or both), and contributed to classical apoptotic cell death *in vitro* (Paddenberg *et al*., 1998; Bendjennat *et al*., 1999; Schmidt *et al*., 2007; Xu *et al*., 2014). In contrast, the cytotoxic nuclease Mpn133 of *M. pneumoniae* was found to bind directly human lung cells and mediate apoptosis through a caspase-independent mechanism. Host cell attachment and Mpn133 internalization is distinct from nuclease activity, and is attributed to a glutamic acid-, lysine- and serine-rich (EKS) region. Mpn133 is distinct not only from the cytotoxic nucleases of *M. gallisepticum*, *M. penetrans*, *M. hyorhinis*, and *M. hyopneumoniae*, but also from its homologue MG_186 of *M. genitalium* by possessing the EKS region. The ability to translocate only the nuclease protein rather than

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263 the entire bacterial cell is a unique feature associated with this motif (Somarajan *et al*.,
264 2010).

265 The genetic disruption by random mutagenesis of multiple *M. gallisepticum* genes
266 identified the uncharacterized lipoprotein MslA as a factor important in virulence, though
267 the mechanism for attenuation of knockout mutants in the Leghorn chicken model was not
268 immediately obvious (Szczepanek *et al*., 2010). The *mslA* gene is part of an operon
269 encoding a predicted nuclease, leading Masukagami *et al*. (2013) to hypothesize that the
270 genetic locus was important for interactions with nucleic acids. Recombinant MslA was
271 found to bind random oligonucleotides composed of either single-stranded RNA, single-
272 stranded DNA, or double-stranded DNA, presumably for nuclease degradation and
273 subsequent transport across the *M. gallisepticum* membrane. Thus, MslA appears to
274 contribute to virulence by conferring an advantage to *M. gallisepticum* rather than inflicting
275 damage on the host cells (Masukagami *et al*. 2013).
276
277 **Cell-activating lipopeptides.** The chance observation that live or heat-killed *Mycoplasma*
278 *orale* could activate cytolytic activity of cultured murine macrophages (Loewenstein *et al*.,
279 1983) led to a number of studies showing that lipoprotein fractions from several other
280 species of mycoplasma have similar capabilities. This has been studied most extensively in
281 *Mycoplasma fermentans*, motivated in part by the early observation that an extract of *M.*
282 *fermentans* induced IL-6 production by murine macrophages and human monocytes
283 (Quentmeier *et al*., 1990), because *M. fermentans* was long suspected to be an agent of
284 rheumatoid arthritis (Jonsson, 1961; Mårdh *et al*., 1973) and high concentrations of IL-6
285 are present in the synovial fluid of many affected individuals. Also, *M. fermentans* was later

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suspected to be a significant co-factor in the progression of AIDS (Montagnier and Blanchard, 1993; Blanchard and Montagnier, 1994). The specific effector identified through fractionation of *M. fermentans* lipophilic proteins was a diacylated 2-kD macrophage activating lipopeptide named MALP-2 (Mühlradt *et al*., 1997). Individual clones of *M. fermentans* differed by as much as 50 fold in the amount of specific activating activity they produced. The single *M. fermentans* gene *malp* in fact encodes an amphiphilic, N-terminal membrane-anchored 428 a.a. precursor lipoprotein called P48 (Kostyal *et al*., 1994; Hall *et al*., 1996), M161Ag (Matsumoto *et al*., 1998) or MALP-404 (Calcutt *et al*., 1999). MALP-404 is post-translationally cleaved to generate the residual diacylated 14 a.a. MALP-2 lipopeptide *S*-[2,3-bisacyl($C_{16:0}$/$C_{18:0}$+$C_{18:1}$)oxypropyl]cysteinyl-GNNDESNISFKEK (Figure 1; Mühlradt *et al*., 1997). The soluble C-terminal fragment is thus permanently released from the mycoplasmal cell surface (Calcutt *et al*., 1999; Davis and Wise, 2002). Candidate orthologs of *malp* are present in *Mycoplasma agalactiae*, *Mycoplasma bovis* and *Mycoplasma gallisepticum* (Rosati *et al*., 1999; Markham *et al*., 2003; Lysnyansky *et al*., 2008), and suspected to occur in certain other mycoplasmas (Hall *et al*., 1999). Similar diacylated lipopeptides *S*-[2,3-bisacyl($C_{16:0}$/$C_{18:0}$)oxypropyl]cysteinyl-GQTDNNSSQSQQPGSGTTNT and *S*-[2,3-bisacyl($C_{16:0}$/$C_{18:0}$)oxypropyl]cysteinyl-GQTN, derived from alleles of the variable lipoprotein *vlp* genes of *Mycoplasma hyorhinis* (Citti *et al*., 2000), were comparable to MALP-2 in their potent capacity to stimulate macrophages (Mühlradt *et al*., 1998). This was significant because *M. hyorhinis* is a proven agent of arthritis in swine, and it was suggested that such lipopeptides may be the cause of mixed inflammatory reactions to many species of mycoplasma. A fibroblast-activating example called LP44, having the N-terminal structure *S*-[2,3-bisacyloxypropyl]-cysteinyl-GDPKHPKSFTEWV-, occurs in *Mycoplasma*

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salivarium (Shibata *et al*., 2000). These lipopeptides are all distinctive in their lack of a third N-terminal fatty acid, which was shown to account for their exceptional potency, versus other bacterial lipopeptides, to stimulate macrophages and several other cell types (Mühlradt *et al*., 1997, 1998; Weigt *et al*., 2003; Link *et al*., 2004; Borsutzky *et al*., 2005; Wilde *et al*., 2007). The lipid moieties are the effective agonists, which specifically engage co-expressed TLR2/TLR6 or orthologs with MyD88-dependent NFκB and MAP protein kinase activation as pro-inflammatory consequences (Garcia *et al*., 1998; Calcutt *et al*., 1999; Takeuchi *et al*., 2000, 2001; Nishiguchi *et al*., 2001; Seya and Matsumoto, 2002; Okusawa *et al*., 2004; Nakao *et al*., 2005; Into *et al*., 2007; Mitsunari *et al*., 2006; Shimizu et al., 2008; Oven *et al*., 2013). The "Multiple-Banded Antigen" (MBA) of *Ureaplasma* spp. is possibly another example occurring in the family *Mycoplasmataceae*. Treatments with a synthetic diacylated N-terminal fragment of MBA, dipalmitoyl-S-glyceryl-cysteinyl-SNSTVKSKLSNQFAKSTDGK, induced the same TLR-dependent pro-inflammatory effects resulting in adverse outcomes of pregnancy in C3H/HeN mice (Uchida *et al*., 2013).

Ruiter and Wentholt (1952) first isolated *M. fermentans* from human patients with ulcerative lesions of the penis. The strains produced abscesses when inoculated into the footpads of mice. Although the organism's pathogenic potential has been investigated extensively since then (Lo *et al*., 1993; Stadtländer *et al*., 1993; Montagnier and Blanchard, 1993; Blanchard and Montagnier, 1994; Hayes *et al*., 1996; Gilroy *et al*., 2001; Yanez *et al*., 2013), no natural disease or persistent immunopathology has been attributed exclusively to any specific activating diacylated or triacylated lipopeptide from either *M. fermentans* or any other species of mycoplasma. Instead, the principal pathogenic effect of exposure to the individual mycoplasmal lipoproteins characterized to date is a transient inflammation

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(Lührmann *et al*., 2002), marked by local infiltration of granulocytes, macrophages and lymphocytes, production of pro-inflammatory cytokines, and complement activation (Deiters and Mühlradt, 1999; Matsumoto and Seva, 1999; Shimizu *et al*., 2008a). Induced apoptosis of the activated cells can be an outcome of NFκB and MAP protein kinase activation (Hall *et al*., 2000; Into *et al*., 2004). Pathogenesis is thought to result not from any single lipopeptide, but instead collectively from the multiple effectors likely present in the lipid-associated membrane fraction of the mycoplasmas (Lührmann *et al*., 2002; Shimizu *et al*., 2007, 2008b). Exposure to MALP-2 alone did stimulate the bone-resorbing activity of osteoclasts in bone, or isolated from bone and cultured on dentine slices, effects thought to mimic the bone-destructive processes in arthritis (Piec *et al*., 1999). Because of its amphiphilic nature, MALP-2 remains *in vivo* at the site where it is either generated or injected. It has thus principally a depot effect, while more soluble mycoplasmal lipopeptides can be expected to circulate and so be more likely to have systemic effects (P.F. Mühlradt, pers. comm.). This property has lead to translational demonstrations of its potential therapeutic utility, such as a mucosal adjuvant or to accelerate skin wound healing (Rharbaoui *et al*., 2002, 2004; Deiters *et al*., 2004). However, when fed a high-fat diet, mice lacking the LDL receptor developed intense atherosclerotic lesions in the aorta following intraperitoneal injection with MALP-2 (Curtiss *et al*., 2012).

Phosphocholine-containing glycoglycerolipids. Reminiscent of the chance discovery of activating lipopeptides in *M. orale*-contaminated macrophage cultures, novel glycolipids were detected in what turned out to be *M. fermentans*-contaminated HTLV-1-infected T_H cell cultures (Matsuda *et al*., 1993, 1995). Fractionation of conditioned culture medium

identified two alkaline-labile glycophospholipids called GGPL-I and GGPL-III that were distinctive by their phosphocholine content. Once again motivated by the suspected associations between *M. fermentans* and rheumatoid arthritis or AIDS, their structures were determined to be 6'-O-phosphocholine-α-glucopyranosyl-(1'-3)-1,2-diacyl-sn-glycerol and 1"-phosphocholine,2"-amino dihydroxypropane-3"-phospho-6'-α-glucopyranosyl-(1'-3)-1,2-diacyl-glycerol, respectively. The structures of GGPL-I and GGPL-III were depicted by Matsuda *et al*. (1994, 1997). The structure of a third example from *M. fermentans* called MfGL-II was shown to be 6'-O-(3"-phosphocholine-2"-amino-1"-phospho-1", 3"-propanediol)-α-D-glucopyranosyl-(1'->3)-1,2-diacyl-glycerol (Zähringer *et al*., 1997). Its structure was depicted by Kornspan and Rottem (2012). Although there are strain differences, these individually constitute between 20% and 35% of the total and thus collectively the vast majority of phospholipids of *M. fermentans*. MfGL-II was shown to effect TNFα release by human monocytes, and to induce protein kinase C activation, nitric oxide production, and prostaglandin E_2 secretion by rat astrocytes or mixed glial cell cultures via pro-inflammatory TLR2- and TLR4-independent mechanisms (Ben-Menachem *et al*., 1998; Brandenburg *et al*., 2003; Sato *et al*., 2010). The terminal phosphocholine moiety is the likely effective agonist (Ben-Menachem *et al*., 1998; Rottem, 2002; Kornspan and Rottem, 2012).

The phosphocholine-containing glycoglycerolipids are antigenic. Rabbit polyclonal anti-*M. fermentans* antiserum stained GGPL-I and GGPL-III (Matsuda *et al*., 1997), and anti-GGPL-III specific antibodies were detected in sera of 29 of 65 HIV-1 infected individuals versus only 2 of 117 healthy controls, as well as 32 of 84 synovial tissue specimens from rheumatoid arthritis patients versus 0 of 30 osteoarthritis or normal controls (Li *et al*.,

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1997; Kawahito *et al*., 2008). Although intradermal or intraperitoneal administration of GGPL-III alone did not cause arthritis or allergic inflammation in mice, it did exacerbate both collagen-induced arthritis and nickel allergy, diseases related to autoantigens or autoantibodies (Sato *et al*., 2010).

Small molecule effectors: hydrogen peroxide. Tang *et al*. (1935, 1936) were among the first to report that certain mycoplasmas produce discoloration of blood pigments when grown in the presence of blood, a quantitative effect associated with erythrocyte hemolysis that could be used to discriminate among strains (Warren, 1942). Somerson *et al*. (1965a,b) and Thomas and Bitensky (1966) showed that a hemolysin produced by *M. pneumoniae*, *Acholeplasma laidlawii*, *M. neurolyticum* and *M. gallisepticum* was dialysable, non-proteinaceous and highy labile. Because catalase and horseradish peroxidase prevented the hemolysis, and a specific catalase inhibitor promoted hemolysis, they tentatively identified this effector as either H_2O_2 or a very low molecular weight organic peroxide. Thus it was speculated that mycoplasmal adhesion to host cells *in vivo* could expose the host to mycoplasmal peroxide and its reactive free radical decomposition products in toxic amounts sufficient to alter local structural integrity, cellular biochemistry and antigenicity (Somerson *et al*., 1965b; Cohen and Somerson, 1967; Lipman and Clyde, 1969).

A peroxide hemolysin is produced by many mycoplasmas, including non-pathogens, in amounts that are species and strain-variable (Cole *et al*., 1968; Sobeslavsky and Chanock, 1968; Brennan and Feinstein, 1969; Johnson and Muscoplat, 1972; Pijoan, 1974; Miles *et al*., 1991; Megid *et al*., 2001; Khan *et al*., 2005; Szczepanek *et al*., 2014). Hydrogen peroxide

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401 synthesis by mycoplasmas is linked to glycerol oxidation (Somerson *et al*., 1965a; Low *et*
402 *al*., 1968; Low, 1971; Miles *et al*., 1991; Vilei and Frey, 2001). The key step is conversion of
403 phosphorylated glycerol to dihydroxyacetone phosphate, a substrate for glycolysis, by the
404 FAD-dependent mycoplasmal enzyme L-α-glycerol-3-phosphate oxidase in a reaction
405 having H_2O_2 as a by-product (Wadher *et al*., 1990; Westberg *et al*., 2004; Bischof *et al*.,
406 2009; Hames *et al*., 2009). This pathway is also present in the mosquito-associated
407 pathogens *Spiroplasma culicicola* and *Spiroplasma taiwanense*, but absent from avirulent
408 *Spiroplasma diminutum* and *Spiroplasma sabaudiense* (Chang *et al*., 2014). Since
409 mycoplasmas lack catalase (except *M. iowae*; Pritchard *et al*., 2014) this might seem
410 potentially suicidal (Brennan and Feinstein, 1969; Lynch and Cole, 1980), but self-injury
411 from cytoplasmic peroxide formation may be limited by a relatively inefficient substrate
412 uptake system that is restricted to passive diffusion mediated by the glycerol facilitator
413 protein GlpF and its accessory proteins (Hames *et al*., 2009; Somarajan *et al*., 2010;
414 Großhennig *et al*., 2013). Glycerophosphocholine, potentially available for example from
415 mammalian host lung cells, is an alternative source of phosphoglycerol for some
416 mycoplasmas and spiroplasmas following uptake via the permease GlpU and its accessory
417 proteins (Schmidl *et al*., 2011; Großhennig *et al*., 2013; Chang *et al*., 2014).

418 For many years the only direct evidence of peroxide as an effector of mycoplasmal
419 virulence was a report that mice depleted of both blood and tissue catalase activity
420 developed *M. pulmonis*-induced pneumonia with faster onset and greater severity than
421 mice lacking only tissue catalase or normal controls (Brennan and Feinstein, 1969).
422 Indirect evidence included the positive correlation between the rate of glycerol oxidation
423 and strain-dependent virulence of *M. mycoides* subsp. *mycoides* SC in cattle (Houshaymi *et*

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al., 1997). This was later attributed specifically to a more efficient glycerol uptake system encoded by the *gtsABC* operon that is present in highly virulent strains but absent from less virulent strains of certain species affiliated with the *M. mycoides* phylogenetic cluster (Vilei and Frey, 2001; Djordjevic *et al*., 2003). This glycerol ABC transporter enables significantly faster production and higher endpoint accumulation of H_2O_2 in mycoplasma culture medium containing a concentration of glycerol equal to that in animal serum. *M. mycoides* subsp. *mycoides* SC need not detoxify this excess peroxide to limit cytoplasmic self-injury because its phosphoglycerol oxidase GlpO is anchored in the mycoplasmal membrane, with surface-exposed epitopes (Pilo *et al*., 2005), so the peroxide it forms is presumably excreted instantly. Studies of primary bovine nasal epithelial cells inoculated *in vitro* with virulent *M. mycoides* subsp. *mycoides* SC indicated that through this excretion mechanism adherent mycoplasmas can expose the host to mycoplasmal peroxide in amounts at least 30 fold greater than that accumulated in culture medium. The model for triggering host cell inflammation by this mechanism that integrates an active glycerol transport and phosphorylation system, glycerol facilitator factor, and glycerol kinase was depicted by Pilo *et al*. (2005). Cytotoxity was directly attributable to GlpO-dependent oxidative damage to host cells (Pilo *et al*., 2005, 2007) and the severity of damage correlated positively with the variable rate of cytoadherence among strains (Bischof *et al*., 2008).

Even species like *M. gallisepticum* and *M. pneumoniae* that have predominantly cytoplasmic glycerol oxidation can liberate peroxide in amounts toxic to cultured fibroblasts or HeLa cells, as demonstrated through *in vitro* infections comparing virulent wildtype to attenuated isogenic knockout mutants of glycerol kinase GlpK, GlpO, phosphoglycerol dehydrogenase GlpD, glycerophosphodiesterase GlpQ, or the GlpF

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accessory proteins Mpn133 and Mpn284 (Hames *et al*., 2009; Schmidl *et al*., 2011; Großhennig *et al*., 2013; Szczepanek *et al*., 2014). A schematic illustration of glycerol metabolism linked to H_2O_2 excretion by *M. pneumoniae* was depicted by Großhennig *et al*. (2013). Genetic complementation restored wildtype cytotoxicity to an attenuated GlpU permease knockout mutant of *M. pneumoniae* (Großhennig *et al*., 2013). However, GlpO and GlpK knockout mutants that were not cytotoxic to co-cultured fibroblasts *in vitro* still caused tracheal lesions in chickens (Szczepanek *et al*., 2014). *Mycoplasma iowae* encodes an active catalase KatE that, when expressed in *M. gallisepticum*, reduced the amount of H_2O_2 accumulated in conditioned broth and also lethality in a *Caenorhabditis elegans* toxicity assay (Pritchard *et al*., 2014). Candidate alternatives to catalase or superoxide dismutase as means of protection from self-peroxidation by such species have been proposed (Chen *et al*., 2000; Jenkins *et al*., 2008; Machado *et al*., 2009; Saikolappan *et al*., 2009).

Small molecule effectors: hydrogen sulfide. Contrary to assumptions based on the effects of catalase and catalase inhibitors on erythrocyte hemolysis by *M. pneumoniae* described above, Großhennig *et al*. (2016) found unexpectedly that a GlpO knockout mutant of *M. pneumoniae*, unable to produce H_2O_2, could still lyse erythrocytes in a blood agar overlay via β-hemolysis. When incubated with the GlpO knockout in liquid suspension, the erythrocytes remained intact but underwent the distinctive discoloration from red to brown of α-hemolysis. From those findings the investigators concluded that H_2O_2 plays only a minor if any role in hemolysis by *M. pneumoniae*. Further, the discoloration of hemoglobin was specifically attributable to cysteine-dependent formation of hydrogen sulfide ions by the mutant. The candidate *M. pneumoniae* cysteine desulfhydrase /

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desulfurase HapE was subsequently identified and characterized, and incubation with purified HapE was sufficient to lyse erythrocytes (Großhennig *et al*., 2016). Homologs of HapE occur widely throughout the genera *Mycoplasma*, *Ureaplasma*, and *Spiroplasma*, and their contribution to virulence of other species remains to be investigated.

Small molecule effectors: ammonia. Ammonia is a by-product of ATP synthesis via arginine hydrolysis by some species of both pathogenic and non-pathogenic mycoplasmas (Schimke and Barile, 1963; Barile *et al*., 1966; Sugimura *et al*., 1993). The highly labile ammonia generated by arginine deiminase and carbamyl phosphokinase in this pathway is potentially toxic through direct chemical reactivity plus the increase in pH that ammonium ions cause in the presence of water. The most direct evidence of mycoplasmal ammonia toxicity is a report that the inflammatory response to cutaneous inoculation of rabbits with viable *M. salivarium* suspended in arginine medium was greater than to *M. salivarium* in arginine-free medium or killed *M. salivarium* (Matsuura *et al*., 1990). In exceptional circumstances, lethal hyperammonaemia has been attributed to *M. hominis* infection in humans (Watson *et al*., 1985; Wylam *et al*., 2013). Members of the genus *Ureaplasma* depend principally on urea hydrolysis to synthesize ATP (Romano *et al*., 1986; Thirkell *et al*., 1989; Smith *et al*., 1993). Indirect evidence that the ammonia produced by urease is toxic includes a report that inoculation with ureaplasmas caused ciliostasis and cytotoxicity within 24 hr in a bovine oviduct explant model, effects that could be simulated by addition of urea and jack bean urease to the culture medium of uninoculated controls (Stalheim *et al*., 1976; Stalheim and Gallagher, 1977). The most direct evidence of ureaplasmal ammonia toxicity is that intraperitoneal injection of the bacterial urease inhibitor flurofamide

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493 protected mice against intravenous challenge with lethal doses of either intact *U.*
494 *urealyticum* or the cytoplasmic fraction of sonicated ureaplasmas, whereas unprotected
495 control mice died within 5 min after challenge (Ligon and Kenny, 1991). Lethal
496 hyperammonaemia syndrome has also been attributed to *U. urealyticum* infection in
497 humans (Bharat *et al*., 2015). Both mycoplasmas and ureaplasmas may limit self-injury
498 from cytoplasmic ammonia by eliminating some of it via citrulline biosynthesis (Schimke
499 and Barile, 1963; Smith *et al*., 1992). A second potentially pathogenic outcome of ammonia
500 release by ureaplasmas is the formation of struvite (NH_4MgPO_4) and precipitation of
501 insoluble struvite crystals at high pH in the urinary tract (Grenabo *et al*., 1988). This has
502 been attributed specifically to ammonia production because it too is preventable by
503 flurofamide and other bacterial urease inhibitors (Takebe *et al*., 1984; Texier-Maugein *et*
504 *al*., 1987; Nagata *et al*., 1995).
505
506 **Candidate Effectors**
507
508 **Glycosidases.** The occurrence of host-derived polysaccharide, glycoprotein or glycolipid
509 degrading enzymes among the mollicutes is intriguing because they are documented
510 virulence effectors in other bacteria, but specific evidence of this has been explored only to
511 a limited extent for mycoplasmas. Activities that have been detected by functional assay or
512 predicted by genome annotation in at least one species of *Mycoplasma* and are associated
513 with virulence in other pathogens include sialidase (11 species), β-galactosidase (two
514 species), N-acetyl-β-hexosaminidase (five species), α-mannosidase (three species),

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hyaluronidase (four species), α-amylase (15 species), and β-glucosidase (11 species). Multiple alleles of each enzyme have been reported among species.

Deglycosylation of host glycoconjugates can be accomplished through either individual or cooperative effects of exoglycosidases, and can lead to highly invasive disease or result in exposure or formation of new host antigens and autoimmune complications of infection (Biberfeld, 1979; Matsushita and Okabe, 2001; King *et al*., 2006). For example, deglycosylation of host biantennary glycoconjugates by the sequential actions of the streptococcal exoglycosidases sialidase (NanA), β-galactosidase (BgaA), N-acetylglucosaminidase (StrH) and mannosidase was depicted by King et al. (2006). β-galactosidase has been shown to play a role in bacterial adherence and serial deglycosylation of the host extracellular matrix (King *et al*., 2006; Limoli *et al*., 2011). N-acetyl-β-hexosaminidase has the potential to alter attachment and dispersion in biofilms produced by several Gram-positive and Gram-negative species (Manuel *et al*., 2007). The relevance of α-mannosidase to bacterial virulence is unclear; however, genes encoding this enzyme are almost exclusively found in pathogenic bacteria rather than commensals (Suits *et al*., 2010). Bacterial hyaluronidases have been implicated in direct tissue damage and sterile inflammation (Horton *et al*., 1998, 1999; Knudson *et al*., 2000; Starr and Engleberg, 2006; Termeer *et al*., 2002). α-glucan degradation by α-amylase is implicated in increased invasiveness, loss of extracellular matrix integrity, and prolonged survivability by increasing nutritional fitness (van Bueren *et al*., 2007; Shelburne *et al*., 2009; Abbott *et al*., 2010). Some *Spiroplasma* spp. possess chitinases that have the potential to injure their arthropod hosts as a consequence of nutrient scavenging from the chitin exoskeleton or

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similar glycoprotein substrates (Gooday, 1999; Alexeev *et al*., 2011; Frederiksen *et al*., 2013).

At least 11 species of mollicutes feature β-glucosidase (enzymatic activity, putative genes, or both), but its potential role in virulence has been explored only for *M. mycoides* subsp. *mycoides*. Strains of *M. mycoides* subsp. *mycoides* displaying different degrees of pathogenicity have corresponding sequence diversity in the β-glucosidase gene *bgl*. An Ala204Val substitution is characteristic of attenuated strains, suggesting that β-glucosidase featuring Val204 could contribute to the disease process. Co-incubation of embryonic bovine lung cells with virulent strains of *M. mycoides* subsp. *mycoides* featuring Val204 resulted in rapid cell death in the presence of exogenous disaccharides, whereas co-incubation of host cells with attenuated strains of *M. mycoides* subsp. *mycoides* possessing Ala204 and the same sugars, or with Val204 strains in the absence of exogenous sugars, did not. However, strains featuring the Val204 allele had lower β-glucosidase activity, suggesting that additional virulence factors may be under catabolite repression. Strains with the Val204 allele were also found to have higher rates of survivability and persistence, which may contribute to the disease process by prolonging bacteremia (Vilei *et al*., 2004; Vilei and Frey, 2007). Further characterization of their glycosidases is thus an area with translational potential for novel strategies to treat or prevent mycoplasmosis.

CAMP factor. Erythrocytes and other cells that have substantial amounts of sphingomyelin in their plasma membranes become sensitized, through exposure to sphingomyelinase, to cooperative lysis by effector molecules collectively referred to as Christie Atkins Munch-Petersen (CAMP) factors (Christie *et al*., 1944; Sterzik and Fehrenbach, 1985). Examples of

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CAMP factors include the excreted streptococcal proteins Cfa and Cfb and their orthologs in other Gram-positive bacteria (Podbielski *et al*., 1994; Gase *et al*., 1999; Sörensen *et al*., 2010), the cholesterol oxidase of *Rhodococcus equi* (Fernánández-Garayzábal *et al*., 1996), and certain pore-forming RTX toxins of Gram-negative bacteria (Frey *et al*., 1994; Jansen *et al*., 1995). The CAMP factors can effect or exacerbate cytolysis in the presence of either host- or polymicrobial community-derived sphingomyelinase (Nakatsuji *et al*., 2010; Lo *et al*., 2011). A CAMP factor(s) was present in strains of *M. fermentans*, *M. hominis*, *M. gallisepticum* and *M. penetrans*, and absent from a strain of *M. pneumoniae* examined (Kornspan *et al*., 2014). In contrast, *M. capricolum*, *M. hyorhinis* and *M. mycoides* subsp. *mycoides* displayed an unusual reverse CAMP phenomenon, which manifested as protection of erythrocytes against lysis *in vitro* by *Staphylococcus aureus* β-hemolysin, and was dependent on mycoplasmal cardiolipin synthetase activity. A strain of *M. penetrans* had both positive and reverse CAMP phenotypes, showing that the mechanisms can be independent. No ortholog of any classical CAMP factor is evident in the mollicute genomes annotated to date. The evidence from *M. pneumoniae* indicates that the CAMP factor of mycoplasmas is not simply hydrogen peroxide, therefore the molecular basis of the cooperative cytolysis observed *in vitro* and its role in effecting mycoplasmal virulence remain to be established.

AMPylators. AMPylation is a form of protein modification achieved by covalent addition of adenosine monophosphate (AMP) to hydroxyl side chains. Bacterial AMPylating enzymes may act as virulence effectors when they are translocated from extracellular bacteria via secretion systems (Roy and Mukherjee, 2009; Woolery *et al*., 2010) or from intracellular

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583 bacteria directly into the host cell cytoplasm (Shin and Roy, 2008). For example, the
584 AMPylators VopS, IbpA, and DrrA that are secreted into eukaryotic cells by *Vibrio*
585 *parahaemolyticus*, *Histophilus somni* and *Legionella pneumophila*, respectively, injure host
586 cells by AMPylating the Rho, Rab, or Arf GTPases that control signaling pathways and other
587 essential host cellular processes. Putative orthologs of FIC-family AMPylators occur in *M.*
588 *alkalescens* and *M. canis* (Brown *et al*., 2012; Manso-Silván *et al*., 2013). Although a role in
589 effecting mycoplasmal virulence remains to be established, their absence from a broader
590 spectrum of mycoplasma species argues against a general function in endogenous
591 metabolic regulation by mycoplasmal AMPylators.
592
593 **Glycophorin A proteinase.** Colonization with hemotropic mycoplasmas (hemoplasmas)
594 results in injury to host erythrocytes and endothelial cells through virulence mechanisms
595 whose effectors are not yet known (Felder *et al*., 2011; do Nascimento *et al*., 2012; Sokoli *et*
596 *al*., 2013). One outcome is excessive eryptosis, the induced death of erythrocytes
597 characterized by cell shrinkage and membrane blebbing that contributes to anemia (Lang
598 *et al*., 2012). Such mechanical properties of the erythrocyte membranes are influenced by
599 integral proteins called glycophorins. Glycophorin A, a heavily sialylated glycoprotein that
600 serves as a receptor for attachment by many species of bacteria, is a substrate for the
601 bacterial enzyme *O*-sialoglycoprotein endopeptidase. Host cell injury via glycophorin A
602 degradation is a putative virulence mechanism of bacteria like *Mannheimia haemolytica*
603 (Abdullah *et al*., 1992), thus this enzyme may also be considered one candidate virulence
604 effector of hemoplasmas. Homologs of glycophorin A protease are annotated also in the
605 genomes of many other species of mollicutes.

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Conclusion

Despite their relative genetic and phenotypic simplicity, mycoplasmas express diverse types of virulence effectors. Examples with profound effects on virulence like the superantigen MAM, the CARDS toxin, and the *gtsABC* operon involved in hydrogen peroxide secretion occur only infrequently. Effectors like sialidase and cytotoxic nucleases are more widely distributed, while diacylated lipopeptides and small-molecule effectors are much more commonly expressed. The challenges remaining as the post-genomic era matures are to elucidate in greater detail those mechanisms of pathogenicity not yet linked to specific virulence effectors, to establish the significance of mycoplasmal orthologs of effectors documented in other bacteria, and to translate this knowledge into intervention strategies effective in reducing the collective burden of human and veterinary mycoplasmosis.

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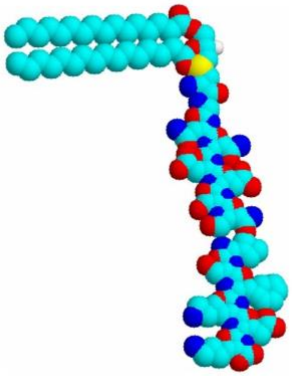
FIGURE

Figure 1.



Structure of the active stereoisomer of MALP-2, S-[2,3-bispalmitoyloxy-(2R)-propyl]-cysteinyl-GNNDESNISFKEK. Used with permission of Peter Mühlradt.