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Wenyi Luo
University of Alabama at Birmingham

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by

WENYI LUO

KEVIN DYBVIG, COMMITTEE CHAIR
DANIEL BULLARD
DAVID E. BRILES
JANET YOTHER
TRENTON R. SCHOEB

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WENYI LUO

GENETICS AND GENOMIC SCIENCES

ABSTRACT

Mycoplasmas cause a group of diseases that are characterized by their chronicity and resistance to treatment. Diseases caused by the murine pathogens *Mycoplasma pulmonis* and *Mycoplasma arthritidis* are often studied as models of human diseases caused by a variety of chronic pathogens that induce a similar pathology. Among several candidate pathogenic factors, superantigens and degradative enzymes such as glycosidases are potentially important but not well characterized.

The *M. arthritidis* mitogen (MAM) is a superantigen secreted by *M. arthritidis*. Its role with respect to arthritogenicity and toxicity is unclear. To improve the efficiency of transformation and hence enhance efforts to generate MAM mutants through transposon mutagenesis, the function of Marth_orf138, a candidate DNA methyltransferase-coding gene that could be a component of a restriction and modification system that served as a barrier to gene transfer, was studied. The expression of Marth_orf138 in *E. coli* protected DNA from digestion by the HhaI restriction enzyme. However, the transformation efficiency of *M. arthritidis* was not significantly affected by modification of plasmids by the HhaI DNA methyltransferase, and existing methods had to be used to obtain MAM mutants. Mutants that overproduced MAM and failed to produce MAM were generated. Mitogenic activity and lethal toxicity in DBA/2J mice correlated with the amount of MAM produced. However, there was no correlation between the severity of arthritis developed as determined by histopathological examination of joint tissue and the

amount of MAM produced. Our results demonstrated that MAM is associated with lethal toxicity but not arthritis in mice.

Glycosidases are potentially important virulence factors but have not been carefully studied in mycoplasmas. By studying the genome sequence of *M. pulmonis*, five genes coding for glycosidases were identified. Cellular localization studies demonstrated that the MYPU_4630 protein was secreted. The enzymatic assay of purified glutathione S-transferase-MYPU_4630 recombinant protein demonstrated that the protein had glycosidic activity for α-linked N-acetylglucosamine (GlcNAc) and N-acetylgalactosamine (GalNAc). MYPU_4630 protein is the first identified glycosidase with activity for GlcNAc and GalNAc in any mycoplasma. Future studies will examine whether mycoplasmal polysaccharides are a substrate for the enzyme and the relevance of the enzyme to pathogenesis.

Keywords: Mycoplasma; Superantigen; *M. arthritidis*; MAM; *M. pulmonis*; Glycosidase

Dedicated to my wife Shaoning Jiang for her unfailing love and continuous support

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TABLE OF CONTENTS

Page

LIST OF TABLES

Tables *Page*

LIST OF FIGURES

Figure *Page*

IDENTIFICATION OF AN ISOSCHIZOMER OF THE HHAI DNA METHYLTRANSFERASE IN *MYCOPLASMA ARTHRITIDIS*

ASSOCIATION OF *MYCOPLASMA ARTHRITIDIS* MITOGEN (MAM) WITH LETHAL TOXICITY BUT NOT ARTHRITIS IN MICE

SECRETED GLYCOSIDASE FROM *MYCOPLASMA PULMONIS*

INTRODUCTION

General characteristics of mycoplasmas

Mycoplasmas are the smallest and simplest free-living organisms (177). They belong to the class *Mollicutes* and are characterized by the lack of a cell wall and a small genome (80). Phylogenetic analysis based initially on rRNA sequences (particularly 16S rRNA) and now on complete genome sequence data supports the model that mycoplasmas are derived from Gram-positive eubacteria (165). One of the major features of mycoplasma evolution is the loss of the cell wall, which makes mycoplasmas devoid of cell wall-associated surface molecules such as teichoic acid. Their single membrane lacks lipopolysacharide (LPS) but harbors abundant surface proteins that extend into the extracellular space (80). Some of these lipoproteins have roles in adherence (19), immune avoidance (80, 195), or activation of macrophages (143). Many of them are encoded by genes containing repetitive DNA sequences (184) and therefore are subject to high-frequency phase or size variation as a result of slipped-strand mispairing during DNA replication (68). The lack of a rigid cell wall makes mycoplasmas pleiomorphic (40). Some species such as *Mycoplasma mobile* and *Mycoplasma pneumoniae* have membrane protrusions composed of a cluster of proteins that are involved in attachment and gliding motility (156). Capsules or capsule-like structures have been observed in several species (6, 97, 158, 160, 183, 206). These structures are likely composed of polysaccharides. However, knowledge of the structure, composition, biosynthesis and pathological roles of mycoplasma capsules is limited. The membrane of mycoplasmas is characterized by the presence of sterols that can account for up to 20% of the total lipid (198). Exogenous sterols are incorporated into the membrane of mycoplasmas and are essential for most

species, which is a unique feature in prokaryotic microorganisms (178, 179).

The other major facet of mycoplasma evolution is the marked reduction of the genome. The genome size of mycoplasmas ranges from 0.58 to 1.8 Mb (80). Their genome is tailored to minimal life requirements (84). Many genes code for proteins that are required for basal metabolism and are therefore indispensible for extracellular living. In *Mycoplasma genitalium*, the mycoplasma species with the smallest known genome, about 382 of the 482 protein-coding genes are essential as determined by global transposon mutagenesis (94). Due to this property, mycoplasmas are considered as important models to study the minimal genomic requirement for a free-living cell to sustain life. The genomic DNA of mycoplasmas is characterized by a low GC content, below 30% with few exceptions, and is manifested as a bias toward the use of A- and U-rich codons. For example, UGA is used as the codon for tryptophan instead of a stop signal (80). As a result of their limited genetic information, mycoplasmas have significantly reduced biosynthetic capabilities as compared to their Gram-positive bacterial relatives (184). Mycoplasmas are thus one of the most fastidious groups of bacteria with a complex nutritional requirement. They lack the major pathways for synthesis of amino acids, nucleic acid precursors, lipids, and vitamins (179). As such, these organic nutrients have to be acquired from the host by the production and secretion of scavenging enzymes (80). They may use various substrates as energy source. ATP is generated by glycolysis in many species, such as *Mycoplasma pulmonis*, while metabolism of arginine generates ATP in *Mycoplasma arthritidis* (173). Even with all their nutrients and energy sources supplied, mycoplasmas still grow slowly, with doubling times in some species as high as 16 hours (168). The strict and varying needs

for nutrients and the long doubling times make their isolation and identification difficult to achieve in clinical settings (168, 182).

Genetic manipulation of mycoplasmas

The role of virulence factors in pathogenesis cannot be definitively studied without mutants that do not produce or overproduce them. However, genetic manipulation of mycoplasmas was difficult and almost impossible until the successful application of polyethylene glycol (PEG) - mediated transformation (78). Even with PEG, transformation frequencies are extremely low, ranging from 10^{-6} to 10^{-8} transformants per CFU (221). Transformation is even more difficult in *M. arthritidis* (220), possibly due to the presence of restriction and modification (R-M) systems. Type I and II R-M systems are common in mycoplasmas and a type III system has been described in *M. pulmonis* (77). One of the major functions of R-M systems is to protect bacteria from infection by bacteriophage (162). However, R-M systems also destroy DNA elements introduced by artificial transformation (230). To avoid R-M activity, it has been reported that DNA must be modified with the AluI methyltransferase prior to being used for transformation of *M. arthritidis* (220). The second hurdle for genetic manipulation of most species of mycoplasma is the lack of homologous recombination (HR)-based methods to specifically target genes for disruption (69). RecA protein promotes single-strand DNA invasion and is required for HR in most systems (124). Specific gene targeting has been reported for a few species of mycoplasma (38, 59, 69, 115, 131, 180). Possibly, these species produce relatively large amounts of RecA and have more active levels of HR. However, the production of RecA in different species and its association with the level of HR has not been studied. Another factor that hampers genetic manipulation is the

inability of most plasmids to replicate in mycoplasmas. *Escherichia coli* plasmids used in cloning are suicide plasmids in mycoplasmas and are lost during cell division. Because the plasmids do not persist, there is little time for recombination between the plasmid and the mycoplasma chromosome to occur. There is only one *Mycoplasma* species that has small cryptic plasmids—*M. mycoides* subsp. mycoides. These plasmids can also replicate in *M. capricolum* but not in other species (123). The reason for their host specificity may be that *M. mycoides* and *M. capricolum* but not other species have *polA*, coding for DNA polymerase I (128, 179), an enzyme required for replication of many plasmids (71, 125, 201). Vectors containing a mycoplasmal chromosomal DNA origin of replication (*oriC*) have been used to enhance the persistence of plasmids in some species (38, 59, 115, 131, 180). Such plasmids are species-specific, and their development is highly dependent on a precise prediction of the replication origin (130). Plasmids containing *oriC* are maintained in low-copy number and can be problematic because of a strong tendency to integrate into the bacterial chromosome at *oriC* site (59).

Due to the reasons aforementioned, transposon mutagenesis is still the most commonly used method to generate mycoplasma mutants (101). Transposons contain one or more antibiotic resistance determinants and a gene coding for the transposase enzyme that catalyzes transposition of the DNA element (138). Because they are usually carried on the plasmids that do not replicate in mycoplasmas, successful transformation is dependent on a transposition event that inserts the transposon into the recipient chromosome. The tetracycline resistance determinant (*tetM*) is widely used as the antibiotic selection marker because of the low level of spontaneous resistance to tetracycline (4, 79). Other selectable markers such as chloramphenicol and gentamicin

resistance determinants are commonly used in mycoplasmas when mutants created with a transposon containing *tetM* are complemented (79, 169). A variable level of background is observed when using chloramphenicol and gentamicin for selection (4, 100). Tn*916* and Tn*4001* are two of the most widely used transposons for mutagenesis (221). They are derived from Gram-positive bacteria and can transpose into the chromosome at a diversity of sites (76, 79, 142). However, both have limitations. Both transposons transpose actively in the mycoplasma chromosome, creating heterogeneity in the cell population when these elements transpose from the initial site of integration to secondary sites in the genome (79, 175). Also, Tn*4001* is bound by insertion sequence elements that sometimes transpose independently of the entire transposon and create additional heterogeneity (139). Newly developed Tn*4001*-based minitransposons have the transposase gene on a plasmid at a site adjacent to but outside of the transposon (175). Insertion of the minitransposon is stable due to the absence of the transposase gene in the mycoplasma genome. Because the transposons insert into the chromosome at essentially random sites, large-scale screening is required to identify the transformants with disruptions in particular genes of interest. In addition to mutagenesis, transposons can be used as vectors to express transformed genes in mycoplamas, as has been successfully used for *M. pneumoniae* (222).

Mycoplasmas as pathogens

Characteristics of mycoplasma infection

Mycoplasmas inhabit a wide range of hosts including plants, insects, animals and humans. They are commensal or occasionally parasitic bacteria and can cause symptomatic infections. In animals and humans, mycoplasmas reside on the surface of

mucosal membranes and cause infections of conjunctivas, airways, joints and the urogenital tracts (179). Diseases induced in animals are of great importance because they cause significant health problems in livestock (66, 141) and experimental animals (31). *M. mycoides* causes contagious bovine pleuropneumonia (CBPP), a cattle disease characterized by lung solidation and subsequent respiratory distress (170) that is associated with a high morbidity and mortality (207). It was identified as the pathogen responsible for CBPP over one hundred years ago, and CBPP is still a major cattle disease in Africa with serious impact on economy (66). Diseases induced in animals by mycoplasmas mimic similar diseases in humans and can be used as models to study their human counterparts. For example, *M. pulmonis* and *M. arthritidis* infections are considered as the most useful models for the study of mycoplasma-induced respiratory and joint infections of humans, respectively (33, 44).

Many different mycoplasma pathogens induce a similar disease course in their respective host. Acute toxic syndrome can be induced experimentally in animals at the onset of infection and can be followed by lethality when inocula of high titers are used (90, 153, 212). If animals survive the acute phase, a characteristically chronic and slowly-progressing disease ensues (43). Atypical pneumonia in humans induced by *M. pneumoniae* can last at least 42 days (144). One study reported that 41% of the pelvic inflammatory disease (PID) patients with *M. genitalium* still tested positive 30 days later with associated endometritis and pelvic pain (99). The pathology of the infection suggests a slow progression of damage to host tissues and is usually associated with persistence of live organisms (57, 65). Prolonged infection can lead to severe sequela. *M. genitalium, M. hominis* and *Ureaplasma urealyticum* are etiological agents of non-gonococcal

urethritis (NGU). Undertreated NGU can progress to PID which can potentially result in infertility (98). Mycoplasmas might be involved in several chronic diseases that have been attributed to autoimmunity, by causing or exacerbating the diseases. *M. pneumoniae* infection has been linked to asthma. *M. pneumoniae* infection may precede the onset of asthma or be associated with exacerbation (117, 161, 203, 204). The association between inflammatory bowel disease (IBD) and mycoplasma infection has also been investigated (34, 185). By using PCR-based techniques, these studies demonstrate the high prevalence of *M. pneumoniae* in IBD. Both studies found a higher detection rate of *M. pneumoniae* in Crohn's disease than in ulcerative cholitis.

Mechanism to prolong persistence

Mycoplasmas possess multiple mechanisms that prolong their persistence in the host by modifying or evading the immune response. Mycoplasmas can suppress the immune system. Depressed cellular immunity and T-cell anergy have been detected in infections by *M. pneumoniae* (187, 213), *M. mycoides* (67) and *M. arthritidis* (58). This immunomodulatory effect may precondition the host immune system to become inert to mycoplasma infection. Some species, such as *M. gallisepticum*, also contain proteins that can break down endogenous peroxide, which is an important component of the host's innate immune response (116). Like other successful pathogens, mycoplasmas have evolved strategies to evade the host immune system. It has been proposed that capsule can protect mycoplasmas from phagocytosis by a passive mechanism (108). A direct inhibitory effect of capsule on macrophage activity has also been reported. Tumor necrosis factor (TNF) and interleukin-1 (IL-1) are produced by activated bronchial alveolar macrophages (BAM) stimulated with unencapsulated *M. dispar* but not

encapsulated *M. dispar* or its purified capsule. BAM that have been treated with purified capsule or encapsulated mycoplasmas lose the ability to be subsequently activated by endotoxin (7). Mycoplasmas can shield themselves from components of the immune system by means in addition to capsule. Surface proteins have been shown to modulate the susceptibility of individual mycoplasma cells to the growth-inhibiting effect of antibody or the killing effect of complement (42, 197). Biofilm structures have been described for several *Mycoplasma* species and have been shown to have a significant contribution to resistance to the stress of heat and drying (148). *M. pulmonis* cells encased in a biofilm are more resistant to lysis by complement and the antimicrobial peptide gramicidin than are dispersed cells (196), suggesting that biofilms contribute to resistance to antimicrobial agents and immune surveillance. Another important mechanism utilized by mycoplasmas to avoid the immune response is antigenic variation (80). Antigenic switching occurs stochastically and creates subpopulations within a cell culture. This ensures the availability of subsets of the population that can survive and continue to propagate in the face of a robust antibody response. Mycoplasmas also harbor shared antigenic determinants with humans. The host immune system may mistakenly recognize these determinants as self-antigens that the immune response ignores (114). Pathogenic mycoplasmas such as *M. pneumoniae* and *M. penetrans* can reside and replicate inside host cells for up to 6 months (62), suggesting another strategy utilized by mycoplasmas to circumvent antibiotic therapy and immune surveillance and hence establish a chronic infection. Contributing to the difficulty of treating infection is the ability of mycoplasmas to rapidly develop resistance to various antimicrobial agents. Since its first appearance in 2000, *M. pneumoniae* resistance to macrolide treatment has

been widely reported in Europe, Asia and the United States (134). Nowadays, as high as 83% of *M. pneumoniae* isolates are resistant to macrolides. Resistance is mediated by point mutations in the 23S rRNA gene.

Virulence factors

Various mycoplasmal factors can have detrimental effects on the host. Due to the difficulty in simultaneously mutating multiple genes, most studies have focused on major virulence factors that when absent significantly reduce the ability of the mycoplasma to cause disease. However, the presence of many accessory factors is necessary for the full presentation of virulence. The attachment of mycoplasmas to host cells is a prerequisite for colonization and pathogenesis. In *M. pneumoniae*, attachment is performed by a well-organized attachment organelle composed of about 100 proteins, among which is the major adhesin P1 (121, 127, 132). Virulent strains have high concentrations of P1 located to the attachment organelle. Strains that do not localize P1 are avirulent, suggesting that P1 localization is associated with virulence (14). P1 alone is not sufficient for adhesion and many accessory factors have to be present to ensure the correct trafficking and localization of organelle proteins (126). A similar attachment organelle is found in *M. genitalium* (26) and *M. gallisepticum* (95). Attachment in other species such as *M. pulmonis* and *M. arthritidis* does not involve an attachment organelle (19, 197). Exopolysaccharides (EPS) play important roles in virulence by modulating properties such as antiphagocytosis (193), antibacteriolytic activity (218), adhesion (171), immune responses and evasion (37, 75), and biofilm production (231). Recently, we demonstrated the presence of extracellular material extending from the surface of *M. pulmonis*. This material is capsule composed of EPS. By lectin staining and tandem mass spectrometry,

we demonstrated the presence of two EPS molecules (EPS-1 and EPS-II) in *M. pulmonis*. Absence of EPS-I is associated with an enhanced ability to form a biofilm but reduced attachment to human lung cells and reduced colonization of the mouse respiratory tract (63).

Mycoplasmas can cause direct damage to host cells by various mechanisms. Species that use arginine as an energy source can cause the inhibition of lymphocyte proliferation in vitro. The inhibition can be relieved by the addition of arginine (176)，suggesting that the damaging effect is achieved by nutrient competition with host cells. Some enzymes produced by mycoplasmas are directly toxic to host cells. Nucleases widely exist in mollicutes (172). In vitro infection has demonstrated that nucleases can alter the metabolism of host nucleic acids and has been associated with chromosome aberration (72). Increased apoptosis and increased sensitivity to inducers of apoptosis have been described in mycoplasma-infected host cells and have been attributed to endonuclease activity or membrane lipoproteins (15, 111, 113, 166, 199). Cellular damage can also be induced by superoxide. The virulence of *M. mycoides* appears to be associated with the production of hydrogen peroxide. The high virulence of the African strain is associated with a strong release of hydrogen peroxide while the less virulent European strain is associated with less hydrogen peroxide production (219). Superoxide anions are produced by many species such as *M. pneumoniae* and markedly inhibit host catalase, leading to oxidative stress and injury to host cells (119).

Mycoplasma infection and the host immune response

Both innate and adaptive host defenses are involved in the response to mycoplasma infection. For pulmonary infection, mucociliary clearance is the first line of defense (181).

Phagocytosis mediated by neutrophils and macrophages is important in clearance of mycoplasmas but opsonization by specific antibodies is generally required (21). Toll-like receptors (TLRs) are also involved in innate defense against mycoplasmas. Lipoproteins such as MG309 of *M. genitalium* (149) and macrophage-activating lipopeptide 2 kDa of *M. fementans* (113) have been demonstrated to interact with TLR-2 and TLR-6. Activation of TLRs is associated with the production of many proinflammatory cytokines including TNF, IL-1 and IL-6. Both antibody and cellular adaptive responses have been depicted in mycoplasma-infected subjects. Antibodies against mycoplasma proteins are protective in some cases but not efficient in others (10, 13, 29, 105, 159, 224). Mycoplasma infection can not only induce T-cell proliferation but also lead to the formation of cytotoxic T cells (188). Complement can be activated by mycoplasma components (122). Activation of complement allows the deposition of complement component C3 on the mycoplasma cell surface, which is a critical factor for host defense (228).

The role of the host immune responses to mycoplasma infection is widely appreciated (108). Proinflammatory cytokines can be induced by components of mycoplasmas as discussed previously. The release of large amount of cytokines appears to correlate with the systemic toxic syndrome during the onset of infection, which will be discussed later. The development of infectious lesions also appears to be directly related to the magnitude of the immune response. It is suggested the formation of the lesions induced by *M. bovis* is majorly dependent on the immune response (110). This process is mainly mediated by antibodies. Hamsters with T-cell depletion have significantly reduced lung histopathology following *M. pneumoniae* infection (210). Similarly, in *M.*

pulmonis-induced murine pneumonia, Th1 and CD8 (+) T-cell activation in the lung plays an important regulatory role in the development of pulmonary disease. Absence of CD8 (+) T cells is associated with dramatically more severe pulmonary disease (118). A clear association between disease susceptibility and cytokine mRNA expression has also been discovered. Various proinflammatory chemokines are produced in the lungs of the susceptible mice and could contribute to the recruitment and maintenance of inflammatory cells at the site of disease (202).

Severe combined immunodeficiency mice inoculated with mycoplasmas develop limited lung pathology but a pronounced systemic infection (83). In immunocompetent hosts, the immune response is possibly the cause of many of the damages outside of the primary lesion. Patients with the *M. pneumoniae* infection can develop various acute extrapulmonary symptoms such as uveitis (70, 225), ocular inflammatory disease (211), conjunctivitis, mucositis (86), rhabdomyolysis (226), hemolytic anemia (32), recurrent arterial thrombosis (227), Stevens Johnson syndrome, neurological abnormalities, arthritis, hepatitis and pericarditis (5). The diversity of these symptoms indicates the underlying immunological basis. Chronic autoimmune diseases are also potentially associated with infection. A study demonstrated a significant detection rate of *M. hominis* in women with systemic lupus erythematosus (SLE), corroborating the association of the infection and SLE (140). Another study reported that primary biliary cirrhosis patients have a significantly increased frequency of *M. pneumoniae*-related antibodies, which occurs early in the disease process and potentially triggers the induction of antimitochondrial antibodies (17). Autoimmunity, possibly due to the modification of self-antigens or molecular mimicry, is proposed to be the underlying mechanism of these

conditions. *M. pneumoniae* can interact with erythrocytes via polysaccharide antigen I. The interaction leads to modification of the self-antigen that induces the production of an anti-I antibody (137). Mycoplasmas can also induce autoimmunity by producing agents that mimic host molecules. Anti-galactocerebroside has been described in patients suffering from Guillain-Barre syndrome secondary to *M. pneumoniae* infection (8). Shared antigens between *M. hyorhinis* and mammalian intermediate filaments and a 24-kDa human cellular polypeptide have also been identified (85, 229).

Superantigens (SAgs)

Microbial SAgs are a group of antigens that have a very strong immune stimulatory effect. They can be produced by bacterial agents such as *Staphylococcus aureus*, *Streptococcus pyogenes* and viral agents such as Epstein-Barr virus and human immunodeficiency virus (135, 136). *S. aureus* can secret the SAg toxic shock syndrome toxin-1 (TSST-1) and the staphylococcal enterotoxins (74) and *Streptococcus pyogenes* can secret streptococcal pyrogenic exotoxins (SPE). SAgs are major virulence factors in these bacteria and are involved in a number of acute diseases including necrotizing fasciitis, food poisoning, and toxic shock syndrome (TSS) (136). The necrotizing fasciitis caused by *S. pyogenes* is mediated by SAgs (35). Ingestion of staphylococcal enterotoxins present in food causes emesis. The food poisoning is associated with the production of several inflammatory mediators but the mechanism is still unknown (74). TSS is an acute and potentially fatal disease induced by SAg-producing strains of *S. aureus* and *S. pyogenes*. In *S. aureus*, TSST-1 is associated with menstrual TSS and approximately 50% of nonmenstrual cases while enterotoxin B and enterotoxin C are associated with 47% and 3% nonmenstrual cases respectively (20). In *S. pyogenes*, TSS

is linked to streptococcal SAgs, in particular, SPE-A (200). TSS is primarily a capillary leak syndrome and typically results in shock and multi-organ failure (74). The mortality is as high as 6% in staphylococcal toxic shock and 30-70% in streptococcal toxic shock (136).

SAgs are also indicated to be involved in chronic conditions, such as arthritis and psoriasis. Although the severity and frequency of infectious arthritis induced by *S. aureus* is closely correlated with the production of TSST-1 (1), the evidence of an association between SAgs and rheumatoid arthritis (RA) is indirect. RA patients have a lower percentage of $V_\beta 14$ positive T cells in the periphery than in synovial fluid. One or a few clones of T cells dominate the $V_\beta 14$ population in synovial fluid of RA patients (167), suggesting the occurrence of clonal expansion, a characteristic of SAgs. T cells isolated from skin lesions of psoriasis patients respond strongly to M proteins of streptococci, consistent with the possibility that streptococcal infection may have a role in psoriasis. A pronounced increase in the number of $V_\beta 2$ T cells is demonstrated in both acute and chronic psoriatic skin lesions, suggesting streptococcal SAgs may be involved in the pathogenesis (217). In other conditions such as ulcerative colitis (194) and encephalomyelitis (EAE) (23), SAgs are not the initiating agents but are potentially involved in relapse or exacerbation. In experimental autoimmune EAE, the SAg staphylococcal enterotoxin B induces exacerbation or relapse of paralytic disease in mice that are in clinical remission following an initial episode of paralysis and triggers paralysis in mice with subclinical disease.

Despite the different origins of these SAgs, they possess common sequence features and structure that enable them to bypass the conventional major histocompatibility

complex (MHC)-restricted antigen processing mechanism (135, 136). SAgs remain unprocessed by antigen-presenting cells (APCs) and bind directly to the α or β chain of MHC II molecules on APCs and sequentially the variable region of the T-cell receptor (TCR) β-chain (V_β). The binding sites are away from conventional peptide-binding sites and hence the binding is not restricted by the antigen specificity of the given TCR. Because each SAg is associated with a characteristic V_β signature, each SAg will trigger activation and expansion of T cells bearing a particular subset of V_β. Since the number of different V_β regions in the human T-cell repertoire is restricted to 24 and one SAg can interact with one or several V_β members, a small amount of SAg is powerful enough to trigger the activation of up to 30% of the whole human T-cell population (136, 208). Once activated, these T cells may continue to proliferate in the presence of autoantigens leading to a chronic autoimmune disorder (191). As a result of SAg-induced T-cell proliferation, large amounts of Th1 proinflammatory cytokines, initially TNF-α, followed by IL6, IFN-γ, and IL2 are released. Additional T cells are recruited to the site of infection and macrophages are activated. Activated macrophages respond with release of cytokines such as IL-1, TNF-α and IL-12 which have key roles in the development of Th1 responses and have been implicated in several experimental autoimmune disease mouse model. The excessive uncoordinated release of proinflammatory cytokines is considered to be responsible for many of the symptoms of acute SAg-induced diseases (136, 191). In addition to T cell and macrophage activation, SAg "bridge" mediated interactions between T helper and B cells can also induce proliferation of the SAg-bearing B cells and polyclonal IgM and IgG production (89). Despite of all the activation activities, SAgs can corrupt the host immune response to infection through the induction

of T-cell anergy. Initial T-cell activation is followed by inhibition of production of some cytokines such as IL-2 and IL-10. A local deficiency of IL-2 could limit the further expansion of antigen-specific T cells, leading to failure of T cells to help establish humoral immunity to SAgs as well as other microbial antigens (135, 136). In addition to direct effects, SAgs can significantly augment the lethality of endotoxins. In rabbits, the lethality can be enhanced up to 100,000 fold by TSST-1 such that the lethal dose of endotoxin can be as low as picograms when SAgs are present (74). This synergistic effect between SAgs and endotoxins might be achieved by SAgs inhibiting the endotoxin clearance of liver (192). However, the role of endotoxin in TSS is still controversial because neutralization of endotoxin does not protect rabbits from toxic shock induced by TSST-1 (150).

M. arthritidis mitogen (MAM) and its potential role in pathogenesis

The involvement of infectious agents in auotoimmune diseases such as RA has been widely proposed (2). However, no strong association between autoimmune diseases and bacterial or viral agents has been found, making a slow-growing or difficult to culture organism such as a mycoplasma the more likely bacterial candidate for having a role in the disease process (186). Several mycoplasmas such as *M. pneumoniae* (41) and *M. fermentans* (93) have been associated with arthritis in humans. A positive association of the mycoplasmas with RA has been reported in a few studies (44, 47). *M. arthritidis* is a natural-occurring arthritogen of rats that causes migratory polyarthritis (102). An experimental polyarthritis can be induced in both rabbits (223) and mice (57) by intraarticular and intravenous injection of live organisms, respectively. The disease in rats is a severe suppurative arthritis. Most rats recover completely from the acute phase of

disease within 6-8 weeks. In contrast, mice develop a more chronic phase of disease that exhibits periods of remission and exacerbation. Joint lesions closely resemble those of human RA, with massive lymphocytic and plasma cell infiltration of synovium, proliferation of synovial membrane with production of multiple villi, and pannus formation leading to destruction of cartilage and bone. Several candidate virulence factors including the adhesion molecules MAA1 and MAA2 (19), a major surface antigen MIA (214), and the SAg MAM (46, 51) have been identified, and their roles in the pathogenesis are under study.

MAM is in many aspects a typical SAg (52). MAM can stimulate mouse lymphocytes that bear functional H-2E molecules of MHC II (49). Responsiveness is also associated with expression of a particular subset of V_β chains present on the TCR (45). Two active domains on MAM have been identified: MAM_{15-31} and MAM_{71-95}. Peptides containing MAM_{15-31} and MAM_{71-95} inhibit lymphocyte proliferation by active MAM in a dose-dependent manner (52). In response to MAM, different cytokine profiles are induced in different strains of mice with type 1 cytokines (represented by IL-2, INF-γ, and TNF-α), a group of proinflammatory cytokines, preferentially increased in C3H/HeJ mice and type 2 (represented by IL-4, IL-5, IL-10, and IL-13) increased in BALB/c mice. IL-12, a cytokine known to promote clonal expansion of Th1 cells, is also induced as the result of macrophage activation. These profiles may predict susceptibility to either arthritis or lethal toxicity (154, 155). B-cell activation induced by MAM has been demonstrated. Activation is dependent on T-helper cells, and polyclonal IgG and IgM antibodies are produced (215, 216). In addition to its ability to stimulate mouse macrophages and lymphocytes, MAM can also stimulate human lymphocytes. It has been

shown that MAM can activate human T cells bearing V_β 17 and that the reaction is dependent on MHC-bearing accessory cells. Human B cells can be activated by MAM, and high levels of IgM and IgG antibodies are released (44, 60). In one study, MAM treatment induced the production of low levels of IgM rheumatoid factor from the lymphocytes of normal or seronegative RA patients (82). Moreover, MAM can affect human NK cells directly by increasing their lytic capacity and indirectly as a consequence of cytokines produced by T cells (61). Similar to T-cell anergy induced by other SAgs, T-cell anergy could be induced with injection of high doses of MAM such that T cells from treated animals lose much of their ability to proliferate in vitro in response to MAM. This anergy state is due to MAM-activated CD4-positive T cells that can suppress the response of normal T cells to mitogens (44).

MAM has a number of unique features that differentiate it from other bacterial SAgs. It is phylogenetically distinct from other SAgs (52). MAM, but not other SAgs, contains a seven-residue lectin legume motif β consensus sequence present in all legume lectins including concanavalin A (52). Unlike most other SAgs, which have a similar three-dimensional structure consisting of a small β-barrel domain and large domain with β-grasp motif, MAM adopts a novel fold composed of two completely α-helical domains (133). It has a strong preference for H-2Eα or HLA-DRα molecules rather than H-2A or HLA-DQ (48). It was recently shown that MAM, unlike other bacterial SAgs, also has contact points with the third complementarity-determining region of the TCR (104). Another major difference is that in proliferation assays, MAM is 10^3- to 10^4- fold more effective for murine cells than are staphylococcal SAgs in respect to the doses required to induce maximal lymphocyte proliferation (54). Besides, MAM can activate macrophages

in the absence of lymphocytes (73) by direct interaction with MHC II molecules (3). A recent study demonstrated that both TLR-2 and TLR-4 are engaged by MAM and their expression upregulated (153). In addition to being a SAg, a recent paper reported that MAM has nuclease activity (72). This suggests that MAM is a scavenging enzyme and it may enhance the fitness of the mycoplasma by providing nutrients or damaging the host tissue by degrading DNA. These unique characteristics demonstrate the novelty of MAM and suggest that further research on its role in the development of SAg-mediated diseases is warranted.

The similarities of conditions associated with MAM to well-characterized SAg diseases suggest that MAM may have an important role in lethal toxicity of mice infected with *M. arthritidis*. Except for staphylococcal food poisoning, acute conditions associated with SAgs all share clinical features such as fever and skin lesions (145). Among these conditions, necrotizing fasciitis is characterized by necrosis of skin and associated structures (87). TSS is characterized by fever, rash, desquamation, hypotension, and involvement of multiple organ systems (129). These symptoms are reminiscence of acute conditions induced by *M. arthritidis* infection. Animals infected with large doses of *M. arthritidis* develop systemic symptoms (155). Lethal conditions similar to TSS (55) and dermal necrosis (53) have been described. They occur only in inbred or congenic mouse strains that express the I-E molecules, indicating they are dependent on MAM. Consistently, MAM induces marked elevation of TNF-α (155), a major mediator of TSS following *S. aureus* and *S. pyogenes* infections (136). In addition to acute diseases, similar to other SAgs, MAM is potentially involved in chronic inflammation. Due to its being the only SAg produced by *M. arthritidis* and its strong mitogenic activity on

splenocytes, MAM has long been considered as the most important factor in arthritogenicity. Like arthritis in mice, arthritis in rabbits induced by *M. arthritidis* is chronic. Despite the persistence of active inflammation for many months after inoculation, *M. arthritidis* antigens were not found in the joint tissues of rabbits after 7 weeks (223), which indicates the presence of a long-term immune disturbance potentially caused by strong immunogenic molecules like MAM. MAM also appears to play important roles in the collagen-induced arthritis (CIA) mouse model. When an experimental autoimmune arthritis is induced by immunization with collagen, MAM can exacerbate the arthritis in mice that were recovering from CIA and trigger arthritis onset in mice suboptimally immunized with type II collagen (50). In addition, based on the potential involvement of SAgs in RA, molecules similar to MAM could be possibly involved in human RA. As described above, MAM can stimulate human lymphocytes. In one study, the predominant T-cell clonotypes identified in the synovium of some RA patients expressed the same sets of V_β regions as expressed by T cell clonotypes that are responsive to MAM (11). In another study, anti-MAM antibodies were detected in RA patients, suggesting that a MAM-like molecule might be associated with RA (190).

The immunological disturbance induced by MAM is probably complex. The mechanism could be one of the following three (46). (1) T cells are activated multiclonally. Cytokines released by activated T cells might be the major mechanism involved in toxic syndrome (155). (2) Polyclonal B-cell antibodies are induced by means of a bridge that links V_β-bearing T helper cells to B cells that carry SAgs. The dual affinity of SAgs for MHC II molecules and selected TCR V gene products could render B cells targets of cognate T cells such that B cells that have received a potent autoantigen-

mediated signal via the surface immunoglobulin receptor will be driven to full activation. The continuous presence of self-antigens leads to the continuous production of autoantibodies (89). (3) Molecular mimicry of certain endogenous antigens may trigger crossreactive autoimmune responses. The occurrence of shared antigens common to *M. arthritidis* and nonhuman hosts has been indicated (27). One group of host antigens that are potentially shared with *M. arthritidis* are minor lymphocytes stimulatory antigens (Mls), antigens expressed on host B cells. These antigens may enable mycoplasmas to become established in their host. MAM might be one of these antigens that have shared epitope with hosts because both MAM and Mls predominantly use the I-E molecule for presentation to T cells. In fact, T-cell hybridomas that express $V_\beta 8.1$ or $V_\beta 6$ react with both MAM and Mls-1^a (46).

Although the role of MAM in host immune disturbance is indisputable, its relative importance in the arthritis and lethal toxicity induced in murine animals has been controversial. Unlike disease induced with *M. arthritidis* by intravenous injection, arthritis could be induced with MAM only by direct injection into joints. Disease induced is transient and lasts only for a few days, which does not reproduce the disease phenotype seen in rats infected with *M. arthritidis* (28). Besides, mouse strains with lymphocytes unresponsive to MAM can develop acute arthritis upon infection with *M. arthritidis* (56), although these lymphocytes might be more responsive to MAM than was realized when the experiments were performed (54). Although studies demonstrate that murine lymphocytes respond to MAM when they have a functional H-2E MHC molecule (48, 49), the incidence of arthritis in H-2E-negative mouse strains can be even higher than in H-2E-positive strains (56). In addition to arthritis, MAM appears to have a more

prominent role in acute disease. Splenocytes from mice bearing a null mutation in TLR-4 are more responsive to MAM. These mice develop an enhanced susceptibility to lethal toxicity induced by *M. arthritidis*. The mortality is so high that the severity of arthritis could not be assessed (155).

Current supportive treatment-based therapy of SAg-mediated diseases is ineffective (106). Attempts are being made to develop therapeutic strategies that counteract SAg production, the action of SAgs on their target cells, or influence the downstream activation of the immune system by SAgs (208). Improvement of treatment will benefit from the development of a model system in which the relationship between disease and SAg has been well characterized. MAM and conditions associated with it is such a model with promise. This model is appealing because both the pathogen and the host are genetically manipulatable. Due to the ambiguous significance of MAM with respect to arthritis and lethal toxicity, its role in disease induced in mice was carefully characterized in the studies described in this thesis.

Glycosidases, potential virulence factors of mycoplasmas

Glycosidases are another group of factors that might be involved in the virulence of mycoplasmas. Glycosidases are enzymes that catalyze the hydrolysis of the glycosidic bond between two adjacent sugars (www.cazy.org). They can be classified to different groups based on various criteria. One of the most useful classification methods for practical purpose is based on the sites in the polysaccharides that are affected, which classifies glycosidases as endoglycosidases or exoglycosidases. Another useful method is based on substrate specificity, which is used in IUB-MB enzyme nomenclature of glycosidases (EC 3.2.1.-) (www.expasy.org/enzyme/3.2.-.-). Glycosidases for each

substrate can be further classified to enzymes specific for alpha linkage or beta linkage based upon the configuration of the anomeric carbon. In bacteria, glycosidases have been detected both intracellularly and extracellularly (12, 18, 36). They are largely involved in nutrient acquisition (92, 205). One of the important glycosidases in bacteria is the enzyme β-galactosidase of *E. coli*. Its coding gene is part of the *lac* operon and expression is controlled in part by lactose or its analogues (189). Systems like this enable bacteria to switch to the use of different nutrients based on availability and are important for bacterial adaptation to various environments. Glycosidases are so important for growth that proteins that inhibit glycosidic activity can significantly inhibit the growth of bacteria (164). In walled bacteria, breaks in the peptidoglycan induced by glycosidases are important for growth, peptidoglycan turnover, cell separation, spore germination, and autolysis (151). Hydrolysis of peptidoglycan mediated by glycosidases may also expose cell adhesion molecules that are otherwise buried. *Lactococcus lactis* secretes a muramidase that can hydrolyse its own peptidoglycan (25). Mutants lacking this enzyme show significantly reduced adherence and biofilm formation (151). Consistant with that, *S. pneumoniae* mutants lacking glucosaminidase LytB fail to separate at the end of cell division (91) and also have reduced colonization of nasopharynx, impaired biofilm formation, and attenuated virulence (96, 152). Glycosidases are important for interactions with host cells. The interaction between glycosidases and host mucin has been well documented. Bacterial subpopulations in the human colon can secret many types of extracellular glycosidases, which may have a major role in degrading mucin in the gut lumen (107). Mucin forms a gel-like structure that is protective for the epithelium. It can trap pathogens and aid in further clearance. Degradation of mucin may facilitate the

adherence, colonization, and spread of pathogenic bacteria, and is an important pathogenic mechanism. Women with bacterial vaginosis have a higher number of mucin-degrading enzymes than normal subjects (163). Similarly, *Pseudomonous aeruginosa*, an important respiratory pathogen in cystic fibrosis patients, produces several glycosidases including those specific for N-acetylglucosamine (GlcNAc) and N-acetylgalactosamine (GalNAc), indicating degradation of mucin might be one of its pathogenic mechanisms (9). Glycosidases are required for full virulence of some bacteria. Deletion of a gene cluster containing a glycosidase in a pathogenic strain of *E. coli* affects the assimilation of seven carbohydrates, decreases growth, and reduces virulence (39).

Glycosidic activities, including β-glucosidase (103) and sialidase (16, 146), have been described in many mycoplasma species. These glycosidases may be involved in a number of activities that are important for survival or presentation of disease. The most obvious function is scavenging of saccharides. Monosaccharides have important roles in the structure and metabolism of mycoplasmas. Glucose is the major energy source for glycolytic mycoplasmas (174, 179). Galactose and GlcNAc are components of EPS (63), and the degradation of host polysaccharide could be one of the means used by mycoplasmas to obtain these monosacchrides. Consistent with that, GlcNAc importation has been demonstrated in *M. pulmonis* (88). Another possible activity of glycosidases is the modification of bacterial EPS. Like the role of glycosidases in other bacteria, secreted glycosidases can possibly degrade mycoplasmal polysaccharides, an activity that might be important for cell division, adherence, or biofilm formation. Similar to glycosidases in other bacteria, mycoplasmal glycosidases might have important roles in interaction with host molecules such as mucin or sialic acid. Neurominidic (sialidic) activity has been

detected for many species such as *M. synoviae* and *M. gallisepticum* (16). Sialidase is generally considered as a virulence factor because it seems to be associated with the spreading of mycoplasma infections. Sialidic activity identified in *M. synoviae* might be associated with virulence because strains isolated from symptomatic birds have a higher sialidic activity than strains from asymptomatic birds (147). *M. alligatoris* has a membrane-bound sialidase, and is rapidly invasive, and has a necrotizing effect. In contrast, *M. crocodylii* lacks sialidase and is less virulent (24). A later study found that sialidase can increase the expression of the apoptosis receptor CD95 in *M. alligatoris*-infected fibroblasts (112). Treatment with sialidase inhibitor protects the fibroblasts by inhibiting CD95 expression.

Although glycosidases are also predicted in species such as *M. arthritidis* (81), we decided to first study them in *M. pulmonis* due to a relatively clear picture of its carbohydrate metabolism and EPS production (63, 88). *M. pulmonis* is the etiologic agent of murine mycoplasmosis, a naturally-occurring respiratory disease in rats and mice (64). The respiratory disease resembles chronic bronchitis, bronchiectasis, and emphysema in humans in morphology and natural history, making it a particularly useful model for study of the pathogenesis of chronic pulmonary inflammation (30). Similar to *M. pneumoniae*, hydrogen peroxide appears to be a virulence factor in *M. pulmonis* (22). Macrophage resistance (109), attachment (209), and mitogenic activity (157) have all been indicated as having a role in pathogenesis. A hemolysin, a set of membrane-associated or secreted nucleases and a potentially secreted protease are predicted as virulence factors (33). However, other than the Vsa proteins, no definitive virulence factors have been identified (197). MYPU_4630 is one of the glycosidases identified in

the *M. pulmonis* genome (33). Because it has fewer TGA codons than the other glycosidase genes of *M. pulmonis* that would need to be converted to TGG to facilitate expression in *E. coli*, MYPU_4630 was chosen for study. MYPU_4630 is annotated as a glucan 1, 6-α-glucosidase. It contains identifiable glycosidase domains but has limited homology to other glycosidases. Its enzymatic specificity cannot be predicted by sequence comparison to other glycosidases.

Major findings presented here

To study the pathogenic importance of MAM in *M. arthritidis*, effort was taken to develop genetic systems that had altered MAM production. Our initial effort to develop mutants using a HR-based knock-out strategy with a plasmid containing an internal fragment of the *mam* gene was unsuccessful. Therefore, we resorted to transposon mutagenesis to generate mutants. As discussed before, *M. arthritidis* is notoriously difficult to transform. Deciphering the mycoplasma's R-M systems seemed to be critical to improve the efficiency of transposon mutagenesis. We have demonstrated previously that the genome of *M. arthritidis* strain 158 is protected from digestion by the AluI restriction endonuclease (220). This is achieved by the modification of cytosine residues at AGCT sequences by a DNA methyltransferase designated MarI. In the present studies, we made an effort to identify the gene coding for MarI. Since Marth_orf138 is the only *M. arthritidis* gene annotated as coding for a cytosine methyltransferase, the activity of Marth_orf138 gene product was studied in *E. coli*. We demonstrated that DNA isolated from *E. coli* cells expressing the Marth_orf138 gene, modified to convert TGA to TGG, was still degraded by the AluI nuclease but had acquired resistance to the restriction endonuclease HhaI (specific for GCGC sequences). Genomic DNA from *M. arthritidis*

was also found to be resistant to HhaI, suggesting Marth_orf138 codes for an isoschizomer of the HhaI DNA methyltransferase, which was designated MarII. However, transformation of *M. arthritidis* with plasmid modified by HhaI methyltransferase did not significantly increase the transformation frequency, indicating that the mycoplasma either lacks a restriction endonuclease that recognizes GCGC sites or the activity is too low to be detected under the assay conditions used.

Our overall objectives are to understand the mycoplasmal factors that contribute to pathogenesis. As described above, pathogenesis is complex, potentially consisting of many proteins and perhaps carbohydrate structures. Our effort has been focused on MAM and the MYPU_4630 product due to their potential importance to virulence and metabolism.

We present here studies to elucidate the role of MAM in disease induced by *M. arthritidis*. This work addresses the questions of whether MAM is the major mitogen secreted by *M. arthritidis* and whether MAM is associated with arthritis or lethal toxicity. Our approach has been to develop mutants that have reduced MAM production or increased MAM production followed by comparison of the virulence of these mutants with the wild-type strain. By screening a transposon library, we identified two independent mutants with a disrupted *mam* gene. Three independent mutants that overproduced MAM were also created by insertion of a transposon containing the *mam* gene under the control of the strong promoter from the *M. arthritidis mia* gene. Loss or overproduction of MAM was confirmed by using mitogen assays. These studies demonstrated that MAM was the major mitogen secreted by *M. arthritidis* and was associated with lethal toxicity but not arthritis in CBA/J and DBA/2J mice. The altered

virulence was most likely due to the gain or loss of mitogenic activity because growth in vitro, colonization of joints, protein profiles, and nuclease activity were unaltered in these mutants. By using the MAM knockout and overexpression mutants developed by us, our collaborator (Hong Mu, University of Utah) was able to confirm that MAM was not required for the induction of *M. arthritidis*-induced arthritis. MAM overproduction was associated with increased toxicity and mortality while MAM knockout was associated with decreased mortality. Additionally, disease severity and progression were heavily dependent upon the MHC II background and TLR responses. The BALB/c mouse ($H\text{-}2^d$ and TLR wild-type) strain was relatively resistant and C3H/HeJ ($H\text{-}2^k$ and TLR-4 mutant) was susceptible to disease induced by *M. arthritidis*.

To study the function of the protein encoded by MYPU_4630, a recombinant protein-based strategy was used. Experiments with a HA-tagged MYPU_4630 protein demonstrated that the protein was secreted. The MYPU_4630 gene was fused with a glutathione S-transferase (GST) tag and expressed in *E. coli*. The identity of the purified protein was confirmed by mass spectrometry. The recombinant protein was demonstrated to be a glycosidase with activity for α-linked N-acetylglucosaminides and N-acetylgalactosaminides. Though β-linked N-acetylglucosaminidase has been demonstrated in *A. laidlawii* (120), the MYPU_4630 protein is the first identified enzyme specific for α-linked amino sugars in any *Mycoplasma*. Future studies will examine whether mycoplasmal EPS is a target for the enzyme and the relevance of the enzyme to pathogenesis.

IDENTIFICATION OF AN ISOSCHIZOMER OF THE HHAI DNA METHYLTRANSFERASE IN *MYCOPLASMA ARTHRITIDIS*

by

WENYI LUO, ANH-HUE T. TU, ZUHUA CAO, HUILAN YU AND KEVIN DYBVIG

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ABSTRACT

The genome of *Mycoplasma arthritidis* strain 158 has modified cytosine residues at AGCT sequences that render the DNA resistant to digestion with the AluI restriction endonuclease. The DNA methyltransferase responsible for the base modification has previously been designated MarI. From the complete genome sequence of *M. arthritidis,* we identify Marth_orf138 as a candidate *marI* gene. Marth_orf138 was cloned in *Escherichia coli* and its TGA codons converted to TGG. DNA isolated from *E. coli* cells expressing the modified Marth_orf138 gene was degraded by the AluI nuclease, indicating that Marth_orf138 does not code for MarI. However, the DNA from *E. coli* was found to have acquired resistance to the restriction endonuclease HhaI. Genomic DNA from *M. arthritidis* was also found to be resistant to HhaI (recognizes GCGC). The *M. arthritidis* isoschizomer of the HhaI DNA methyltransferase, coded by Marth_orf138, is designated MarII. Transformation of *M. arthritidis* was not significantly affected by modification of plasmid at HhaI sites, indicating that the mycoplasma lacks a restriction endonuclease that recognizes GCGC sites.

INTRODUCTION

Mycoplasma arthritidis is a naturally-occurring arthritogen of rats, and experimental arthritis can be induced in mice by intravenous injection (Cahill *et al.*, 1971; Cole & Cassell, 1979). Studies on the basic biology and pathogenic mechanisms of *M. arthritidis* are hampered by inefficient transformation protocols. Electroporation has yielded negative results. A barrier to gene transfer has been attributed to the MarI restriction enzyme, an isoschizomer of AluI (recognizes AGCT) because transformation has been described previously as successful only when plasmid DNA was modified in vitro at AGCT sites by using the AluI DNA methyltransferase (M.AluI) (Voelker & Dybvig, 1996).

Genomic DNA of *M. arthritidis* is resistant to digestion with the AluI nuclease due to the modification of AGCT sites by the MarI DNA methyltransferase (M.MarI) (Voelker & Dybvig, 1996). The complete genome sequence of *M. arthritidis* was examined for potential M.MarI genes. The only predicted cytosine-specific DNA methyltransferase and obvious M.MarI candidate identified was the product of the Marth_orf138 gene. Experiments were undertaken to examine DNA methyltransferase activity associated with Marth_orf138. Unexpectedly, we found that Marth_orf138 codes for an isoschizomer of the HhaI (recognizes GCGC sites) DNA methyltransferase (M.HhaI) and not M.MarI. We show that genomic DNA from *M. arthritidis* has modified GCGC sites. In vitro modification of plasmid DNA with M.HhaI had little affect on the frequency of transformation of *M. arthritidis*, suggesting that the mycoplasma lacks an isoschizomer of the HhaI nuclease.

MATERIALS AND METHODS

Bacterial strains used

M. arthritidis strain 158-1 is a filter clone of strain 158 (Voelker & Dybvig, 1996). Mycoplasmas were cultured in EA (agar) or EB (broth) at 37°C as described previously (Voelker & Dybvig, 1998; Voelker *et al.*, 1995). For antibiotic selection in *M. arthritidis*, medium was supplemented with tetracycline (5 ug ml^{-1}). *E. coli* One Shot TOP10 (Invitrogen, Carlsbad, CA) grown in Luria-Bertani medium was used as the host strain to construct plasmids with ampicillin selection at 100 ug ml^{-1}.

Expression of Marth_orf138 in *E. coli*

The gene Marth_orf00138 including its promoter region was amplified from strain 158 by PCR using primer F-KpnI: TATAGGTACCTGCAAAATATGCTATTCGG (KpnI recognition site is underlined) and R-EcoRI: GAAAATGCTTTGAATTCGGAAGG (EcoRI recognition site is underlined). The PCR product was digested with KpnI and EcoRI to generate staggered ends and inserted into plasmid pUC18 (Invitrogen) that had been linearized by digestion with KpnI and EcoRI. In mycoplasmas, UGA codons specify tryptophan. To convert Marth_orf138 UGA codons to UGG, a PCR-based multiple mutation reaction (MMR) was performed according to a protocol published previously (Hames *et al.*, 2005). Forward primer F-KpnI, reverse primer R-EcoRI, and 5' terminal phosphorylated mutation primers CTAATGAATGGGACAAGCATGCTCAAGAAGTG and CTGATTTTTATCTAATTCATCCATCTTGGAATCAAGC (underlined nucleotides refer to base triplets that were converted from TGA to TGG) were used for MMR. All PCR reactions were performed using iProof high fidelity DNA polymerase (Bio-Rad,

Hercules, CA). A MMR product of the expected size was inserted into the KpnI/EcoRI site in pUC18. The final plasmid designated pMarII was sequenced to confirm that the UGA codons had been converted to UGG.

Restriction digestion

Genomic DNA was isolated with Easy-DNA kit (Invitrogen) according to manufacturer's instruction. Plasmid DNA was isolated with Qiagen miniprep kit according to manufacturer's instruction. Three ug DNA was incubated with 10 units AluI or HhaI restriction enzyme at 37°C overnight. Restriction products were resolved on agarose gels and visualized by ethidium bromide staining.

Sequence analysis of the predicted Marth_orf00138 protein

Sequence alignment of the predicted protein of Marth_orf00138 (accession number YP_001999779) and M.HhaI (accession number P05102) was performed with AlignX program of VectorNTI suite 10.3.0 (Invitrogen) using the default setting. Conserved amino acids and motifs were as described (Cheng *et al.*, 1993; Posfai *et al.*, 1989).

DNA methylation and transformation of *M. arthritidis*

DNA was modified by incubation with 20 units of M.AluI, 100 units of M.HhaI or both at 37°C overnight. DNA modifications were confirmed by digestion with the corresponding restriction enzymes followed by electrophoresis. Transformation of *M. arthritidis* was performed according to protocols as described previously (Voelker & Dybvig, 1996).

Statistics

Statistical analyses were performed in SigmaStat version 3.1 (Systat Software, San Jose, CA). One-way ANOVA test was used to analyze the transformation results of *M.*

arthritidis 158 and t-test was used to analyze the transformation results of *M. arthritidis* 158-1. $P < 0.05$ was used as the criterion of significance.

RESULTS AND DISCUSSION

Identification of an isoschizomer of M.HhaI

It was previously concluded that the M.MarI DNA methyltransferase of *M. arthritidis* modifies cytosine residues at the sequence AGCT, rendering DNA resistant to the AluI nuclease (Voelker & Dybvig, 1996). The Marth_orf138 gene product was chosen for study because it is the only annotated cytosine-specific DNA methyltransferase predicted from the complete *M. arthritidis* genome sequence (Dybvig *et al.*, 2008). BLAST analysis of the non-redundant protein sequences indicated that the Marth_orf138 gene product was most closely related (E value of 7e-150) to M.HhaI from *Haemophilus parahaemolyticus* (accession number P05102). Overall, the Marth_orf138 gene product has 75% amino acid sequence identity with M.HhaI (Dybvig *et al.*, 2008). An alignment of M.HhaI and the Marth_orf138 gene product reveals conservation of ten conserved motifs (Fig. 1) that form the core structure of the protein surrounding the active site cleft (Cheng *et al.*, 1993; Posfai *et al.*, 1989). A single target recognition domain was identified in the Marth_orf138 gene product within a moderate length of variable region between the conserved VIII and IX motifs.

To investigate its function, the Marth_orf138 gene was cloned into *E. coli* on plasmid pUC18 and its TGA codons converted to TGG. The AluI restriction enzyme readily degraded genomic and plasmid DNA isolated from *E. coli* cells expressing the TGG-modified Marth_orf138 gene, suggesting that the gene did not code for M.MarI (Fig. 2). However, DNA from cells expressing the modified gene was found to be resistant to the

HhaI nuclease, indicating that Marth_orf138 codes for a DNA methyltransferase that modifies the sequence GCGC. At present, the *M. arthritidis* gene that codes for M.MarI remains unknown. Attempts to identify potential *marI* genes by various strategies such as BLASTp analysis of *M. arthritidis* sequences with query sequences from M.AluI, M.HhaI and the Marth_orf138 gene product were unsuccessful.

Experiments were undertaken to determine whether *M. arthritidis* genomic DNA has modified GCGC sites. *M. arthritidis* genomic DNA was found to be resistant to cleavage by the HhaI nuclease, indicating that GCGC sites are modified (Fig. 3). The DNA was also resistant to cleavage by the AluI nuclease, confirming the previous report of modified AGCT sites. *M. arthritidis* DNA was readily cleaved by NheI, a nuclease with specificity for GCTAGC sites (data not shown). NheI cleaves unmodified GCTAGC sites but does not cleave if either C nucleotide in the recognition sequence is modified to 5-methylcytosine. Therefore, not all GC dinucleotides are modified in the *M. arthritidis* genome, and the Marth_orf138 gene product is likely specific for the sequence GCGC as suggested by its strong homology to M.HhaI.

DNA methylation by M.AluI and M.HhaI is not required for transformation of *M. arthritidis* strain 158

Experiments were undertaken to compare the effect of modification of DNA with M.AluI, M.HhaI, or both on transformation. Plasmid pTF85 contains the minitransposon Tn*4001TF1* that was used to construct a transposon library with disruption of over 200 of the predicted 635 protein-coding regions of *M. arthritidis* (Dybvig *et al.*, 2008). *M. arthritidis* strain 158 was transformed with pTF85, with care taken to ensure equal amounts of DNA per transformation. The transformation frequency was compared

between mycoplasmas transformed with unmethylated pTF85, pTF85 methylated with M.AluI, pTF85 methylated with M.HhaI, or pTF85 methylated with both M.AluI and M.HhaI (Table 1). None of the plasmid modifications resulted in statistically significant differences in transformation frequency. Similar to the results obtained for strain 158, transformation of *M. arthritidis* strain 158-1 with M.AluI-modified DNA also yielded transformants at a frequency that was not statistically different from that obtained with unmethylated DNA.

The role of DNA methylation in the transformation of *M. arthritidis* needs to be reevaluated. Previously, it was concluded that modification of DNA with M.AluI was required for transformation of *M. arthritidis* 158-1 with the Tn*916*-containing plasmid pAM120 (Voelker & Dybvig, 1996). However, the results of the current study demonstrate that transformation of strains 158 and 158-1 with DNA modified by M.AluI, or M.HhaI, or a combination of both yielded transformation frequencies that were not statistically different from transformation with unmodified DNA. Possibly, these strains of mycoplasma lack restriction nucleases specific for AGCT and GCGC sequences, and the M.MarI and M.MarII enzymes have roles other than to protect DNA from the activity of restriction endonucleases. Alternatively, AGCT and GCGC restriction activity may be present but only at a low level when using the growth and transformation conditions of this study.

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ACKNOWLEDGEMENTS

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Table 1. Transformation frequency using unmodified and modified pTF85 DNA

Mycoplasma strain	pTF85 modification	Transformants per CFU[a]
158	None	$1.3 \times 10^{-7} \pm 1.4 \times 10^{-7}$ (n = 6)[b]
158	M.AluI modified	$2.7 \times 10^{-7} \pm 2.0 \times 10^{-7}$ (n = 4)
158	M.HhaI modified	$2.1 \times 10^{-7} \pm 1.4 \times 10^{-7}$ (n = 3)
158	M.AluI and M.HhaI modified	$4.1 \times 10^{-7} \pm 2.4 \times 10^{-7}$ (n = 3)
158-1	None	$7.4 \times 10^{-8} \pm 6.6 \times 10^{-8}$ (n = 4)
158-1	M.AluI modified	$1.4 \times 10^{-7} \pm 1.3 \times 10^{-7}$ (n = 4)

[a]Transformation frequency reported as mean ± standard deviation.

[b]n refers to the number of times the experiment was performed. None of the differences between groups was statistically significant.

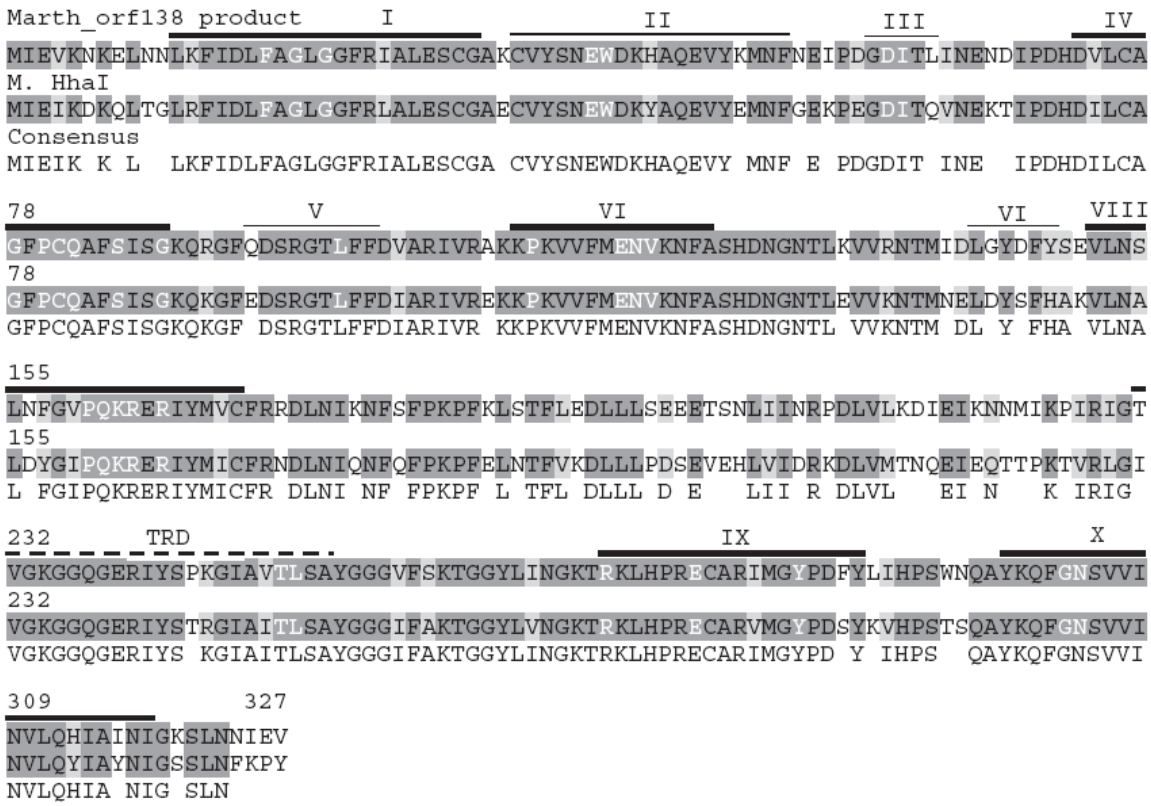
FIGURE LEGENDS

Fig. 1. Sequence alignment of the Marth_orf138 gene product with M.HhaI of *Haemophilus parahaemolyticus* (Caserta *et al.*, 1987; Cheng *et al.*, 1993). The numbers indicate the amino acid position. Solid bars above the sequence delineate the boundary of the motifs with thick bars indicating highly conserved motifs and thin bars represent less conserved motifs. The dashed bar above the sequence represents the TRD. Black font with dark grey background indicates identical amino acids. Black font with light grey background indicates a block of similar amino acids. White font indicates conserved or invariant amino acid residues in 5-methylcytosine methyltransferases.

Fig. 2. Ethidium bromide-stained agarose gel of total DNA of *E. coli* harboring pUC18 (pUC) or pMarII (pMa) digested with HhaI (H) or AluI (A). As controls, DNA samples were also incubated with HhaI restriction buffer (Hb) and AluI restriction buffer (Ab) in the absence of nucleases.

Fig. 3. Ethidium bromide-stained agarose gel of genomic DNA of *M. arthritidis* 158 (M) and *E. coli* (E) digested with HhaI (H) and AluI (A). DNA samples were also incubated with HhaI restriction buffer (Hb) and AluI restriction buffer (Ab) respectively to exclude the presence of nuclease in DNA samples.

Fig. 1:


Marth_orf138 product
I
II
III
IV
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M. HhaI
MIEIKDKQLTGLRFIDLFAGLGGFRLALESCGAECVYSNEWDKYAQEVYEMNFGEKPEGDITQVNEKTIPDHDILCA
Consensus
MIEIK K L LKFIDLFAGLGGFRIALESCGA CVYSNEWDKHAQEVY MNF E PDGDIT INE IPDHDILCA
78
V
VI
VI
VIII
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GFPCQAFSISGKQKGF DSRGTLFFDIARIVR KKPKVVFMENVKNFASHDNGNTL VVKNTM DL Y FHA VLNA
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155
LDYGIPQKRERIYMICFRNDLNIQNFQFPKPFELNTFVKDLLLPDSEVEHLVIDRKDLVMTNQEIEQTTPKTVRLGI
L FGIPQKRERIYMICFR DLNI NF FPKPF L TFL DLLL D E LII R DLVL EI N K IRIG
232
TRD
IX
X
VGKGGQGERIYSPKGIAVTLSAYGGGVFSKTGGYLINGKTRKLHPRECARIMGYPDFYLIHPSWNQAYKQFGNSVVI
232
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VGKGGQGERIYS KGIAITLSAYGGGIFAKTGGYLINGKTRKLHPRECARIMGYPD Y IHPS QAYKQFGNSVVI
309
327
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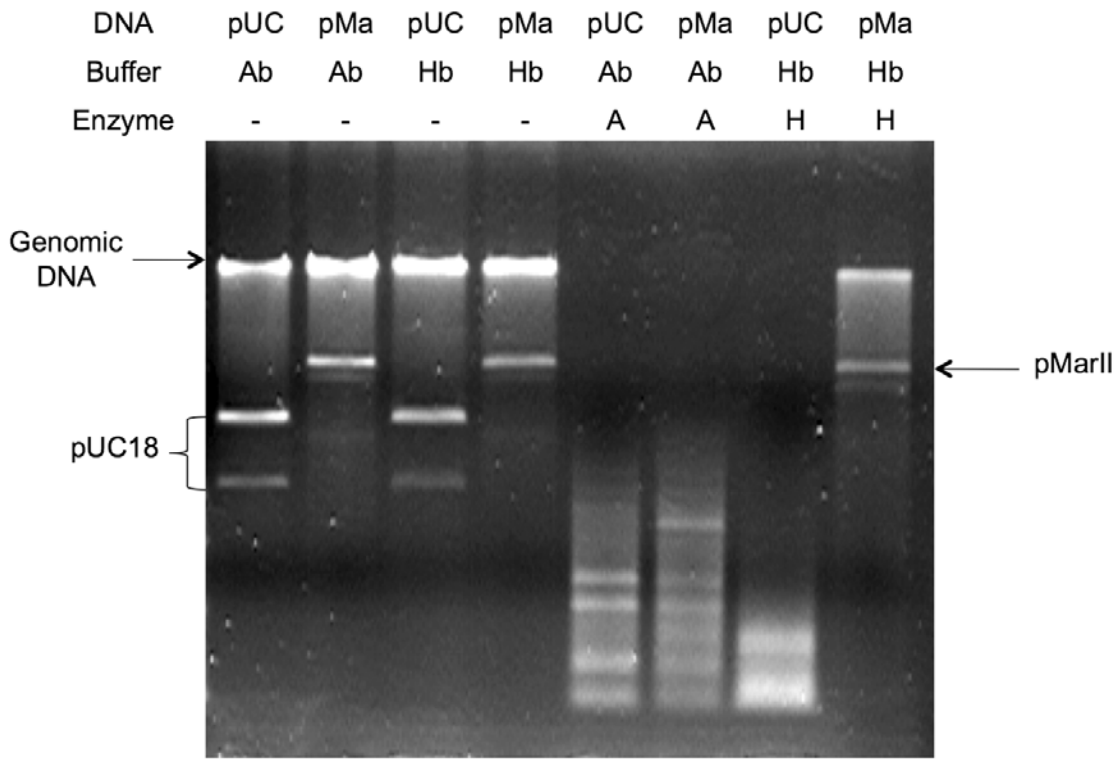
NVLQYIAYNIGSSLNFKPY
NVLQHIA NIG SLN

Fig. 2:


DNA
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pMa
pUC
pMa
pUC
pMa
pUC
pMa
Buffer
Ab
Ab
Hb
Hb
Ab
Ab
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H
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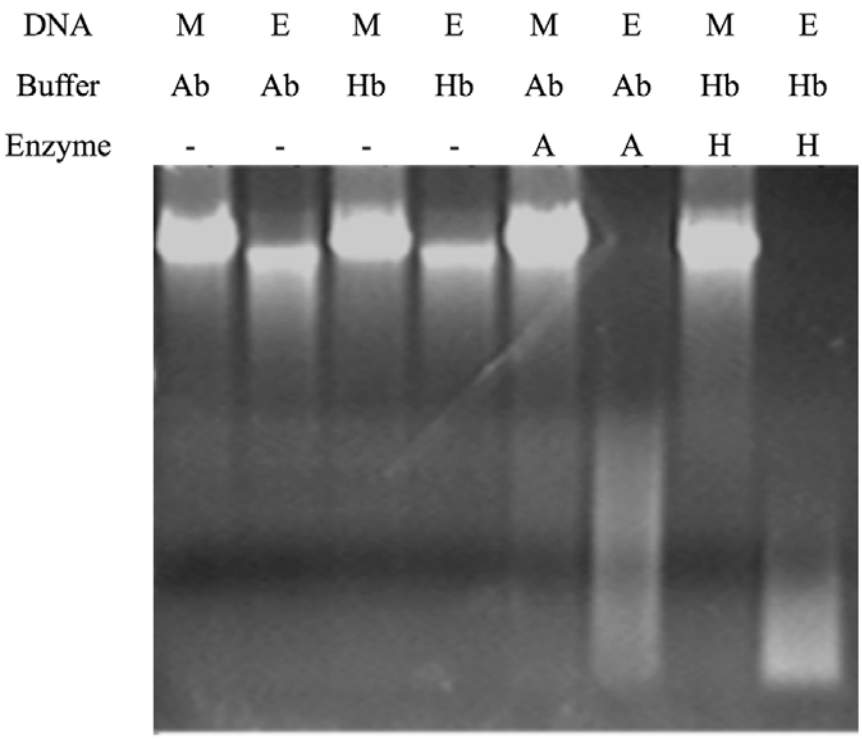
Genomic DNA
pUC18
pMarII

Fig. 3:



ASSOCIATION OF *MYCOPLASMA ARTHRITIDIS* MITOGEN (MAM) WITH LETHAL TOXICITY BUT NOT ARTHRITIS IN MICE

by

WENYI LUO, HUILAN YU, ZUHUA CAO, AND TRENTON R. SCHOEB, MICHELE MARRON AND KEVIN DYBVIG

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ABSTRACT

Mycoplasma arthritidis induces an acute to chronic arthritis in rodents. Arthritis induced in mice histologically resembles human rheumatoid arthritis and can be associated with lethal toxicity following systemic injection. The *M. arthritidis* superantigen (MAM) has long been implicated as having a role in pathogenesis, but its significance with respect to toxicity and arthritogenicity in mycoplasma-induced disease is unclear. To study the pathogenic significance of MAM, *M. arthritidis* mutants that overproduced or failed to produce MAM were developed. MAM overproduction and knockout mutants were more or less mitogenic, respectively, than the wild-type strain. The degree of mitogenic activity correlated with lethal toxicity in DBA/2J mice. In contrast, histopathological studies detected no correlation between MAM production and the severity of arthritis induced in DBA/2J and CBA/J mice.

INTRODUCTION

Mycoplasma arthritidis is a naturally-occurring arthritogen of rats (12). Experimental arthritis can be induced in mice by intravenous injection (4) and rabbits by intraarticular injection (48). Disease in rats and mice is associated with persistence of viable organisms in the joints, but disease in rabbit persists even in the absence of viable organisms (12). Arthritis induced in mice is chronic and relapsing and histologically resembles human rheumatoid arthritis (RA) (10, 22). *M. arthritidis* secretes a superantigen (SAg), *M. arthritidis* mitogen (MAM), which is a potent activator of murine and human T cells (14, 23). MAM is in many aspects a typical SAg. It does not bind to the antigen groove of the major histocompatibility complex (MHC) molecule as do conventional antigens, and T-cell recognition of MAM is not MHC-restricted (11). The presence of a functional H-2E molecule is required to present MAM to T cells (14) with presentation mediated by Eα-containing molecules (13). T-cell responses to MAM stimulation are clonally expressed (16). Responsiveness is associated with expression of particular subsets of V_β chains present on the T-cell receptor (TCR).

MAM has unique features that differentiate it from other bacterial SAgs. Sequence analysis of MAM revealed that it is phylogenetically distinct (17). MAM, but not other SAgs, contains the consensus legume lectin motif that is important for T-cell activation by concanavalin A. Unlike most other superantigens, which have a similar three-dimensional structure consisting of a small β-barrel domain and a large domain with a β-

grasp motif, MAM adopts a novel fold composed of two completely α-helical domains (33). It interacts with the third complementarity-determining region (CDR3) of the TCR (31), a feature of nominal peptide antigens rather than superantigens. It is much more mitogenic for murine cells than are staphylococcal SAgs (19). Both Toll-like receptor 2 and 4 (TLR2 and TLR4) on macrophages are engaged by MAM (34). At least one of these TLRs must be present for macrophage activation by MAM. It was also suggested that TLR4 signaling down regulates the MAM/TLR2 inflammatory response. The interaction between MAM and TLRs of macrophages and the subsequent release of cytokines appears to determine the susceptibility of mice to *M. arthritidis*-induced disease. The cytokine profile induced by MAM in C3H/HeJ mice, a strain bearing a missense mutation in TLR4, is different from that in C3H/SnJ mice, a strain bearing an intact TLR4 (36). As a result, these strains of mice display different susceptibility to disease induced by *M. arthritidis*. MAM is the first superantigen described to have DNase activity (26). These unique characteristics demonstrate the novelty of MAM and warrant further research on its role in the development of superantigen-mediated diseases.

The role of MAM in the pathogenesis of rodent arthritis is unclear. Lymphocytes from some strains of mice that are resistant to *M. arthritidis*-induced arthritis respond to MAM (14, 21, 35). Arthritis in rats can be induced by direct intraarticular injection of MAM, but the arthritis induced does not reproduce the disease phenotype induced by infection with live organisms (5). Significant toxicity but not necessarily significant arthritis has been associated with MAM (18, 20, 36). In this study, we developed mutants

that failed to produce MAM or overproduced MAM with the goal of elucidating the role of MAM in disease induced by *M. arthritidis*.

MATERIALS AND METHODS

Strains of bacteria and medium. Mutants of *M. arthritidis* strain 158 (8, 43) were developed in this study (Table 1) by using the minitransposon Tn*4001TF1* as an insertional mutagen and as a vector. Strain TnCtrl was developed as a control that has the empty minitransposon vector inserted at an intergenic position in the mycoplasma genome. *M. arthritidis* was cultured in EA (agar) or EB (broth) at 37°C as described previously (44, 46). For antibiotic selection in *M. arthritidis*, medium was supplemented with 5 ug of tetracycline/ml. *E. coli* One Shot TOP10 (Invitrogen, Carlsbad, CA) grown in Luria-Bertani medium was used as the host strain to construct plasmids, with tetracycline selection at 10 ug/ml. DNA from *Mycoplasma pulmonis* strain CTp12 was used as a control for some PCR experiments (40).

Development of *mam* knockout mutants. Oligonucleotides used for plasmid construction and PCR reactions are listed in Table 2. To minimize mutations caused by PCR amplification, Expand High Fidelity^{Plus} PCR system (Roche Applied Science, Indianapolis, IN) was used for all plasmid construction. Plasmid pMT85 carrying mini-Tn*4001* was provided courtesy of Dr. R. Herrmann (53). To construct a mini-Tn*4001* carrying a marker that would function in *M. arthritidis*, a 2.3-kb region of transposon Tn*916* carrying the *tetM* gene was amplified by PCR using primer pair TetM-F-Bam and

TetM-R-Bam. The product was cloned into the BamHI site of the mini-Tn*4001* portion of pMT85 to generate Tn*4001TF1* in plasmid pTF85 (Fig. 1A).

To obtain mutants that fail to produce MAM, a transposon library was constructed using minitransposon Tn*4001TF1*. Mycoplasma strain 158 was transformed using the polyethylene glycol-mediated method as described previously (45). Plasmid pTF85 does not replicate in mycoplasmas, and transformants are obtained only when the minitransposon transposes into the mycoplasma chromosome. Individual transformant colonies were picked and grown at 37°C in 1 ml broth containing tetracycline. Each culture was frozen at -80°C in broth supplemented with 15% glycerol.

The genomic location of the minitransposon was determined for each transformant by PCR amplification of the junction between the transposon and the adjacent genomic DNA. The sequence of the PCR product was compared to the complete genome sequence for identification of the transposon insertion site. The inverse PCR conditions and primers were similar to those used to map the transposon location in transformants of *M. pulmonis* (41) except that genomic DNA was digested with Sau3AI instead of NlaIII and inverse PCR amplifications were performed with primers iPCR-F and iPCR-R. The inverse PCR product was purified from an agarose gel and the nucleotide sequence determined using the primer iPCR-S. Two mutants, KO1 and KO2, were identified that had the minitransposon inserted within the *mam* gene (Table 1).

Development of *mam* overexpression mutants. To construct mutants that overproduce MAM, the *mam* gene was inserted downstream of a strong promoter derived from the *M.*

arthritidis mia gene (Fig. 2A). The *mia* promoter was thought to have strong activity because MIA is one of the most abundant proteins produced by *M. arthritidis* strain 158 (43). The *mia* promoter including its ribosomal binding site was amplified by PCR by using the primer pair mia-PF-Not and mia-PR-Eco and inserted into plasmid pCR2.1-Topo (Invitrogen), to generate plasmid Topo-*mia*. The *mam* coding region including its signal peptide coding sequence was amplified from strain 158 genomic DNA using primer pair mam-F-Eco and mam-R-Not and inserted into a separate pCR2.1-Topo vector, resulting in plasmid Topo-*mam*. The fragment containing the *mia* promoter was excised from Topo-*mia* by digestion with NotI and inserted into the NotI site of pBluescript to generate plasmid pBS-*mia*. The fragment containing the *mam* coding region was excised from Topo-*mam* by digestion with EcoRI and inserted into EcoRI site of pBS-*mia*. The resulting plasmid, pBS-*mia-mam,* has the *mam* coding region immediately downstream of the *mia* promoter. The *mia-mam* construct was excised from pBS-*mia-mam* by digestion with NotI and inserted into the NotI site of the Tn*4001TF1* portion of pTF85. Two constructs were obtained, Tn*4001TF1-mia-mam*1 has the *mia-mam* gene in the forward orientation and Tn*4001TF1-mia-mam*2 has *mia-mam* in reverse orientation in the transposon. The resulting plasmids, pTF85-*mia-mam*1 and pTF85-*mia-mam*2, were confirmed to be absent of any mutations by DNA sequence analysis and transformed into wild-type *M. arthritidis* 158 by the PEG method.

The genomic location of the minitransposon carrying the *mia-mam* gene was determined to identify transformants that might have the minitransposon inserted into an

innocuous site that would not adversely affect the virulence of the mycoplasma. The protocol for identifying the nucleotide insertion site of Tn*4001TF1-mia-mam*1 or Tn*4001TF1-mia-mam*2 in transformants of 158 was the same as described above for mapping the location of Tn*4001TF1*. Three independent overexpression mutants, Over1, Over2 and Over3, each with the transposon inserted at what appeared to be an innocuous site were chosen for further study.

Reverse transcriptase (RT)-PCR. Bacteria were grown to stationary phase, harvested by centrifugation and washed once in 1 x phosphate-buffered saline (PBS). Genomic DNA was isolated by using the EasyDNA kit (Invitrogen) according to manufacturer's instruction. Total RNA was isolated using RNAqueous-4PCR kit (Applied Biosystems/Ambion, Austin, TX) according to manufacturer's instruction. TURBO DNase (Ambion) was used to remove DNA contamination present in the samples. One-step RT-PCR was performed using the MessageSensor RT kit (Ambion) and SuperTaq DNA polymerase (Ambion) according to manufacturer's instructions. A 117-bp fragment covering a portion of the sequence downstream of the transcription start site of the *mia* promoter and *mam* coding region of the exogenous *mia-mam* transcripts was amplified using mia-RT-F and mia-mam-RT-R. A 91-bp fragment covering portion of natural *mam* promoter and *mam* coding region of the endogenous *mam* transcripts was amplified with mam-RT-F and mia-mam-RT-R (Fig. 2B). Transcripts of both *mia-mam* and *mam* were amplified in the same reaction. Reactions without reverse transcriptase or RNA template were used as negative controls. RNA samples were reverse transcribed 15

minutes at 42 ºC. cDNA samples were pre-denatured 5 minutes at 95 ºC, followed by 35 cycles of denaturation at 95 ºC for 15 seconds, annealing at 45ºC for 40 seconds, and extension at 72 ºC for 60 seconds. Final extension was performed at 72 ºC for 10 minutes. PCR products were electrophoresed in 10% polyacrylamide gels and visualized by ethidium bromide staining. Experiments were repeated three times to ensure the same trend was reproduced.

Mitogen assays. Cultures of *M. arthritidis* were grown in sterile microcentrifuge tubes to stationary phase, and CFU was assayed for each culture. *M. arthritidis* culture supernatant filtrates (MAS-F) was obtained by centrifuging the fresh culture at 12,000 x *g* for 15 minutes to remove cells, followed by filtration of the supernatant through a 0.2 uM sterile filter (VWR International, West Chester, PA). The filtrates were dialyzed overnight in Slide-A-Lyzer 7K MWCO Dialysis Cassettes (Pierce, Rockford, IL) against 1 x PBS at pH 7.2 to remove toxic metabolites that might interfere with the assay. Filtrates were stored at -80°C. Prior to performing mitogen assays, filtrates were adjusted to normalize the filtrates to the culture that had the least CFU concentration. For some assays the normalized filtrates were diluted 10 times with 1 x PBS (10 x dilutions).

Splenocyte suspensions were prepared from two DBA/2J or CBA/J mice by pressing spleens through a 70 uM NITEX fabric (Sefar Filtration, Depew, NY) into Hank's Buffered Salt Solution (HBSS) (Invitrogen/Gibco). Red cells were lysed in 10 ml Gey's solution (6). After red cell lysis, splenocytes were washed and counted using a Z2 Coulter Counter (Beckman Coulter, Fullerton, CA). Cells were harvested and suspended

at a concentration of 5 x 10^6 cells/ml in RPMI 1640 (Invitrogen/Gibco) containing 10% fetal bovine serum (Hyclone, Logan, UT) plus 0.05 mM 2-mercaptoethanol and 2 mM L-glutamine (Invitrogen) (RPMI-2ME).

The ability of MAS-F to stimulate T cells was assessed by the dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA). 100 ul of splenocyte suspension was dispensed into each well of a 96-well flat bottom cell culture plate (Corning, Corning, NY). 5 ul of diluted MAS-F was added to test wells. As a negative control, 5 ul of dialyzed mycoplasma culture medium was added to wells in place of MAS-F to measure the background fluorescence. Positive controls consisted of adding 5 ul of 10 ug/ml phorbol 12-myristyl 13-acetate **(**PMA) (Sigma) and 5 ul of 20 ug/ml A23187 ionophore (Sigma) instead of MAS-F. The total volume of each well was adjusted to 200 ul with RPMI-2ME. All test samples and controls were analyzed in triplicate. After incubation at 37 °C in a humidified 5% CO_2 atmosphere for 24 hours, cell proliferation was assessed using the DELFIA cell proliferation kit (PerkinElmer) according to manufacturer's instruction. Briefly, cells were labeled with 5-bromo-2-deoxyuridine **(**BrdU) by adding 20 ul of 100 uM BrdU labeling solution to each well and re-incubated for an additional 8 hours. The cells were fixed for 30 minutes at room temperature and stained with 100 ul of 0.5 ug/ml anti-BrdU-Europium working solution. After incubation at room temperature for 2 hours, cells were washed 4 times and fluorescence was induced by adding 200 ul DELFIA inducer to each well. After 15 minutes incubation, the Europium-fluorescence was measured in a Wallac 1420 multilabeled counter (PerkinElmer) in its

Time-Resolved Fluorometry (DELFIA)-Europium mode. Undiluted filtrates were used for the mitogenic comparison between wild-type *M. arthritidis*, the TnCtrl strain, and the *mam* knockout mutants KO1 and KO2. Because of the high level of superantigen activity produced by the Over1, Over2 and Over3 overexpression mutants, it was necessary to assay 10 x diluted filtrates to compare wild-type *M. arthritidis*, TnCtrl, and the *mam* overexpression mutants. Each experiment was repeated twice to ensure the same trend was obtained.

Animal experimentation. In preparation for infection of animals, cells were harvested by centrifugation, washed once in fresh EB medium, suspended in EB with 15% glycerol, and stored at -70°C. Before inoculation, an aliquot was thawed and assayed for CFU.

The authors' institutional review boards approved all animal studies. Female CBA/J (H^k, E_α^+, 6 to 8 weeks old) and DBA/2J mice (H^d, E_α^+, 6 to 8 weeks) were purchased from Jackson Laboratory (Bar Harbor, Maine). CBA/J or DBA/2J mice were infected with 4 x 10^8 CFU or 1 x 10^9 CFU, respectively, by tail vein injection. For CBA/J mice, animals were infected in groups of 5. Arthritis was assessed by histological analysis of the joints (wrist and ankle) tissue at two weeks postinoculation. The virulence of wild-type *M. arthritidis* strain 158, the TnCtrl, the two independent *mam* knockout mutants KO1 and KO2, and the three independent *mam* overexpression mutants Over1, Over2 and Over3 were assessed in CBA/J mice. A negative control consisted of the injection of mice with sterile EB. Similar experiments were performed in DBA/2J mice infected in groups of 8, but with a more comprehensive assessment of the induced disease. Visible

arthritis in wrists, ankles, metatarsal and metacarpal joints was scored as 0-3 and digits were scored as 0-1.5 based on the severity of inflammation. Deaths in each group were recorded and compared with statistical analysis. The body weight of each mouse was measured and recorded. Other manifestations of the disease such as dermal necrosis and paralysis of the legs were also observed and recorded. Arthritis in DBA/2J mice was also assessed by histological analysis of the wrist and ankle joints tissue at two weeks postinoculation. For these experiments, the virulence of the transposon control, the *mam* knockout mutant KO1 and the overexpression mutant Over1 were assessed. Again, one group of mice was injected with sterile EB as a control.

Histopathological evaluation of arthritis. Wrist and ankle joints were excised from mice sacrificed 2 weeks postinoculation. The joints were fixed in 70% alcoholic formalin, demineralized, sectioned at a thickness of 5 um, and stained with hematoxylin-eosin. The severity of arthritis was assessed by a pathologist (T. R. S.) who was not provided with the identity of the experimental groups. For each joint, the following lesion characteristics were scored 0, 1, 2, or 3 for absent/normal, mild, moderate, or severe: Joint cavity exudate, synovial and capsular inflammation, synovial and capsular fibrosis, articular cartilage erosion, intra-articular bone destruction/remodeling, ankylosis, extra-articular osteomyelitis, periostitis and periosteal bone proliferation, tendon sheath exudate, tendon sheath inflammation, and tendon sheath fibrosis. Distribution (single or multiple joints and/or tendon sheaths) of the above changes was also scored 0, 1, 2, 3, or 4. The total score for the joint was calculated as the product of the extent score and the

sum of the component scores, with scores for cartilage destruction, osteomyelitis, and periostitis weighted by multiplying by 2 since bone lesions indicate severe disease. The total score for the mouse was the sum of the individual joint scores.

Growth in vitro and colonization of mouse joints. The protocol for measuring the growth of mutants in vitro is adapted from that described elsewhere (27). Briefly, fresh mycoplasma cultures were diluted 1000 times in EB in a sealed sterile tube and incubated at 37°C. Cultures were sampled every 2 hours and continued until stationary phase was reached. At each time point, 50 ul samples removed for CFU analysis. Growth curves were plotted as the log CFU versus time to calculate the doubling times of wild-type and mutant strains.

The level of colonization or load of *M. arthritidis* in the joints was also studied. DBA/2J mice were infected with 1 x 10^9 CFU in groups of 3. Mice were sacrificed at three weeks. Ankle and wrist joints were collected under sterile condition with the fur removed. Joints from each mouse were minced in 1 ml EB and sonicated in a Model 250/450 Sonifier (Brandson, Danbury, CT) at power level of 5 and duty cycle of 50% for 45 seconds. The suspension was subjected to CFU analysis. Some of the mycoplasmas recovered from the joints were analyzed as described below to assess the stability of the transposon insertion in the genome.

Stability of transposon integration. To study the stability of the mini-Tn*4001* transposon in the mycoplasma genome, mycoplasmas recovered from animal joints were grown in EB medium without antibiotic selection and genomic DNA isolated. Inverse

PCR (procedure as described above) combined with DNA sequencing was firstly performed on the recovered TnCtrl isolates to confirm the presence of single copy of the transposon in the genome. Regular PCR with primers mam-F-Eco and iPCR-R was used to confirm the presence of the transposon in the *mam* coding region of the KO1 mutant. Regular PCR reactions using primers flanking transposon insertion sites were then performed to exclude the presence of any revertants in these isolates that would arise from loss of the transposon. Primer pair mam-F-Eco and mam-R-Not and primer pair Tn-F and Tn-R were used to assay for loss of the transposon from its resident location in the KO1 mutant and the TnCtrl strain, respectively. Genomic DNA from wild-type 158 was used throughout as a control.

Protein profiles. Cultures (10 ml) of *M. arthritidis* were grown until stationary phase was reached. Cells were harvested, washed twice in 1 x PBS, and suspended in Western blot lysis buffer (52). The samples were boiled at 95°C for 10 minutes, and the protein concentration quantified using a BCA Protein Assay Kit (Pierce). 30 ug of protein from each strain was resolved on a 4-15% gradient SDS-PAGE ready gel (Bio-Rad). The separated proteins were transferred to a nitrocellulose membrane (Whatman). The major antigens of *M. arthritidis* were detected on the immunoblots using previously described serum and methods (43).

Nuclease assays. DNase assays were performed as described elsewhere (26). Briefly, genomic DNA was isolated from *E. coli* One Shot TOP10 by using the EasyDNA kit. Three ug of *E. coli* genomic DNA was incubated at 37°C for 30 minutes with EB or

diluted MAS-F from TnCtrl, KO1, or Over1. Ten-times serial dilutions of MAS-F of KO1 were used to digest the genomic DNA from *E. coli*. The MAS-F of TnCtrl, KO1, and Over1 was diluted with the dilution factor at which no DNase activity was detected in the MAS-F of KO1. As a control, a sample of genomic DNA was incubated with 7 units of DNase I (Qiagen, Hilden, Germany). To each reaction, 5 ul EB medium or diluted filtrates and 8 ul RDD buffer (Qiagen) were added. The total volume for each reaction was adjusted to 35 ul with water. DNA degradation was analyzed by agarose gel electrophoresis followed by staining with ethidium bromide.

Statistical analysis. All statistical analyses were performed in SigmaStat version 3.1 (Systat Software, San Jose, CA). Mitogen assay data were normally distributed and had equal variance and were analyzed by one-way ANOVA. Pairwise comparison was performed using Tukey and Student-Newman-Keuls tests. Only the results that were confirmed significant by two tests were reported as significant. Kruskall-Wallis one-way ANOVA test was used to analyze the histopathological results. Survival test was performed using Log-Rank Test and Gehan-Breslow Test and confirmed significant by both tests. Pairwise comparison of survival rates between groups was performed using Holm-Sidak Test. T-test was used for the comparison of the load of mycoplasma in the joints of infected animals. For all tests, $P < 0.05$ was used as the criterion of significance.

RESULTS

MAM knockout mutants. From a library of *M. arthritidis* strain 158 mutants created by using the minitransposon Tn*4001TF1* as an insertional mutagen, two independent

mutants with disruptions in the *mam* gene were isolated (Fig. 1). The full-length MAM protein has 238 amino acids. In the knockout mutants KO1 and KO2, the truncated *mam* gene product would have a length of only 42% (101 amino acids) or 30% (72 amino acids) of the total MAM protein (Table 1). Truncated MAM proteins from both mutants lack amino acids 71-95, a domain critical for lymphocyte activation (17).

MAM overproduction mutants. To overproduce MAM, the *mam* coding region was inserted downstream of the strong promoter isolated from the *M. arthritidis mia* gene. Transformants containing the *mia-mam* gene cloned into Tn*4001TF1* were screened to identify those that had the minitransposon inserted into the mycoplasma genome at a site that was likely to be innocuous (Fig. 2A, Table 2). Strain Over1 has the transposon integrated in an intergenic region downstream of ORF459, 460-bp upstream of the next gene, ORF462, which codes for a hypothetical lipoprotein. Strain Over2 has the transposon integrated within an intergenic region downstream of ORF109, 159-bp upstream of ORF110, which codes for a predicted ABC transporter ATP-binding protein. Based on its location, it is unlikely that the transposon in either mutant would alter the expression of neighboring genes. Strain Over3 has the transposon inserted into the 3' end of the *mspD* gene, coding for a large surface protein of 2592 amino acids (28). Over3 would produce a MspD protein 91% the length of the wild-type protein. The Msp proteins are a 12-member family of predicted phase-variable surface proteins that may have redundant functions, and it is not known which of the *msp* genes is expressed in the parent strain. We view it unlikely that Over3 would have altered virulence due to

disruption of the 3' terminus of *mspD*.

To compare the expression of exogenous *mia-mam* and endogenous *mam*, RT-PCR experiments were designed to amplify *mia-mam* and endogenous *mam* in the same reaction. Conventional PCR with DNA template demonstrated that the primers chosen amplified *mia-mam* and the native *mam* genes with comparable efficiency. About 10 times more *mia-mam* transcripts were detected than endogenous *mam* in the overexpression mutants (Fig. 2B). Controls demonstrated that no RT-PCR product was detected in reactions in which reverse transcriptase or RNA template was omitted (data not shown).

Mitogenic activity. Splenocytes from both CBA/J and DBA/2J mice were used to study the mitogenic activity of MAS-F. Using undiluted filtrates, the overexpression mutants stimulated the splenocytes to such high levels that the cells proliferated and died during the 24-hour incubation period before assaying for the incorporation of BrdU as a measure of T-cell stimulation. This suggested a level of mitogenic activity higher than controls stimulated with PMA and the calcium ionophore A23187, which lacked significant cell death. Using undiluted filtrates, the wild-type 158 strain stimulated splenocytes from CBA/J and DBA/2J mice as predicted. The mitogenic activity of the TnCtrl strain was indistinguishable from that of the 158 parent. In contrast, the KO1 and KO2 mutants lacked mitogenic activity, stimulating splenocytes to about the same degree as controls that were incubated with mycoplasma growth medium (EB) only (Fig. 3A and 3C). Accordingly, there is no evidence of a T-cell mitogen secreted by *M.*

arthritidis other than MAM. Using 10 x diluted filtrates (Fig. 3B and 3D), significant increased mitogenic activity was detected in each of the *mam* overexpression mutants compared to wild-type *M. arthritidis* and TnCtrl. No difference in mitogenic activity was detected between the three *mam* overexpression mutants. These results indicate that the MAM protein produced from *mia-mam* acts as a mitogen stimulating splenocytes from CBA/J and DBA/2J mice.

MAM is not required to cause arthritis in CBA/J mice. The ability of MAM knockout and overexpression strains to induce arthritis in CBA/J mice was compared to the wild-type and TnCtrl strains. Mice were sacrificed two weeks postinjection and the wrists and ankle joints were collected for histopathological studies. Lesions induced by all strains of *M. arthritidis* were primarily synovitis and tenosynovitis with profound synovial proliferation and accumulation of polymorphonuclear leukocytes and mononuclear cells in synovia and tendon sheaths (Fig. 4A). Modest neutrophil and fibrin exudates in the articular lumina were also observed in some cases. Cartilage damage and osteomyelitis, which occur in arthritis induced in rats by *M. arthritidis* (22), were not observed. No arthritis (median arthritis score of zero) was induced by EB medium, consistent with previous studies (42). The median arthritis score induced by the different strains of mycoplasma was compared. Mice infected with the parent 158 strain, *mam* knockout mutants, or overexpression mutants all had mild to moderate arthritis. The median arthritis score for mice infected with the wild-type strain 158, TnCtrl, KO1, KO2, Over1, Over2, and Over3 strains was 1.1, 2.4, 3.1, 1.2, 2.9, 2.9, and 1.9, respectively (Fig. 4B).

No significant differences in arthritis score between any of the groups infected with mycoplasma were detected by statistical analysis, indicating that MAM is not associated with arthritogenicity in CBA/J mice.

MAM is associated with mortality but not arthritis in DBA/2J mice. A more comprehensive study of the association between MAM and disease was performed in DBA/2J mice, which have a defect in the C5 component of the complement system that may contribute to the high susceptibility of these animals to infection. For these experiments, the severity of disease induced by KO1, Over1, TnCtrl and sterile EB medium was determined by assessment of clinical arthritis, histopathological examination of the joints, body weight, and signs relating to toxicity including time of death. No arthritis developed in mice injected with EB. Throughout the two-week course of infection, all groups of mice infected with mycoplasma exhibited similar dynamic arthritic scores (Fig. 5A). All groups of infected animals had a similar number of inflamed joints (Fig. 5B). The experimental protocol was to collect wrist and ankle joints at two weeks postinoculation for histopathological analysis, but 8/8 mice infected with Over1 and 4/8 mice infected with TnCtrl died prior to the date of sacrifice. Histological analysis was limited to the surviving animals, 8/8 mice injected with EB medium, 8/8 mice infected with KO1 and 4/8 mice infected with TnCtrl. No arthritis was induced by EB medium. All of the infected mice had mild to moderate arthritis. Lesions did not differ from those in CBA/J mice. The median arthritis score of animals infected with

TnCtrl or KO1 was 8.6 or 9.7, respectively, a difference which is not statistically significant (Fig. 5C).

Though there were no significant differences in arthritis detected between the *mam* mutants and TnCtrl, significantly different mortality was observed (Fig. 6A). This finding was first noted in a small pilot study in which we found that mice infected with Over1 were more prone to die than mice infected with KO1 (data not shown). In follow up experiments, 6/8 mice infected with Over1 died at day 3 and the remaining two died by day 6. Half of the mice infected with the TnCtrl strain died, all at day 7 or later. No mice infected with KO1 died. A comparison of the survival rates between the three groups yielded a statistically significant result. Consistent with the mortality data, significant greater weight loss occurred in mice infected with TnCtrl than with KO1. The weight loss in these two groups of mice was comparable for the first 6 days, but the weight loss for mice infected with KO1 subsequently leveled off while weight loss for mice infected with TnCtrl continued (Fig. 6B). Additionally, more severe ruffling of fur, lethargy and seizures were observed in mice infected with TnCtrl compared to mice infected with KO1. Although similar dermal necrosis developed under the neck skin in the first week, the dermal necrosis developed in mice infected with KO1 was shorter in duration and began to recede starting at day 7. For mice infected with TnCtrl, the severity of dermal necrosis was constant until the end of experiment. Three mice infected with TnCtrl exhibited paralysis in both hind legs one day before their death, which was never observed in mice infected with KO1 or in CBA/J mice. No disease was

induced by EB medium under any conditions.

Transposon integrations are stable in vivo. The loss of the transposon vector would lead to reversion of the mycoplasma's phenotype that might interfere with the interpretation of in vivo results. It was anticipated that the vector would be maintained in the genome because of its being a minitransposon, but the stability of Tn*4001TF1* during infection had not been previously examined. A PCR-based assay was performed to confirm the presence and location of the transposon, excluding the possibility of transposon loss or jumping, in *mam* mutants. Seven isolates from each of 3 DBA/2J mice that had been infected with the KO1 or TnCtrl were isolated from the joints of the animals at 21 days postinoculation. For each of these strains of mycoplasma, all 21 isolates had the transposon located at the same genomic site as did the inoculum. Using primers that bound to mycoplasma DNA that flanked the location of the transposon, no PCR product was obtained that would be indicative of even a minor subpopulation of template lacking the transposon. All evidence suggests that Tn*4001TF1* is stably integrated into the mycoplasma genome.

MAM does not affect growth of the mycoplasma or colonization of mouse joints. It has been reported that mice bearing different MHC molecules have a different ability to clear *M. arthritidis*, resulting in different susceptibility to *M. arthritidis*-induced toxicity and death (20). It has also been reported that *Escherichia coli* producing the MAM protein has reduced growth (26). Because the possibility exists that MAM might alter growth or affect clearance, experiments were performed to study whether the load of

mycoplasma in the joints was altered in the mutants. The *mam* knockout mutants and the three *mam* overexpression mutants had the same growth parameters as did wild-type *M. arthritidis* 158 and TnCtrl with a doubling time of about 2 hours (Fig. 7A). Colonization of *M. arthritidis in vivo* was assessed by measuring the CFU recovered from the joints of infected mice. For this purpose, DBA/2J mice were infected with 1 x 10^9 CFU. Joints were collected 3 weeks postinoculation and assayed for CFU. A similar number of mycoplasmas were recovered from the mice infected with the KO1 mutant and TnCtrl, suggesting that the *mam* mutant survives in the joints as efficiently as does TnCtrl (Fig. 7B).

The antigenic profile of phase-variable genes and DNase activity of mycoplasma filtrates is not altered in *mam* mutants. Several potential phase- or length-variable genes have been identified in the *M. arthritidis* genome (28). Some of these genes encode surface proteins that might conceivably affect virulence. For example, the MIA protein has been implicated as being associated with virulence of *M. arthritidis* (43). To examine the antigenic profile of wild-type and mutant strains, Western blots of *M. arthritidis* proteins were reacted with serum from mice that has been infected with *M. arthritidis* strain 158L3-1. The protein profiles including the MIA protein were essentially identical among all mutants and wild-type strains (data not shown).

MAM reportedly has DNase activity (26). Being a nuclease provides MAM with another possible mechanism to affect fitness in the host. Because several *M. arthritidis* genes are predicted to code for nucleases, the absence of MAM may not substantially

reduce the total DNase activity of mycoplasma filtrates. To examine whether the DNase activity of filtrates correlated with MAM, genomic DNA from *E. coli* was digested with serial 10-fold dilutions of MAS-F from strain KO1. DNase activity was detected in 10^4-diluted MAS-F. When diluted 10^5, no DNase activity was detected in MAS-F prepared from strains TnCtrl, KO1, or Over1. We conclude that MAM is not the major nuclease present in preparations of MAS-F.

DISCUSSION

MAM has been studied for many years but its definitive role in murine disease has remained unclear. Especially unclear is the arthritogenicity of MAM. All strains of *M. arthritidis* produce MAM, even strains that fail to induce significant arthritis (10, 46, 51). Some studies have indicated that arthritis developed only in strains of mice that had macrophages with a functional H-2E molecule to present MAM to T cells (14). However, mouse strains lacking H-2E can develop acute arthritis, and the incidence of arthritis in H-2E-negative strains at times can be higher than in H-2E-positive strains (21). One difficulty in sorting out the literature is that for some strains of mice, the lymphocytes might be more responsive to MAM than was realized at the time the experiments were performed (19). Similarly, there is no clear association between lymphocyte response to MAM and arthritis in rats. Athymic nude rats can develop polyarthritis in response to *M. arthritidis* infection (3). Although collagen-induced arthritis in mice can be triggered or exacerbated by systemic administration of MAM (15), arthritis in rats could only be induced with purified MAM by direct injection into the joints (5). The disease induced

was transient and characterized by hypertrophy and hyperplasia in the subsynovium. In contrast, rats infected with live *M. arthritidis* organisms develop a purulent, sustained disease phenotype characterized by infiltration of the synovium and subsynovium with polymorphonuclear neutrophilic leukocytes. Unlike the unclear relationship between arthritis and MAM, a strong association between toxicity and MAM has been found. Toxic shock syndrome (20) and dermal necrosis (18) associated with *M. arthritidis* infection are dependent on MAM since they occur only in mouse strains that have the appropriate MHC haplotype to respond to MAM. The association between toxicity and MAM was revisited recently in a study in which C3H/HeJ mice, which lack functional TLR4, were found to be more susceptible to toxic death induced by infection of live *M. arthritidis* organisms than C3H/HeSnJ mice, which have an intact TLR4 (36). The different susceptibility to toxic death was attributed to different cytokine profiles induced by MAM, but no clear prediction as to the susceptibility to arthritis could be made because many of the C3H/HeJ mice died.

To study the role of MAM in disease induced by *M. arthritidis*, we developed stable mutants that failed to produce or overproduced MAM and demonstrated that MAM is the major mitogen secreted by *M. arthritidis*. The absence of MAM led to significantly reduced mitogenic activity while excessive MAM production led to significantly enhanced mitogenic activity. We demonstrated that MAM was not associated with arthritis but was associated with toxic syndrome and mortality. All the mycoplasma isolates recovered from animals infected with mutants of *M. arthritidis* harbored a single

copy of the same transposon in the same genomic location as in the inoculum. No revertants, emerging mutants or contamination of other mutants was detected. The altered virulence in these mutants was associated with the level of mitogenic activity. Mutants and wild-type controls had no apparent differences in growth and had essentially the same antigenic profile.

Although the mitogenic activity of MAM is associated with lethal toxicity, the mechanism is unclear. Recent studies suggest that the cytokines elicited by *M. arthritidis* play a key role. The elicitation of type 1 cytokines and TNF-α is associated with host susceptibility to *M. arthritidis*-induced disease while a predominant induction of type 2 cytokines is associated with host resistance (34-36). Although one report (35) demonstrated that C3H/HeJ, a strain of mouse mainly secreting type 1 cytokines in response to MAM, is more susceptible to *M. arthritidis*-induced arthritis than BALB/c, a strain of mouse mainly producing a type 2 cytokine response to MAM, the difference in arthritis severity may not be due to the cytokines that are produced. Different responses other than cytokines may be elicited by MAM in mouse strains that have different genetic backgrounds. Also, all the cytokine changes discovered were systemic. It is unclear whether local cytokine expression, especially the cytokines in the joints, differs between strains of mice. Mu et al. compared infection in C3H/HeJ and C3H/SnJ mice, which differ only at the lps^d gene, encoding TLR4 (36). These mice exhibited different susceptibility to toxicity and death induced by MAM as a result of the different cytokine profiles elicited. C3H/HeJ mice have a spontaneous mutation in lps^d gene that results in

the dominant secretion of type 1 cytokines, making these mice more prone to toxicity and death. The different cytokine profiles can be attributed to the different status of Toll-like receptors (34). Though TLR4 and TLR2 are both engaged by MAM, TLR4 appears to be an antagonist of TLR2 activity since absence of TLR4 leads to over activation of TLR2 and subsequent release of type 1 cytokines. The systemic effects of MAM seen in this current study are reminiscent of these previous studies. DBA/2J mice infected with the *mam* overexpression mutant had significantly increased mortality while mice infected with the *mam* knockout mutant had no mortality and reduced symptoms of toxicity such as fatigue, ruffled fur, dermal necrosis and weight loss. Further studies are needed to examine the cytokine profiles elicited during infection with the *mam* overexpression and knockout mutants to investigate the cytokine responses that are associated with lethal toxicity.

Although MAM conceivably may contribute in minor respects to arthritis induced by *M. arthritidis*, it is clear that MAM is not a major arthritogenic factor for CBA/J and DBA/2J mice because MAM is not required to cause arthritis in either strain. Mice infected with *mam* knockout mutants developed moderate arthritis indistinguishable from mice infected with control strains. The virulence of *M. arthritidis* is probably a multifactorial process (51). Several potential virulence factors have been identified in *M. arthritidis*. We no longer believe that bacteriophage MAV1 is associated with virulence (7, 8). MAA1 and MAA2 are two adhesion molecules that have been identified in *M. arthritidis*, involved in attachment to host lung cells in vitro (47, 49, 50). Rats that were

actively immunized with MAA1 or MAA2 proteins or passively immunized with antibodies against these proteins were partially protected from *M. arthritidis*-induced arthritis (51). However, all *M. arthritidis* strains have the ability to adhere to rat cells in vitro regardless of their virulence (51), and whether the MAA proteins have a role in mouse arthritis is unknown. Another candidate virulence factor is the MIA protein, which was the only noted antigenic difference between a spontaneous mutant (strain 158-1) of *M. arthritidis* 158 that has a drastic reduction in arthritogenicity (43). Further studies are needed to clarify the role of MIA in pathogenesis.

Our studies demonstrate the advantage of using Tn*4001TF1* rather than previously described derivatives of Tn*4001* as a mycoplasma mutagen. Tn*4001* transposes actively in mycoplasmas. Revertants can arise at a high frequency and complicate phenotypic analysis. Tn*4001TF1* lacks the transposase gene, which is located elsewhere on pTF85, eliminating many problems associated with instability of Tn*4001* (37). Indeed, our results suggest that Tn*4001TF1* is stably integrated in the *M. arthritidis* genome even during growth in animals for a period of at least 3 weeks. Analysis of the transposon libraries suggests that Tn*4001TF1*inserts into the mycoplasma genome at essentially random sites (30). Unlike libraries constructed using Tn*4001*, no examples were encountered in which more than one copy of Tn*4001TF1* was inserted in the genome of a single transformant.

We also demonstrated that Tn*4001TF1* is a useful vector for mediating expression of cloned genes in mycoplasmas. Previous research has demonstrated that MIA is an

immunodominant protein of *M. arthritidis* (43). By RT-PCR, we demonstrated that the strength of *mia* promoter is roughly 10 times that of natural *mam* promoter. A stretch of 8 A nucleotides is found in the putative -35 and -10 regions of the *mia* promoter. We were initially concerned that phase variation due to slipped-strand mispairing within this poly(A) region might affect the spacing between the -10 and -35 sites, resulting in phase variation of promoter activity. RT-PCR experiments and mitogen assays performed on different stocks of the overexpression mutants that had been passaged *in vitro* did not detect any loss of *mia-mam* expression. Animal experiments demonstrated that overproduction of MAM leads to enhanced virulence, suggesting that *mam* is overexpressed in vivo from the *mia-mam* promoter. Therefore, the frequency of phase variation in *mia* promoter activity, if any, would be low.

Whether MAM-like SAgs are involved in human disease is unclear. *M. arthritidis* antigens have been detected in human patients (9). Antibody against MAM has also been detected in sera from patients with rheumatic diseases (39). The major T-cell subset identified in the synovium of several RA patients expressed the exact V_β domain that has been found to be MAM-responsive (1, 32). It has also been demonstrated that MAM can stimulate human T-cell proliferation (2), promote human B-cell differentiation (24), and upregulate human NK-cell activity (25). Rheumatoid factors can be produced by human B cells in response to MAM stimulation (29). Therefore MAM-like SAgs have the potential to perturb human immunity and contribute to disease. As demonstrated in our study, an acute severe toxic syndrome is associated with MAM. This is a reminiscence of

acute diseases of human induced by other SAgs (38). Therefore, MAM-like SAgs, when encountered *in vivo*, may cause similar clinical syndromes or exacerbate the disease by further disturbing the immune system. The studies on MAM and other virulence factors of *M. arthritidis* will provide clues to the role of superantigens in inflammatory diseases in human and give insight to the pathogenesis of human rheumatic diseases, which would lead to novel therapeutic targets for these diseases.

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Table 1. *M. arthritidis* strains used in this study

Strain	Parent	Transposon	Transposon position[a]	Disrupted gene product	Truncation of gene product[b]
158	NA	NA	NA	NA	NA
TnCtrl	158	Tn*4001TF1*	37911	NA	NA
KO1	158	Tn*4001TF1*	38870	MAM	101/238
KO2	158	Tn*4001TF1*	38784	MAM	72/238
Over1	158	Tn*4001TF1-mia-mam2*	398742	NA	NA
Over2	158	Tn*4001TF1-mia-mam1*	95959	NA	NA
Over3	158	Tn*4001TF1-mia-mam1*	412672	MspD	2353/2592

[a]The nucleotide positions correspond to the completed *M. arthritidis* genome sequence (GenBank accession number CP001047).

[b]Number of amino acids in truncated product / number of amino acids in full-length product.

Table 2. Oligonucleotides used in this study

Oligonucleotide	Sequence[a]
TetM-F-Bam	GGTTGTGGATCCTTGTGGGTACTTTTAGGGC
TetM-R-Bam	CTTATTGGATCCGAAACCATATTTATATAACAAC
iPCR-F	CTGATTCTGTGGATAACCGTATTACCGC
iPCR-R	CAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTA
iPCR-S	TTTGAGTGAGCTGATACCGCTCGC
mia-PF-Not	CGGCGGCCGCAACCTTTGGAGATTGCTTC
mia-PR-Eco	CATTATTTTATTGTGAATTCCTTTTC
mam-F-Eco	CACAAATTTAAAAATCATAAGGAATTCAAAAATGAAAAC
mam-R-Not	CGGCGGCCGCCCCCAACCTTTTAGATTG
mia-RT-F	AAATTTATTAAAACAGGTAACTCCG
mam-RT-F	GCTGTGAAAATGAAATTCTTCAC
mia-mam-RT-R	GCTAAAGTGACGGTTGCG
Tn-F	TTTAGCAAATCATATATAAAAGCGCG
Tn-R	TTTTAAATAGTTTCTTTGTTTACCCGC
Over1-F	ACTACCGAAGGCGAAACCGC
Over1-R	ACATCTTCGCAAGAAGCCGC

[a]Underlined nucleotides correspond to restriction sites used for cloning of PCR products.

FIGURE LEGENDS

Fig. 1. (A) Schematic of plasmid pTF85 illustrating transposon Tn*4001TF1* with the direction of gene (open lines) transcription indicated by arrows. *tetM*, tetracycline resistance marker; *gm-r*, gentamicin resistance marker; *tnp*, transposase gene; *ori*, origin of plasmid replication; B, BamHI restriction sites; N, NotI site into which the *mia-mam* gene was inserted for overexpression studies; S, rightward most Sau3A1 site in Tn*4001TF1* (other Sau3A1 sites not shown). The orientation of the primers iPCR-F, iPCR-R, and iPCR-S used for inverse PCR to map the genomic location of the transposon are indicated. (B) Agraose gel of direct PCR products obtained from amplification of genomic DNA isolated from the *mam* knockout mutants KO1 and KO2 and using the mam-F-Eco and mam-R-Not primers that flank *mam*. Genomic DNA from wild-type *M. arthritidis* 158 and *M. pulmonis* strain CTp12 was used as positive and negative control, respectively. PCR reactions amplifying the *mia* promoter with primers mia-PF-Not and mia-PR-Eco were performed to serve as quality controls of DNA template preparation. The direct PCR product of the *mam* gene and the *mia* promoter is 805 bp and 369 bp, respectively.

Fig. 2. (A) Schematic of the *mam* (top) and *mia-mam* (bottom) genes showing the locations of primers used for RT-PCR. mam-F, primer mam-RT-F; R, primer mia-mam-RT-R; mia-F, mia-RT-F; E, EcoRI restriction site used for combining *mia* promoter with *mam* coding region. (B) Agarose gels of RT-PCR and regular PCR products obtained from amplification of the *mam* (91 bp product) and *mia-mam* (117 bp product) genes

using the three primers mia-mam-RT-R, mam-RT-F and mia-RT-F mixed together. Top: template was wild-type 158 (WT) or Over1 genomic DNA. Bottom: template was Over1 genomic DNA (Over1 PCR), Over1 RNA (Over1 RT), Over2 RNA (Over2 RT), or Over3 RNA (Over3 RT).

Fig. 3. The mitogenic activity of *mam* knockout and overexpression mutants on splenocytes of CBA/J (panels A and B) and DBA/2J (panels C and D) mice. T-cell proliferation was represented as fluorescence counts. Data have had background (splenocytes stimulated with EB) subtracted. Experiments were repeated twice to ensure the same trend was obtained. The data represent the mean of 3 replicates. Standard deviation was indicated with error bars. Undiluted filtrates were used for the mitogenic comparison between wild-type, transposon control, and *mam* knockout mutants (panels A and C). 10 x diluted filtrates were used for the mitogenic comparison between wild-type, TnCtrl, and *mam* overexpression mutants. * represents results that are statistically significant.

Fig. 4. Arthritis induced in CBA/J mice. (A) Representative histopathology of arthritis induced in CBA/J mice: a, 4 x amplification of normal metacarpal joints from a control mouse injected with EB medium; b, 20 x amplification of synovium of a control mouse injected with EB, showing normal synovial cells; c, 4 x amplification of metacarpal joints of a mouse inoculated with a mutant, showing moderate synovitis (s) and tendonitis (t) with exudates (e) of neutrophils and fibrin in the joint space; d, 20 x amplification of

synovium of a mouse inoculated with a mutant, showing moderate synovitis with cell proliferation (cp) and exudate of neutrophils and fibrin (e) overlying synovial cells. (B) Histological scores of CBA/J mice infected with *M. arthritidis*. The box encompasses the 25^{th} through the 75^{th} percentiles of data range (interquartile range (IQR)). The solid line in the box shows the median. The whisker shows up to 1.5 times the IQR. "x" designates data points outside the whisker.

Fig. 5. Arthritis induced in DBA/2J mice. (A) Average subjective scores for visible arthritis. Visible arthritis developed in wrists, ankles, metatarsal and metacarpal joints was scored as 0-3 and digits were scored as 0-1.5 based on the severity of inflammation. (B) Average number of joints involved. Standard deviation was presented as error bars. (C) Histological scores of arthritis in joints (wrist and ankle) tissue collected at two weeks postinoculation. The box encompasses the 25^{th} through the 75^{th} percentiles of IQR. The solid line in the box shows the median. The whisker shows up to 1.5 times the IQR. "x" designates data points outside the whisker.

Fig. 6. Toxic effect of *M. arthritidis* on DBA/2J mice. (A) Survival curve plotted as survival rate versus time (days) over 2-week period. Groups of mice infected with EB and KO1 were combined as MAM (-) group. The difference between groups was statistically significant. (B) Weight change as a function of time over a 2-week period. Standard deviation was presented as error bars. Mice infected with Over1 are not presented due to early death.

Fig. 7. The effect of MAM on the growth of *M. arthritidis in vitro* and mycoplasma load *in vivo*. (A) 40-hour growth curves of *M. arthritidis* strains. (B) CFU recovered from the ankle and wrist joints at 3 weeks postinoculation from DBA/2J mice infected with 1×10^9 *M. arthritidis*. The log of the total CFU recovered from each mouse is presented.

Fig. 1:

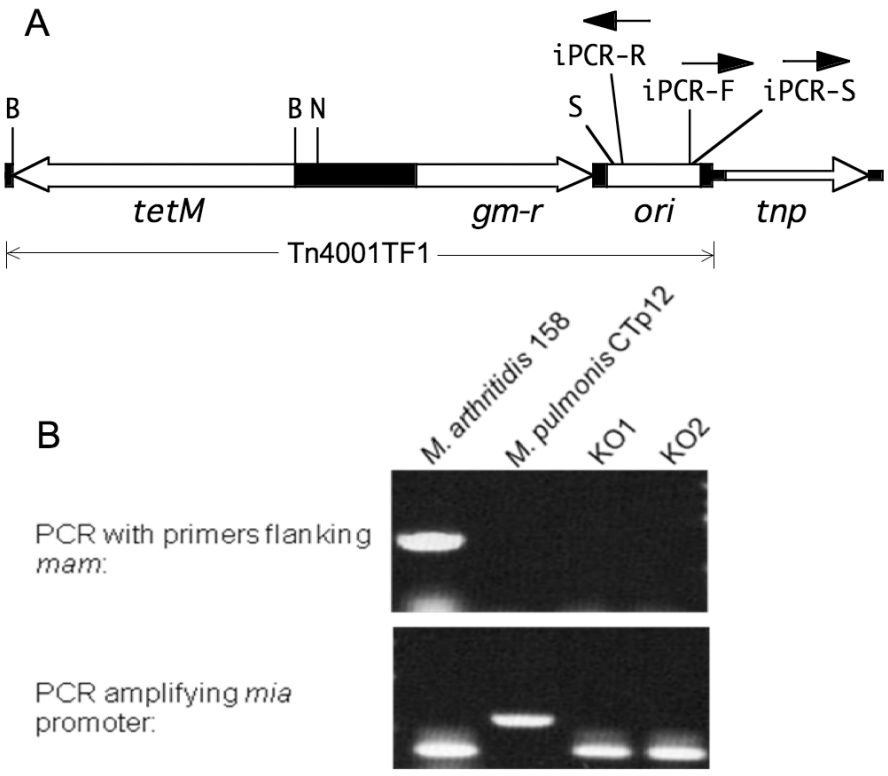
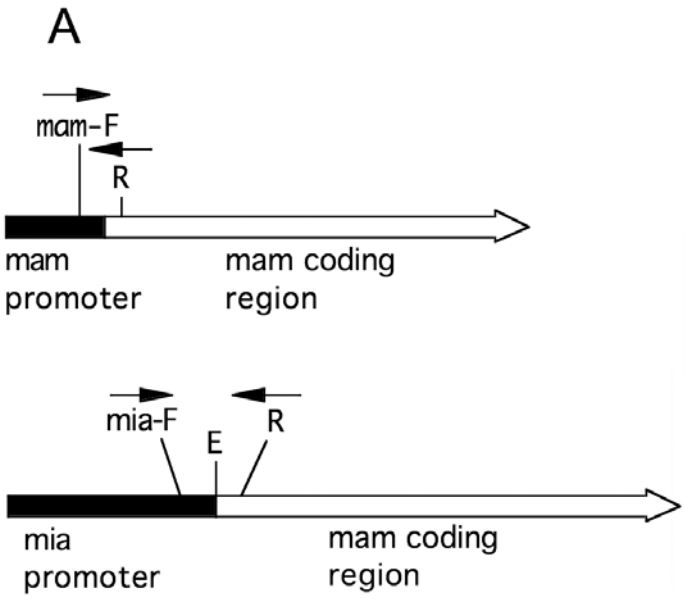

A
iPCR-R
iPCR-F
iPCR-S
B
B N
S
tetM
gm-r
ori
tnp
Tn4001TF1
B
M. arthritidis 158
M. pulmonis CTp12
KO1
KO2
PCR with primers flanking mam:
PCR amplifying mia promoter:

Fig. 2:


A
mam-F
R
mam
promoter
mam coding
region
mia-F
E
R
mia
promoter
mam coding
region

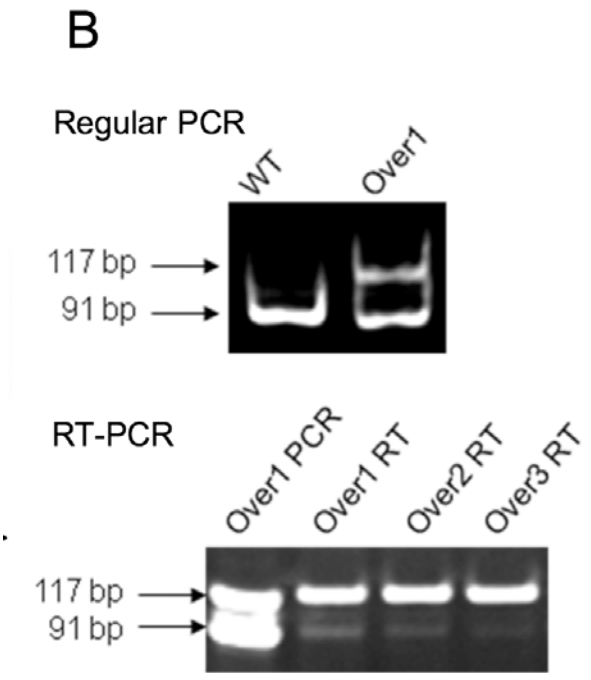

B
Regular PCR
WT
Over1
117 bp
91 bp
RT-PCR
Over1 PCR
Over1 RT
Over2 RT
Over3 RT
117 bp
91 bp

Fig. 3 A and B:

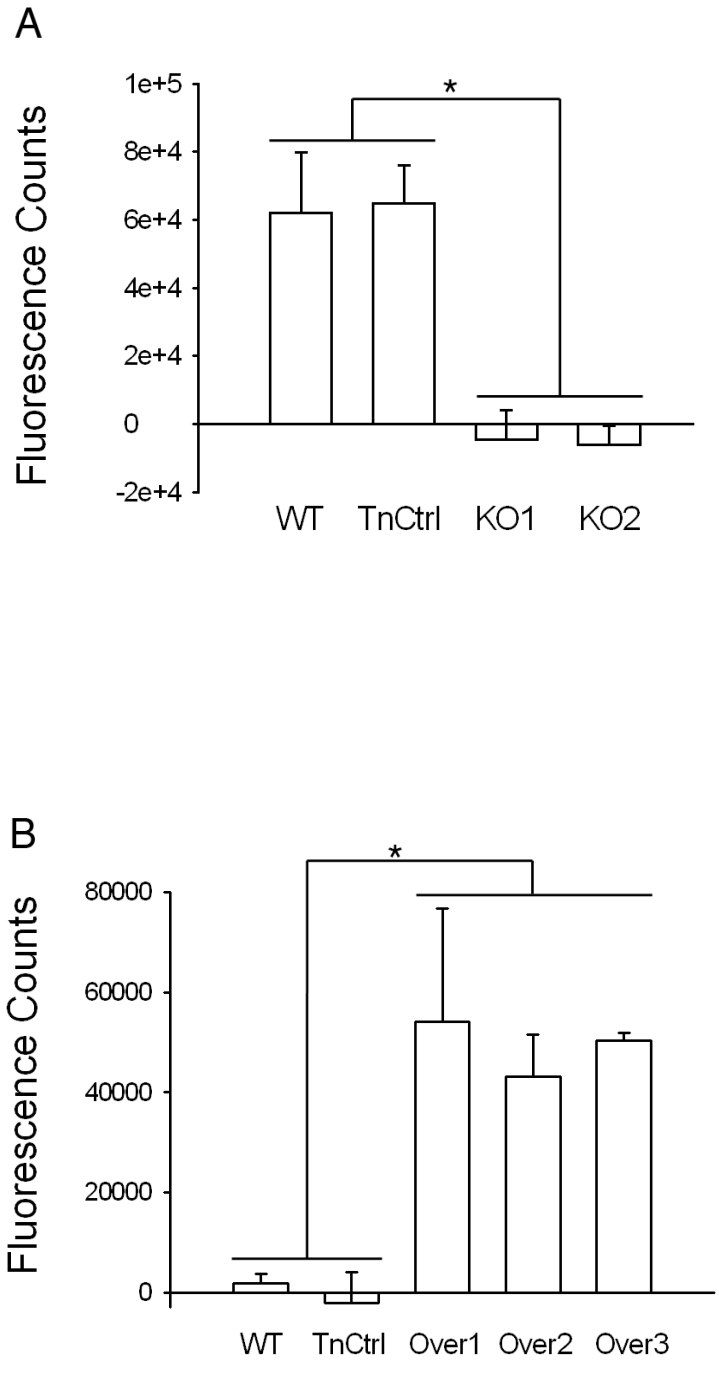

A
Fluorescence Counts
1e+5
8e+4
6e+4
4e+4
2e+4
0
-2e+4
*
WT
TnCtrl
KO1
KO2
B
Fluorescence Counts
80000
60000
40000
20000
0
*
WT
TnCtrl
Over1
Over2
Over3

Fig. 3 C and D:

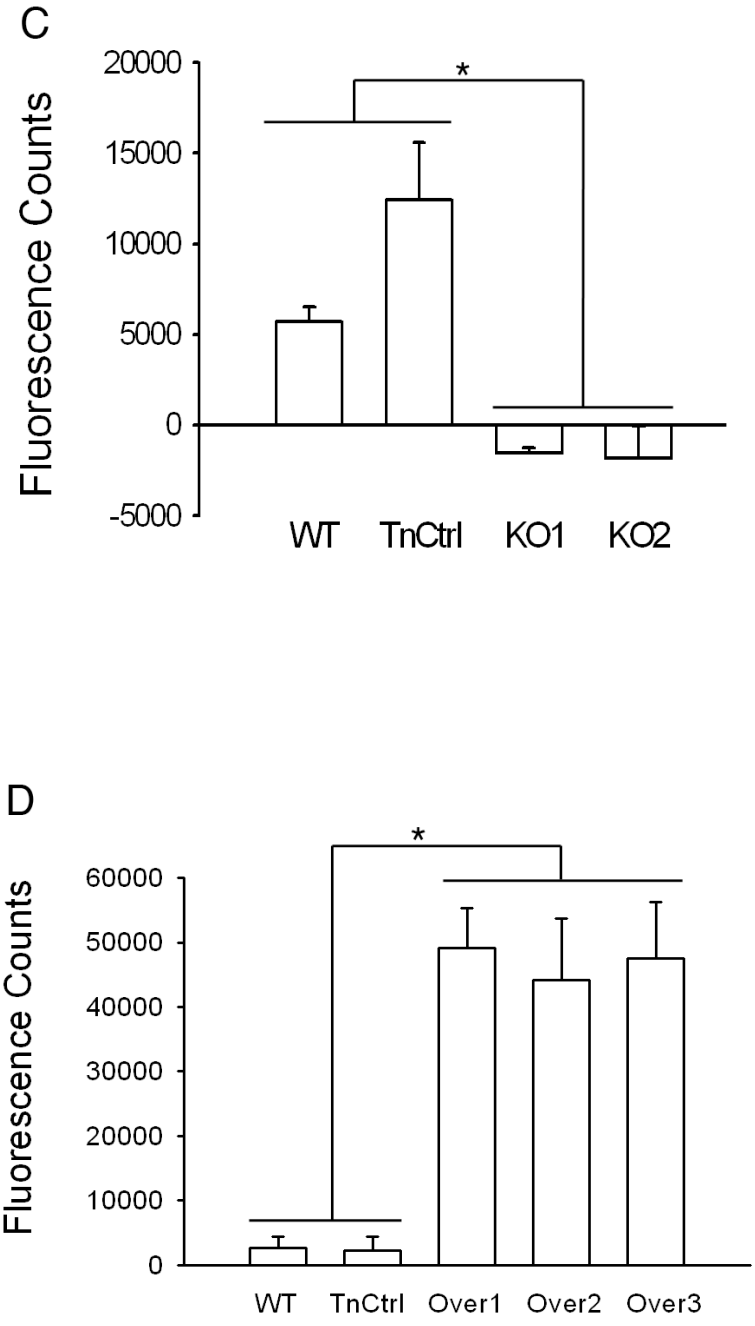
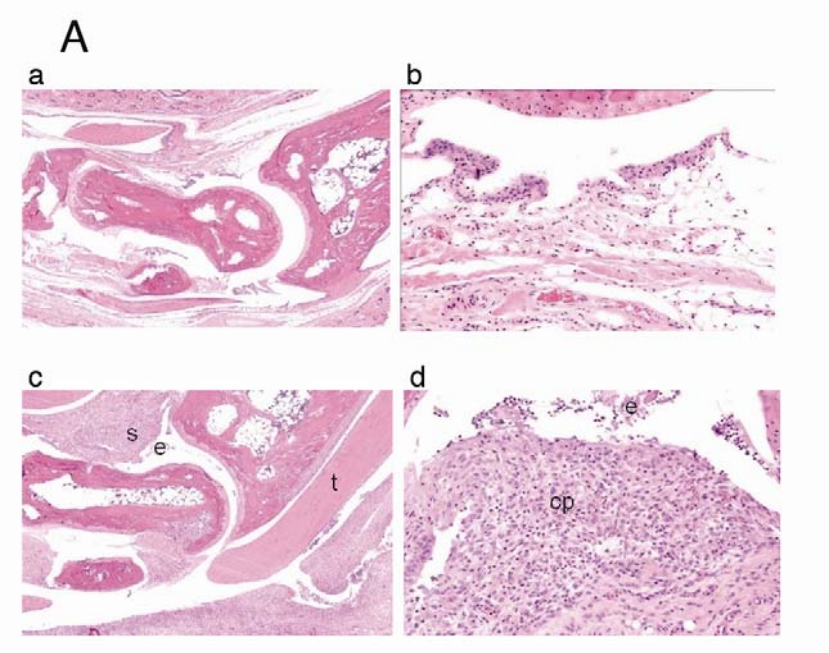

C
Fluorescence Counts
20000
15000
10000
5000
0
-5000
*
WT
TnCtrl
KO1
KO2
D
Fluorescence Counts
60000
50000
40000
30000
20000
10000
0
*
WT
TnCtrl
Over1
Over2
Over3

Fig. 4:


A
a
b
c
d
s
e
t
e
cp

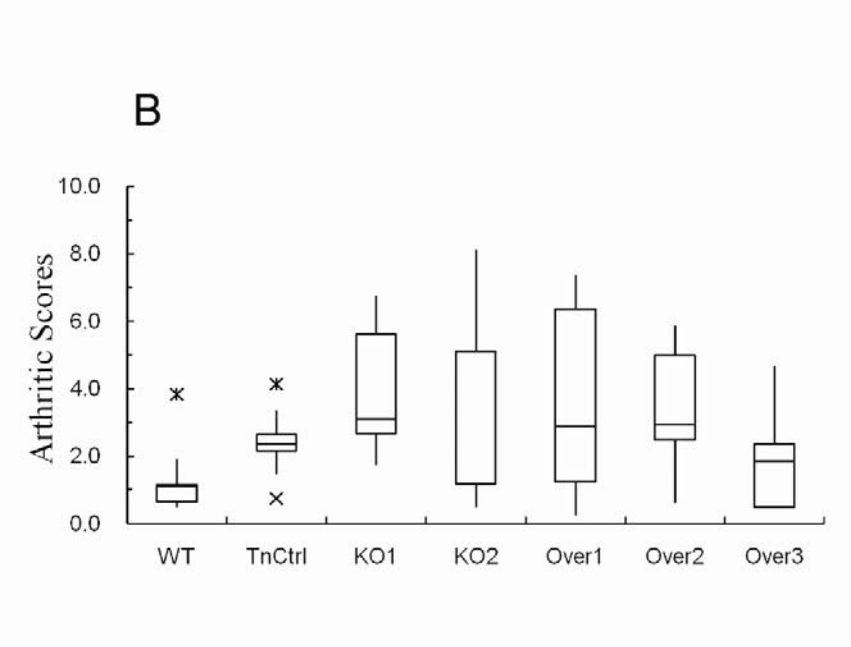

B
Arthritic Scores
10.0
8.0
6.0
4.0
2.0
0.0
WT
TnCtrl
KO1
KO2
Over1
Over2
Over3

Fig. 5:

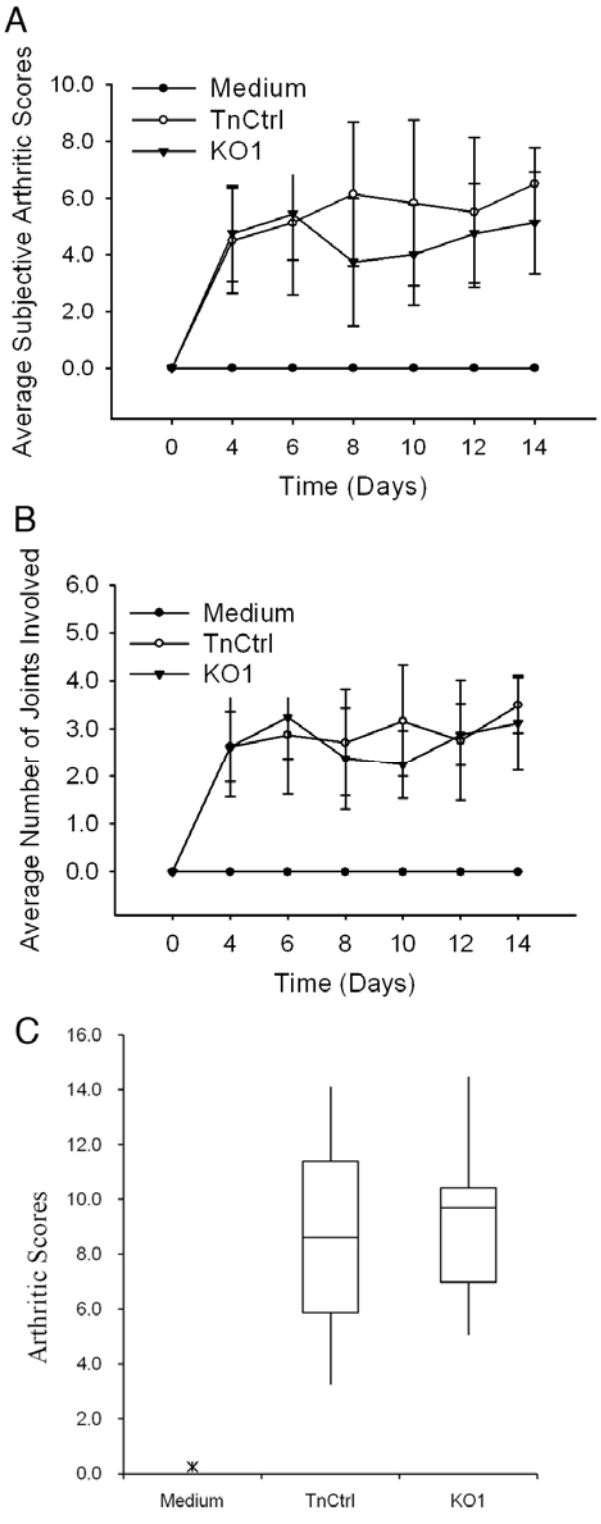

A
Average Subjective Arthritic Scores
10.0
8.0
6.0
4.0
2.0
0.0
Medium
TnCtrl
KO1
0
4
6
8
10
12
14
Time (Days)
B
Average Number of Joints Involved
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Medium
TnCtrl
KO1
0
4
6
8
10
12
14
Time (Days)
C
Arthritic Scores
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
Medium
TnCtrl
KO1

Fig. 6:

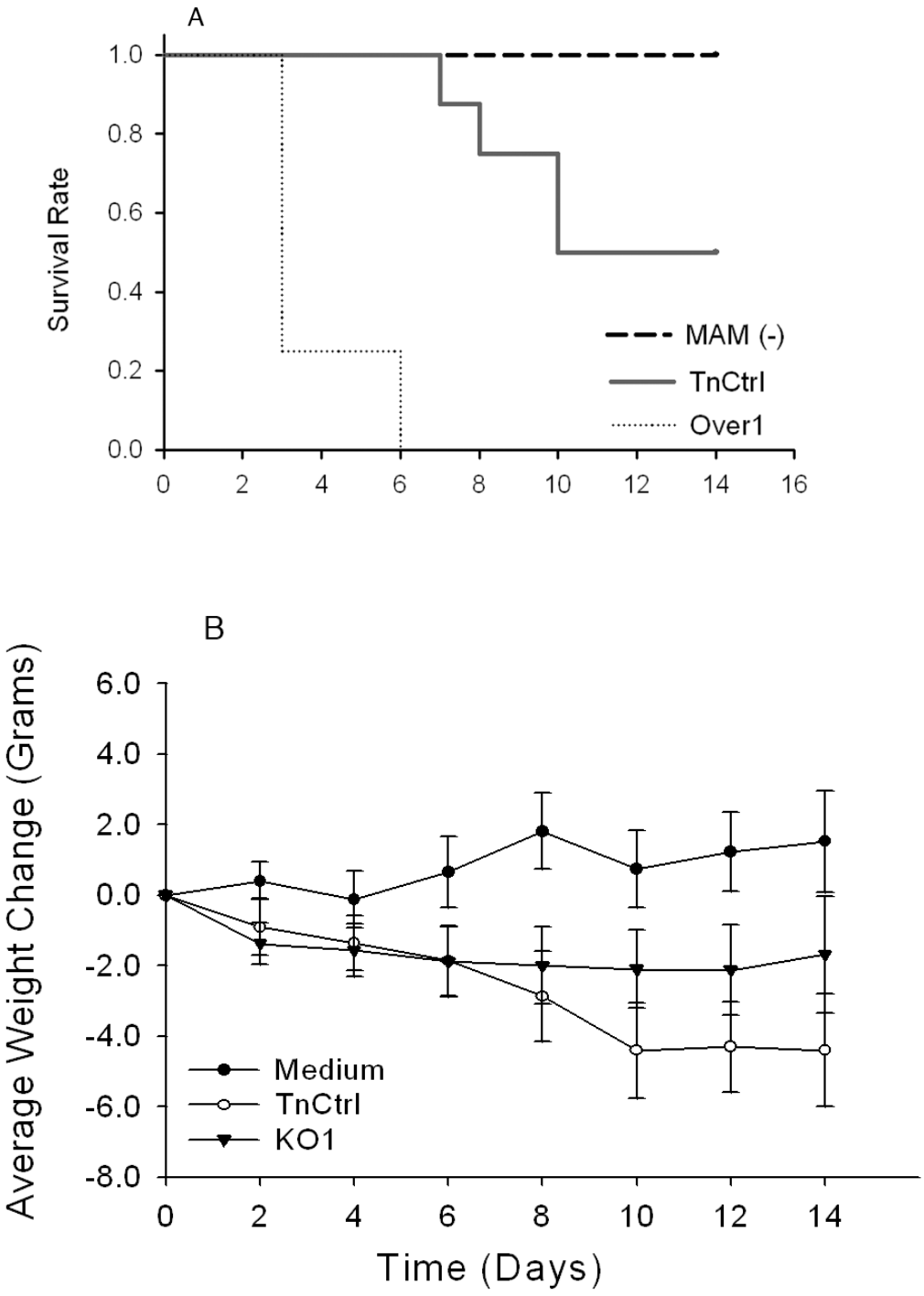
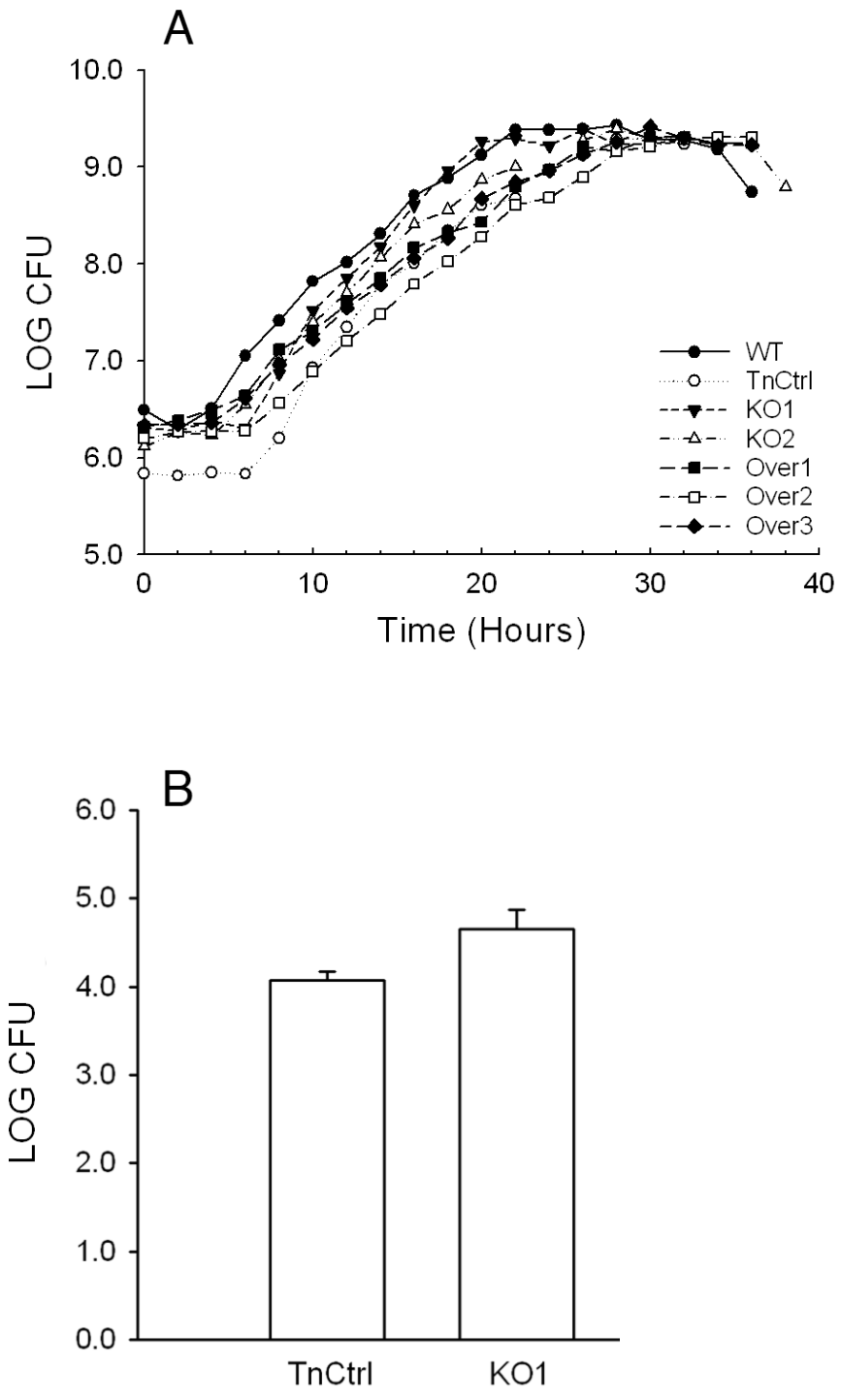

A
Survival Rate
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
12
14
16
MAM (-)
TnCtrl
Over1
B
Average Weight Change (Grams)
6.0
4.0
2.0
0.0
-2.0
-4.0
-6.0
-8.0
0
2
4
6
8
10
12
14
Time (Days)
Medium
TnCtrl
KO1

Fig. 7:


A
10.0
9.0
8.0
7.0
6.0
5.0
LOG CFU
0
10
20
30
40
Time (Hours)
WT
TnCtrl
KO1
KO2
Over1
Over2
Over3
B
6.0
5.0
4.0
3.0
2.0
1.0
0.0
LOG CFU
TnCtrl
KO1

SECRETED GLYCOSIDASE FROM *MYCOPLASMA PULMONIS*

by

WENYI LUO, JIAHUI TAO, HUILAN YU, BINGDONG SHA AND KEVIN DYBVIG

In preparation for *FEMS Microbiology Letter*

Format adapted for dissertation

ABSTRACT

The protein coded by the MYPU_4630 gene is one of several predicted glycosidases in *M. pulmonis* and is annotated as a glucan 1, 6-α-glucosidase. Although the protein lacks a classical signal peptide sequence, cellular localization studies with hemaglutinin-tagged MYPU_4630 protein demonstrated that the glycosidase was secreted. The enzymatic assay of purified glutathione S-transferase-MYPU_4630 recombinant protein demonstrated that the glycosidase had activity for N-acetylglucosamine and N-acetylgalactosamine. Possible roles for the MYPU_4630 protein in pathogenesis are discussed.

INTRODUCTION

Glycosidases are common enzymes produced by almost all prokaryotes with important roles in carbohydrate metabolism through the hydrolysis of the glycosidic bonds between two monosaccharides. They exist intracellularly (Bailey & Bourne, 1961) or extracellularly (Beylot, *et al.*, 2001, Chiarezza, *et al.*, 2009) and are mostly involved in nutritient scavenging (Tailford, *et al.*, 2007, Gilbert, 2008). They also can have roles in cell division, adhesion, biofilm formation, and interactions with the host (Hoskins & Boulding, 1981, Mercier, *et al.*, 2002). In some bacteria such as *Streptococcus pneumoniae*, glycosidases are indicated as being major virulence factors. (Gosink, *et al.*, 2000, Moscoso, *et al.*, 2006).

Mycoplasmas are the smallest self-replicating organisms (Razin, 1987). They evolved from Gram-positive bacteria (Oshima & Nishida, 2007) by loss of a cell wall and most of the genome (Dybvig & Voelker, 1996). Due to their limited genetic information, mycoplasmas rely heavily on the host for many nutrients such as amino acids, nucleic acid precursors, lipids, carbohydrates, and vitamins (Razin, *et al.*, 1998). Consistent with that, several glycosidases including β-glucosidase (Henrikson, *et al.*, 1964), sialidase (Bercic, *et al.*, 2008, May & Brown, 2009) and β-N-acetylglucosaminidase (Kahane, *et al.*, 1990) have been described in mycoplasmas.

M. pulmonis is the pathogen of murine mycoplasmosis (Davidson, *et al.*, 1988). This disease is slowly progressing and histologically resembles human pneumonia (Cassell, 1982). Exopolysaccharide (EPS) structures have recently been identified and are possible virulence factors (Daubenspeck, *et al.*, 2009). The *M. pulmonis* genome codes for several predicted glycosidases. In addition to scavenging function, these enzymes could possibly

degrade mycoplasmal EPS (Mercier, *et al.*, 2002) or host molecules such as mucin (Hoskins & Boulding, 1981). The MYPU_4630 gene is annotated as coding for a glucan 1, 6-α-glucosidase. To study the function of the MYPU_4630 protein, we engineered a glutathione S-transferase (GST)-tagged MYPU_4630 protein. The recombinant protein had glycosidic activity for α-linked N-acetylglucosaminides and N-acetylgalactosaminides. Additional experiments indicated that the protein was secreted.

MATERIALS AND METHODS

Bacterial strains used and transformation of *M. pulmonis*

M. pulmonis CTG is derived from strain CT and is considered as the wild-type strain (Simmons, *et al.*, 2004). Mycoplasmas were cultured in HA (agar) or HB (broth) at 37°C as described previously (Davidson, *et al.*, 1988). For antibiotic selection in *M. pulmonis*, medium was supplemented with tetracycline (5 ug ml^{-1}). *E. coli* strains One Shot TOP10 and BL21 Star DE3 (Invitrogen) grown in Luria-Bertani (LB) medium were used as hosts to construct plasmids and express recombinant protein, respectively, with tetracycline selection at 10 ug ml^{-1}. Transformation of CTG was performed as described previously (Dybvig & Alderete, 1988, Dybvig, *et al.*, 2000).

Promoter prediction and amino acid sequence analysis

The promoter analysis was performed with BPROM, an online bacterial promoter prediction tool at http://linux1.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb. The amino acid sequence analysis was performed using protein BLAST available from http://blast.ncbi.nlm.nih.gov/Blast.cgi.

Construction of hemaglutinin (HA) and glutathione S-transferase (GST)-tagged MYPU_4630 proteins

To construct the HA-tagged MYPU_4630 protein, the MYPU_4630 coding region and its predicted promoter region were amplified by PCR with primers GCGAATTCAAATGATTGAAAAGTCAGTTGG (EcoRI site is underlined) and GCG CGG CCG C*GG CAT AAT CCG GCA CAT CAT AAG GGT A* TT TGT TTT GG (NotI site is underlined and the sequence coding for HA tag is italicized) and inserted into the EcoRI/NotI site of the transposon portion of plasmid pTF85 (Luo*, et al.*, 2008). The sequence of the resulting plasmid was confirmed by DNA sequencing and transformed into *M. pulmonis* CTG.

To construct the GST-tagged MYPU_4630 protein, the coding region of the gene MYPU_4630 was amplified from CTG by PCR using the primer pair CACCATGAAAAATTCAAAGTATATTTTTTATCAAC and TTAGTATTTGTTTTGGAAAAATAAATTAGTG. The PCR product was inserted into plasmid pET100 (Invitrogen). To convert MYPU_4630 TGA codons, which encode tryptophans in mycoplasmas but are stop codons in *E. coli*, to TGG, a PCR-based multiple mutation reaction was performed according to a protocol published previously (Hames*, et al.*, 2005). Forward primer GCGGATCCATGAAAAATTCAAAGTATATTTTTTATCAAC (BamHI site was underlined), reverse primer GCGAATTCTTAGTATTTGTTTTGGAAAAATAAATTAGTG (EcoRI site was underlined), and 5' terminal phosphorylated mutation primers GAAAATCTATGGACTTTAAACAATAATTCAAAAAAG,

CTAGTTATTTGGTCTTCAAAATTAGTTGATTTTAAAAAAC,

GAAGAGCACTTTCACTTTGGGAAAAAAAC,

GTTATCCAATGATTTGGACTAAAAATCAGCTTG, and

GTTAATTCTCAATGGGAAGATTCGAGATCAATTTTTC (underlined nucleotides refer to base triplets that were converted from TGA to TGG) were used for MMR. All PCR reactions were performed using iProof high fidelity DNA polymerase (Bio-Rad, Hercules, CA). An MMR product of the expected size was inserted into the BamHI/EcoRI site of plasmid pGEX 2TRS, which contains a GST tag upstream of the multiple cloning site such that the MYPU_4630 coding sequence would be fused in frame with the GST tag. The final plasmid was sequenced to confirm that the TGA codons had been converted to TGG and that no other mutations had been introduced.

Cellular localization

Signal peptide and transmembrane domains were predicted by using Phobius, a combined online transmembrane topology and signal peptide predictor at http://phobius.sbc.su.se/index.html. The probability of secretion was predicted with SecretomeP 2.0, a prediction tool for non-classical protein secretion at http://www.cbs.dtu.dk/services/SecretomeP-2.0, with the Gram-positive option selected. Proteins with SecP scores greater than 0.5 were predicted to be secreted.

The cellular localization of MYPU_4630 was further examined by studying the HA-tagged MYPU_4630 protein. Mycoplasma cells were grown in 500 ml HB medium to late log phase. Cells were harvested by centrifugation, washed in HB, and suspended in 5 ml HB. Protein secretion was followed by incubation at 37 ºC and sampling the culture every 2 hours. The cells and the supernatant of the samples were separated by

centrifugation. Cell lysates were prepared as described (Xu, *et al.*, 2006). Samples (cell lysates or supernatants) were electrophoresized in a 4-15% precast SDS-PAGE gel (Bio-Rad, Hercules, CA). The Precision Plus protein kaleidoscope standards were used to indicate the molecular weight of loaded proteins (Bio-Rad). Proteins were transferred to nitrocellulose membranes (Whatman, GE, Piscataway, NJ) and reacted with mouse anti-HA antibody (Cell signaling, Danvers, MA) and anti-mouse IgG (Cell signaling). An 82-kDa HA-tagged IKKα was used as the positive control and IKKα without the HA tag was used as the negative control. The same experiment was repeated on mycoplasmas cultured in CMRL culture medium (Invitrogen) containing 0.5% glucose.

Recombinant protein purification and identification by mass spectrometry

The plasmid containing the GST-tagged MYPU_4630 gene was extracted from *E. coli* One Shot TOP10 and transformed into *E. coli* BL21 Star DE3. The cells were grown at 37ºC in LB to OD_{600} of 0.6 and induced with 1 mM isopropyl β-D-thiogalactoside for 6 h at 18ºC. The cells were harvested and lysed. Purification was achieved by using a GST column (Amersham Biosciences, Piscataway, NJ). The eluted proteins were divided into 100 µl aliquots and stored at -80ºC in 20% glycerol. The proteins from one aliquot were resolved in a SDS-PAGE gel and stained with Coomassie bright blue. The protein concentration was estimated according to the intensity of the staining. The purified recombinant protein was trypsin digested in gel and sequenced with ABI 4000 Qtrap from Applied Biosystems (Invitrogen). The matched protein was identified by MASCOT at http://www.matrixscience.com.

Measurement of glycosidic activity of recombinant protein

Glycosidic activity was measured with *p*-nitrophenyl conjugated substrates except for α-D-N-acetylneuraminic acid which was conjugated to 4-methylumbelliferyl. All substrates were purchased from Sigma (St. Louis, MO). The *p*-nitrophenyl or 4-methylumbelliferyl absorbs light at a characteristic wave length when it is released from the sugar by hydrolysis of the glycosidic bond. The substrates tested and reaction condition for each substrate are listed in Table 1. For each substrate, a test group and a control group were set up. The former contained both the substrate and the purified recombinant protein. The latter contained substrate and deionized water or buffer in place of the purified protein (for α, β-glucoside and α-D-N-acetylneuraminic acid) or purified protein added after the reaction had been stopped according to the protocol of the substrate's supplier. The enzymes β-glucosidase, β-galactosidase, and β-N-acetylglucosaminidase were included as positive controls. Experiments were repeated at least twice. The absorbance was measured at 400 nm for all the *p*-nitrophenyl conjugated substrates. For 4-methylumbelliferyl labeled α-D-N-acetylneuraminic acid, the reaction was excited at 340 nm and emission was measured at 430 nm. The glycosidic activity was calculated as $((A_{Test}-A_{Blank})$ * total volume of assay * dilution factor)/(milimolar extinction coefficient of *p*-nitrophenyl or 4-methylumbelliferyl * time of the assay * volume of enzyme used).

Statistics

Statistical analyses were performed in SigmaStat version 3.1 (Systat Software, San Jose, CA). T-test was used to analyze the glycosidic activity results of MYPU_4630. $P < 0.05$ was used as the criterion of significance.

RESULTS AND DISCUSSION

Amino acid sequence analysis of MYPU_4630 protein

MYPU_4630 was originally annotated as a glucan 1,6-α-glucosidase. It is one of five glycosidases predicted from the *M. pulmonis* genome (Chambaud, *et al.*, 2001). The other four predicted glycolsidases are the products of MYPU_1030, annotated as an oligo-1,6-glucosidase, MYPU_6320, annotated as a lipoprotein with α-amylase 3 activity (1,4-α-D-glucan glucanohydrolase), MYPU_6330, annotated as an oligo-1,6-glucosidase, and MYPU_6440, annotated as an α-dextrin endo-1,6-α-glucosidase (pullulanase). Blast analysis demonstrated that the MYPU_4630 protein had sequence similarity to glucosidase, α-amylase, and trehalose-6-phosphate hydrolase from different bacteria with the highest similarity to a predicted membrane bound oligo-1,6-glucosidase of *Mycoplasma arthritidis* 158L3-1 (26%) and a predicted α-glucosidase of *Mycoplasma mobile* (24%). The MYPU_4630 is located just downstream of the MYPU_4620 gene which codes for a conserved hypothetical protein. Although the intergenic region between these two genes is only 4 nucleotides, they may not form an operon because analysis using BPROM predicted a MYPU_4630 promoter inside the MYPU_4620 coding sequence.

Cellular localization of MYPU_4630 protein in *M. pulmonis*

A combined transmembrane topology and signal peptide prediction tool was used to predict the cellular localization of MYPU_4630 and the other four predicted *M. pulmonis* glycosidases. No signal peptide or transmembrane domain was detected in any of these glycosidases, suggesting the proteins are unlikely to be classically secreted or membrane associated.

Many secreted proteins in bacteria do not contain signal peptides. These proteins can account for up to 18% of the total secreted proteins (Tjalsma*, et al.*, 2004). Although no motif can be identified, non-classically secreted proteins can be distinguished from cytoplasmic proteins by properties such as amino acid composition, secondary structure and disordered regions (Bendtsen*, et al.*, 2005). Analysis using SecretomeP 2.0 predicted that all five of the putative glycosidases of *M. pulmonis* were secreted (Table 2), though the SecP score for the MYPU_4630 protein was barely above the significance criterion.

To examine the cellular localization of the MYPU_4630 protein, a C-terminal HA-tagged protein was developed. The presence of HA-tagged MYPU_4630 protein in HB culture supernatant and cell lysates was examined on Western blots reacted with HA antibody (Fig. 1). Protein reacted with HA antibody was only detected in supernatant but not in cell lysates. The amount of protein present in the supernatant after 2 or 4 hours incubation at 37 ºC was significantly higher than that in the supernatant at 0 hours. The data showed that MYPU_4630 was an expressed gene coding for a secreted protein found in the culture supernatant but not in cell lysates. The same experiment was performed again under conditions in which cells were suspended in CMRL medium (a medium without protein components) instead of HB and similar result was obtained (data not shown). The secretion level in CMRL was significantly lower than in HB, indicating the efficient secretion of MYPU_4630 protein is dependent on nutrients present in HB but not in CMRL. Because no motif indicative of a classified secreted protein was identified, the secretion mechanism for MYPU_4630 is probably non-classical and worthy of further study.

Expression and purification of a recombinant GST-MYPU_4630 protein

To study the function of the MYPU_4630 protein, a recombinant GST-MYPU_4630 protein was created and purified. The eluted proteins were resolved on a SDS-PAGE gel and stained with Coomassie bright blue. Only one protein product, corresponding to the molecular weight of the MYPU_4630 protein, was detected (Fig. 2). The identity of the protein product was studied by mass spectrometry. All 32 fragments that met the criteria for protein certainty matched the sequence of the MYPU_4630 protein, suggesting that the recombinant GST-MYPU_4630 protein is the only protein present in the elution.

Substrate specificity of the recombinant GST-MYPU_4630 protein

A colorimetric-based strategy was used to study the enzymatic specificity of the recombinant protein. Both α- and β-linked glucosides, galactosides, N-acetylglucosaminides, and rhamnosides were firstly tested. The purified protein had no glycosidic activity for any of these glycosides except α-linked N-acetylglucosaminides (Fig. 3), which agrees with the annotation of MYPU_4630 protein as an alpha linkage-specific glycosidase. The calculated activity of this enzyme was 22 unit/mg (one unit will hydrolyze 1.0 μmole of α-N-acetylglucosaminides per minute at pH 4.0 at 37 °C). N-acetylglucosamine (GlcNAc) is structurally similar to other amino sugars such as N-acetylgalactosamine (GalNAc) and N-acetylneuraminic acid. The N-acetylneuraminic acid is the substrate of sialidase, an enzyme detected in several species of mycoplasma and considered as a virulence factor (May & Brown, 2009). Therefore, experiments were performed to examine whether MYPU_4630 had glycosidic activity towards these amino sugars. MYPU_4630 exhibited glycosidic activity toward α-N-acetylgalactosaminides but not N-acetylneuraminic acids, with the activity for α-N-acetylgalactosaminides being

even higher than for α-N-acetylglucosaminides (Fig. 3). We did not demonstrate whether MYPU_4630 was an endoglycosidase or exoglycosidase or whether it was specific for sugars of 1-4 or 1-6 linkage.

Alpha-N-acetylglucosaminidase and α-N-acetylgalactosaminidase belong to the glycoside hydrolase family 89 and 101 (www.cazy.org) and are responsible for hydrolysis of α-linked GlcNAc and GalNAc, respectively. GlcNAc is often the terminal sugar of an oligosaccharide of a glycoprotein (amigo.geneontology.org). An α-linked GlcNAc has been identified as a moiety of mucin derived from mammalian gastric gland mucous cells (Ishihara, *et al.*, 1996). GalNAc is also present in mucin. An endo-α-N-acetylgalactosaminidase can liberate the O-glycosidically linked galactosyl β 1-3 GalNAc disaccharide from mucin glycoprotein (Katayama, *et al.*, 2005). Mucins are a family of large, heavily glycosylated proteins produced by many epithelial tissues in vertebrates. They are a major component of mucus and have important innate immune functions by trapping and removing pathogens (Voynow & Rubin, 2009). Successful mucosal pathogens usually develop strategy to subvert or avoid the mucin barrier (Linden, *et al.*, 2008). Despite its unclear pathogenic importance, both α-N-acetylglucosaminidase and α-N-acetylgalactosaminidase have been identified in pathogenic bacteria such as *Clostridium perfringens* (Ficko-Blean, *et al.*, 2008) and *Streptococcus pneumonia* (Gregg & Boraston, 2009). *M. pulmonis* may adapt a similar strategy to invade respiratory epithelium to cause disease. Mucin is one of many possible targets of the secreted MYPU_4630 protein. Degrading host-derived mucin might be a mechanism to scavenge sugars and have a role in pathogenesis. Its α-N-acetylgalactosaminidase activity might release O-linked glycan while its α-N-acetylglucosaminidase activity together with other

glycosidases might further degrade released polysaccharides. In addition to mucins, heparan sulfate is possibly another target. The human genetic disease mucopolysaccharidosis IIIB is caused by the accumulation of heparan sulfate due to a deficiency in α-N-acetylglucosaminidase (Malinowska, *et al.*, 2009), demonstrating the presence of α-GlcNAc in heparan sulfate. Heparan sulfate proteoglycans have an important role in cell growth by binding and regulating growth factors (Spivak-Kroizman, *et al.*, 1994, Forsten, *et al.*, 2000). They are also involved in inflammation by binding chemokines to prolong the white-cell attracting activity (Schenauer, *et al.*, 2007).

The presence of terminal GlcNAc in EPS of *M. pulmonis* was suggested because the EPS can be stained with GS-II lectin (Daubenspeck, *et al.*, 2009). Perhaps the EPS of *M. pulmonis* is the target of its own glycosidases. If this is the case, the MYPU_4630 protein and other glycosidases may have a role in the turnover and maintenance of EPS and impact processes such as cell division, adhesion and biofilm formation (Mercier, *et al.*, 2002). A GlcNAc transport system has been identified in *M. pulmonis*. (French, *et al.*, 2008). Therefore, the GlcNAc released from EPS or host molecules such as mucin or heparan sulfate can be imported into the cytoplasm.

The MYPU_4630 protein is the first glycosidase ever identified in mycoplasmas with activity for GlcNAc and GalNAc. Glycosidases in *M. pulmonis* might be involved in virulence as they are thought to be for other bacteria (Gosink, *et al.*, 2000, Chouikha, *et al.*, 2006). However, MYPU_4630 alone might not have a significant impact on virulence because the five glycosidases predicted from the genome sequence might have overlapping activities or act in combination.

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Table 1. Substrates used for enzymatic specificity and reaction conditions

Substrates	Buffer	Cofactor	PH	Temperature (°C)
α-D-glucoside	57 mM potassium phosphate	0.1 mM glutathione	6.8	37
β-D-glucoside	80 mM sodium acetate		5.0	37
a-D-galactopyranoside	80 mM potassium phosphate		6.5	25
β-D-galactopyranoside	87 mM sodium acetate	0.11% BSA[a] 43.5 mM NaCl	4.4	25
α-D-N-acetylglucosaminide	100 mM citrate 100 mM sodium phosphate	0.03% BSA	4.0	37
β-D-N-acetylglucosaminide	45 mM citrate	0.01% BSA[a] 90 mM NaCl	5.0	25
α-L-rhamnopyranoside	50 mM potassium phosphate		7.0	25
α-D-N-acetylgalactosaminide	100 mM citrate 100 mM sodium phosphate	0.03% BSA	4.0	37
α-D-N-acetylneuraminic acid	250 mM sodium acetate		5.5	37

[a] BSA stands for bovine serum albumin.

Table 2. Prediction of cellular localization of glycosidases in *M. pulmonis*

Glycosidases coding gene	SP and TM[a]	SecP score[b]
MYPU_1030	No	0.87
MYPU_4630	No	0.53
MYPU_6320	No	0.78
MYPU_6330	No	0.68
MYPU_6440	No	0.70

[a] Prediction of signal peptide and transmembrane domain by Phobius. SP, signal peptide. TM, transmembrane domain.

[b] SecP score as predicted by SecretomeP 2.0.

FIGURE LEGENDS

Fig. 1. Western blot of the supernatant and cell lysates of *M. pulmonis* producing HA-tagged MYPU_4630 protein using anti-HA antibody. The symbol "+" designates the lane for the positive control and "-" designates the lane for the negative control. The molecular weight of the positive control HA-tagged IKKα is 82 kDa and the molecular weight of the HA-tagged MYPU_4630 protein is 63 kDa.

Fig. 2. SDS-PAGE gel of eluted proteins stained with Coomassie bright blue.

Fig. 3. MYPU_4630 protein releases α-linked GlcNAc and GalNAc. 100 µl of 0.1-0.4 mg/ml purified protein was used for the enzymatic assay. The values on y-axis indicate the absorbance at 410 nm. The control groups contain the purified protein added only after the reaction had been stopped. Standard errors are indicated as error bars.

Fig. 1:

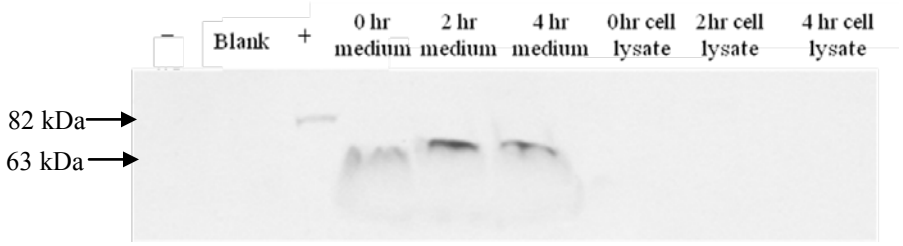
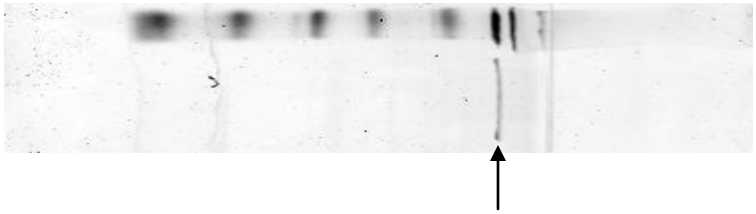
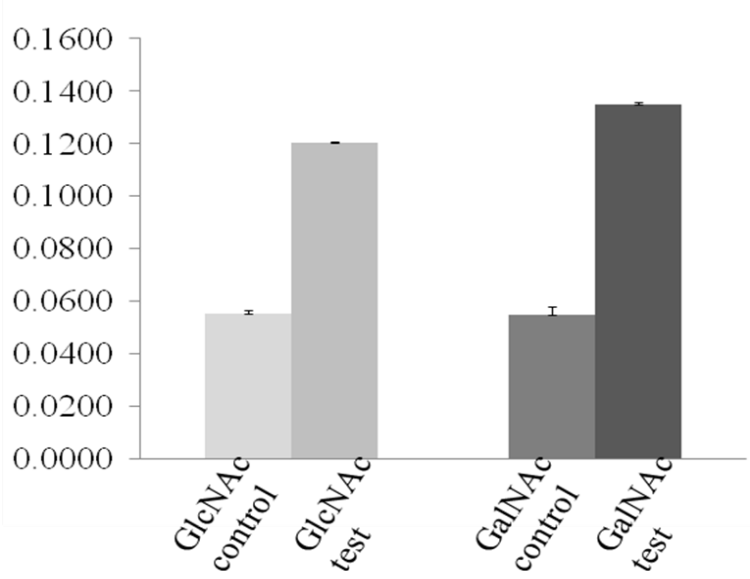

–
Blank
+
0 hr medium
2 hr medium
4 hr medium
0hr cell lysate
2hr cell lysate
4 hr cell lysate
82 kDa
63 kDa

Fig. 2:



GST-tagged MYPU_4630 protein, 72 kDa

Fig. 3:


0.1600
0.1400
0.1200
0.1000
0.0800
0.0600
0.0400
0.0200
0.0000
GlcNAc control
GlcNAc test
GalNAc control
GalNAc test

SUMMARY AND DISCUSSION

Major discoveries

The pathogenesis of mycoplasmal infection is complex, involving multiple virulence factors. Our effort has been focused on the characterization of two candidate virulence factors. One is the *M. arthritidis* mitogen (MAM) and the other is the glycosidase encoded by MYPU_4630 in *M. pulmonis*.

As a superantigen and strong T-cell mitogen, MAM has long been considered as the major factor associated with arthritis induced by *M. arthritidis*. However, its role with respect to arthritis and lethal toxicity had not been definitively studied. To test the role of MAM in disease, we developed mutants that did not produce MAM or overproduced MAM. T-cell proliferation assays demonstrated that MAM was the major mitogen secreted by *M. arthritidis*. Animal experiments demonstrated that lethal toxicity was positively correlated with mitogenic activity. In contrast, the severity of arthritis induced by MAM knockout and overproduction mutants was similar and comparable to the arthritis induced by wild-type *M. arthritidis*. The level of the mitogenic activity produced did not correlate with other potential factors such as growth of the mycoplasmas, colonization of the joints, expression profiles of mycoplasma proteins, and the mycoplasma's secreted nuclease activity. Nuclease assays also demonstrated that MAM was not the major nuclease secreted by *M. arthritidis*. Combined with studies performed by our collaborator Hong Mu at the University of Utah, we clearly demonstrated the association between MAM and lethal toxicity and presented evidence of the mechanism being interactions between MAM, MHC II and TLRs. This is consistent with the model that both MHC II and TLRs (TLR-2 and 4) are engaged by MAM. When MHC II, TLR-2

and 4 are present, type II cytokines are secreted and mice exhibit a mild disease. TLR-4 appears to be a negative regulator of TLR-2. When TLR-4 is absent, type I proinflammatory cytokines are secreted and mice present a more severe lethal toxicity (12).

As another group of potential virulence factors, glycosidases of *M. pulmonis* were studied. Five glycosidases have been predicted from the genome sequence. Because MYPU_4630 contained the least number of TGA codons that would need to be converted to TGG for expression in *E. coli*, the protein coded by MYPU_4630 was firstly studied. By studying the MYPU_4630 protein fused with a HA tag, we demonstrated that the protein was secreted into the supernatant of the culture medium. Further analysis of a GST-MYPU_4630 recombinant protein demonstrated that this protein was a glycosidase with activity for α-linked GlcNAc and GalNAc.

Transposon library usage in *M. arthritidis*

Many genes of mycoplasmas have diverged too far from those of other bacteria to predict function through informatics. The identification of proteins that are involved in important physiological or pathological processes will rely heavily on the study of transposon mutants. As mentioned previously, a stable transposon library has been developed in *M. arthritidis* by using a minitransposon (4). The transposon's location in each transformant was mapped by inverse PCR, and disruption of the nonessential genes verified by direct PCR. In this library, the transposon was mapped to 1,113 genomic locations, disrupting 218 different genes. However, this library has not reached saturation. Because the genome size of *M. arthritidis* (820 kb) (4) is comparable to *M. pulmonis* (963 kb) (1), it should possess a similar number of essential genes, which is estimated to

be greater than 271 but less than 382 (5). Of the 635 protein-coding regions of *M. arthritidis* (4), the number of nonessential genes should be between 253 and 364. Many genes of interest have not yet been disrupted. Therefore, the transposon library needs to be expanded. Ideally, the expansion should continue until the number of disrupted genes does not increase as additional mutants are analyzed.

Complementation of the mutant gene with its wild-type copy is needed to confirm that the altered phenotype is caused by transposon disruption and not other mutations elsewhere in the genome. For complementation, a transposon with an antibiotic resistance maker other than the tetracycline marker carried on the original transposon has been developed and successfully applied in *M. arthritidis*.

Strategies on the tools to improve the genetic manipulation in *M. arthritidis*

Due to the low efficiency of transformation that perhaps was the result of a restriction-modification (R-M) system serving as a barrier, part of our initial effort was directed toward understanding R-M systems in *M. arthritidis*. This effort led to the discovery of an isoschizomer of the HhaI DNA methyltransferase. Another DNA methyltransferase that protects genomic DNA of *M. arthritidis* from digestion with the AluI restriction endonuclease has yet to be discovered. Due to the fact that modification of plasmids by either AluI methyltransferase or HhaI methyltransferase could not increase the transformation efficiency, the role of isoschizomers of the AluI and HhaI restriction enzymes in inhibiting transformation of *M. arthritidis* may not be as important as originally thought.

Further effort needs to be taken to understand the mechanisms underlying the low transformation efficiency in *M. arthritidis*. The barrier to transformation could be non-

specific nucleases that are either membrane bound or secreted, R-M enzymes that have not been identified, or surface proteins such as MIA. To seek for factors that might impede the transformation of *M. arthritidis*, pools of library mutants could be transformed, selecting for transformants using chloramphenicol resistance as the marker. Mutants that are transformed readily would predominate in the transformant pool. The functions of genes disrupted in these mutants could be predicted with bioinformatics tools and the mechanisms examined accordingly. The discoveries from these studies will possibly be used to develop novel strategies to improve the transformation efficiency for *M. arthritidis* and other mycoplasmas.

Homologous recombination (HR) enables targeted gene disruption and would greatly enhance the efficiency of mycoplasma genetic manipulation if it could be made to work. HR-based mutagenesis using suicide vectors has not been successful in most mycoplasmas, but *oriC*-based plasmid has been successfully used for targeted mutagenesis in several species as described previously. One study demonstrated that HR of *oriC* plasmids was able to disrupt a gene in *M. pulmonis*, but the mutant cultures retained a cell population that had an intact wild-type copy of the gene no matter how hard the investigators tried to filter clone the cultures (3). The most probable explanation is that the incompatibility of two *oriC* sequences in one chromosome makes the integration unstable and that a subpopulation of cells exists that has lost the integrated plasmid. Currently, the same group is trying to counter select for the presence of *oriC* such that mutants experiencing a second crossover event can be selected (3, 15). These mutants would contain the disrupted gene but not a second copy of *oriC* and therefore would be more stable. *M. arthritidis* has lower RecA activity than *M. pulmonis*

(unpublished data), which explains the failure of our initial HR-based knockout strategy. RecA overexpression might theoretically be able to enhance HR but is apparently lethal when attempted in *M. pulmonis* (unpublished data). No *oriC* region was recognized in the *M. arthritidis* genome sequence. Because *oriC* function is species specific (9), *oriC* plasmids derived from other species should not work in *M. arthritidis*. As such, the *oriC* of *M. arthritidis* has to be identified independently by using similar methods as described for *M. pulmonis* (3).

Role of MAM in pathogenesis

The role of MAM in modulating host cytokine profiles is clear. However, the cytokine profiles induced by whole *M. arthritidis* organisms have not been studied. Many mycoplasma components especially lipoproteins have significant immunomodulatory activity (7, 10). Therefore, the cytokine profiles in response to infection of *M. arthritidis* are probably different from those induced by purified MAM. The former could be studied by enzyme-linked immunosorbent assay or real-time PCR of cytokine mRNAs. Although MAM affects the cytokine profiles of the host (11), whether these cytokines determine the severity of the infection is not clear. The effect of cytokines in disease severity could be studied in C3H/HeJ mice with cytokine production blocked by antibody or small interference RNAs. If infection of *M. arthritidis* in these mice leads to a less severe disease, the role of cytokines in pathogenesis will be confirmed.

Although MAM is associated with lethal toxicity, systemic injection of purified MAM did not lead to lethal toxicity in mice (13). Therefore, either live organisms need to be present to replenish the protein continuously, or the pathogenic effect of MAM requires other mycoplasmal components. A family of lipoproteins has been predicted

from the genome of *M. arthritidis* (4). They are likely candidate components that act synergistically with MAM. As discussed previously, SAgs can significantly enhance the lethality of endotoxin. Although mycoplasmas do not have LPS, their lipoproteins have classical endotoxic properties with activity comparable to LPS including the stimulation of synthesis and secretion of cytokines (6, 14). Mycoplasmal components involved in lethal toxicity could be discovered with similar methods described later for screening of arthritogenic factors, but higher titers of inocula are required. Another aspect about MAM that deserves further study is its secretion mechanism. Although MAM contains a signal peptide sequence, we failed to identify a type I signal peptidase from the proteins predicted from the *M. arthritidis* genome sequence. Possible ways to study the secretion of MAM will be discussed later in this section. The demonstration of lethal toxicity induced by MAM similar to that induced by other SAgs makes *M. arthritidis* infection of mice a relevant model to test the prevention or treatment of diseases associated with SAgs. A large scale drug screening can be performed and candidate drugs characterized individually for their potency, efficacy, and side effects. The potential application of SAgs in the therapy of tumors has been proposed elsewhere (19). The effect of MAM on tumor progression can be studied by injecting mice bearing tumor implants with purified MAM or attenuated strains of *M. arthritidis*. Due to the different structure of MAM compared to other SAgs, MAM might be efficacious for different types of tumors or could be used as an alternative when tumors become resistant to other SAgs.

Arthritogenic factors in *M. arthritidis*

As a model to study arthritis induced by mycoplasmas and a potential model of human rheumatoid arthritis, factors that are associated with arthritogenicity in *M.*

arthritidis need to be further characterized. Other than MAM, MAA1, MAA2 and MIA are the only proteins that have been studied experimentally. MAA1 and MAA2 are involved in adherence of *M. arthritidis* to host cells (17). Protective immunity can be elicited by them in rats, indicating their role in pathogenesis (18). MIA is another potential virulence factor. Rats infected with *M. arthritidis* produce abundant antibodies against MIA. A shorter form of MIA is produced by the more arthritogenic strain 158, a longer form of MIA is produced by the less arthritogenic strain 158-1 (2). With the completion of *M. arthrititis* genome sequence, several other potential virulence factors are revealed. Examples include a peptide methionine sulfoxide reductase (MsrA), which is required for full virulence in *M. genitalium*, and a potential hemolysin (4).

The availability of a robust transposon library in *M. arthritidis* facilitates high through-put screening of virulence factors. The screening of virulence factors in vivo is aided by the clear presentation of the disease phenotype in rats (2). Unlike disease in mice, rats develop severe arthritis which is acute for about a 2-week period following postinoculation. The rapid disease presentation would facilitate quick screening of the library. In *M. arthritidis*-infected rats, the swelling of involved joints is readily visible. The changes in joint circumference can be measured with calipers. The loss of mobility can be scored consistently. The weight of the infected rats correlates well with the severity of arthritis as virulent strains are always associated with prominent weight loss. Therefore, histopathological studies are unneeded to gauge virulence unless ambiguous results occur.

To screen for virulence factors of *M. arthritidis*, rats could be systematically infected with mutants of *M. arthritidis* by tail infection. All the parameters mentioned above

would be measured and arthritogenicity compared to the wild-type *M. arthritidis* strain. Mutants that have reduced arthritogenicity are expected to be identified in some library members. Although relatively unlikely, mutants with enhanced arthritogenicity are also possible. The arthritogenicity of these mutants and of complemented mutants can be further studied in larger groups of animals. Due to the complexity of pathogenesis, it is likely that multiple factors with unrelated functions will be identified as involved in pathogenesis. Virulence factors that have been confirmed by animal experimentation could be examined further to reveal the mechanism by which they are associated with arthritogenicity.

Previous unpublished studies demonstrated that the low virulence of *M. arthritidis* strain 158-1 was associated with rapid clearing during the first few hours of infection. Because macrophages have important roles in protection of murine animals from infection by *M. pulmonis* (8), possibly they are important in the clearance of *M. arthritidis*. Therefore, the ability of macrophages to eliminate *M. arthritidis* strain 158-1 and its parental strain 158 should be evaluated. A significant level of killing of 158-1 would indicate that the mutant has a defect in antiphagocytosis. Similarly, the effect of other immune components in clearance can be studied and their roles confirmed in mice that are deficient in these components. Although MIA is suspected to be responsible for the difference in arthritogenicity of 158 and 158-1, its pathogenic importance has not been confirmed. With the expansion of the transposon library and progress in HR-based methods of mutagenesis, it may be possible to get a mutant with a disrupted *mia* gene. Also, the whole genome of 158-1 can be sequenced and the role of any mutations that are detected can be studied.

Glycosidases

The full spectrum of substrates for the MYPU_4630 glycosidase is unknown. As discussed previously, the enzyme can likely remove glycomoieties from host proteins such as mucin and heparan sulfate. Mucin or heparan sulfate could be co-incubated with wild-type *M. pulmonis* or a mutant in which the MYPU_4630 gene has been disrupted, and the monosaccharides released studied by thin layer chromatography. Alternatively, hydrolysis of terminal GlcNAc could be studied by electrophoresis of the proteins after co-incubation with the glycosidase followed by lectin staining. Although we demonstrated GlcNAc is a component of *M. pulmonis* EPS-II, we do not know whether the GlcNAc in EPS-II is α-linked and would be a substrate of the MYPU_4630 glycosidase. To examine this possibility, the wild-type *M. pulmonis* could be treated with purified MYPU_4630 enzyme and the EPS examined by lectin staining. If the EPS is indeed a substrate, the lectin binding will be decreased or eliminated. Similarly, treating a biofilm of *M. pulmonis* with purified MYPU_4630 protein may cause dissolution of the biofilm due to the degradation of EPS.

Mutants in each of the five predicted glycosidases have been identified from the transposon library of *M. pulmonis*. More than one mutant is available for each glycosidase, which provides a way to study their enzymatic specificity by identifying substrates that can be hydrolyzed by the wild-type strain but not a mutant. If the enzymatic activity for the glycosidase is too low to detect, a recombinant protein could be expressed in *E. coli* and purified protein used for the above analysis. The cellular localization or secretion of the glycosidases could be studied by using a strategy similar to the one used here for the MYPU_4630 protein. The demonstration of MYPU_4630 as

a secreted protein suggests the reliability of SecP scores as a predictor of non-classical secreted proteins. The other *M. pulmonis* glycosidases have even higher SecP scores, indicating they are secreted proteins. The enzymatic targets of these glycosidases could be studied by using a strategy similar to that described above for MYPU_4630.

The role of glycosidases in virulence could be studied by comparing the pathogenic effect of the mutants with wild-type *M. pulmonis*. The presence of several glycosidases suggests that any single glycosidase might not have a significant impact on virulence. The study of the importance of these glycosidases in virulence might require the development of double or triple knockout mutants in which multiple glycosidases are absent. Improvement of homologous recombination methods of mutagenesis will facilitate this process. Additionally, glycosidases in other mycoplasmas can also be studied with similar strategies.

Secreted proteins

Many secreted proteins in bacteria are important for virulence (16) but have not been studied in mycoplasmas. To study the secreted proteins of *M. pulmonis* and *M. arthritidis*, all of the proteins present in the culture supernatant could be characterized with two-dimensional SDS-PAGE gel electrophoresis stained with sensitive dyes followed by mass spectrometric analysis. The secretion of individual proteins could be confirmed by using the fusion protein strategy taken for MYPU_4630. The secretion mechanisms for MAM in *M. arthritidis* and the glycosidases in *M. pulmonis* could be studied by screening the transposon library for mutants that fail to secrete these proteins. Mutants with disruptions in genes coding for ABC transporters will be given high priority. These studies would not only shed light on the understanding of pathogenesis, they might also lead to the

discovery of novel protein secretory pathways and give insight into the secretion mechanisms of non-classical secreted proteins in other bacteria.

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APPENDIX

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) APPROVAL FOR ANIMAL STUDIES

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

DATE: June 16, 2009

TO: Dybvig, Kevin F.
KAUL-720A 0024
934-9327

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: Mechanisms of Mycoplasma-Induced Arthritis
Sponsor: NIH
Animal Project Number: 090607530

On June 16, 2009, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

Species	Use Category	Number in Category
Mice	C	140
Mice	A	20

Animal use is scheduled for review one year from June 2009. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 090607530 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.

Institutional Animal Care and Use Committee
B10 Volker Hall
1670 University Boulevard
205.934.7692
FAX 205.934.1188

Mailing Address:
VH B10
1530 3RD AVE S
BIRMINGHAM AL 35294-0019