GENETIC AND BIOCHEMICAL ANALYSIS OF CYTADHERENCE-RELATED OPERONS IN *MYCOPLASMA PNEUMONIAE*

by

ROBERT HENDREN WALDO, III

(Under the Direction of Duncan Charles Krause)

ABSTRACT

Cytadherence or attachment to host tissue is a crucial event in the pathogenesis of infections by *Mycoplasma pneumoniae*, a common agent of respiratory disease in humans. A large group of cytadherence-accessory proteins work together to localize the major adhesin P1 to the polar attachment organelle in a functional form. The *hmw* gene cluster, which contains the genes encoding cytadherence-accessory proteins P30, HMW1, and HMW3 in addition to other possible cytadherence proteins, was found to be transcribed from four different promoters in a series of overlapping transcripts that may impact the regulation of genes in the cluster. In another cluster, the *p1* operon, genes MPN140-142 were transcribed as polycistronic message. Recombinant genes of the *p1* operon encoding the P1 adhesin and the proteins B and C were delivered and expressed in non-adherent mutants lacking some or all of these proteins. Some but not all translation of the gene encoding B/C (MPN142) was linked to the translation of the gene encoding P1 (MPN141). Furthermore, in the absence of P1, B/C were unstable and little or no protein was detected. This linkage of P1/B/C at transcription, translation, and protein stability sheds light on relationships between P1/B/C on the genetic and

biochemical level and supports the view of these three proteins as integral members of a single major adhesin complex.

INDEX WORDS: *Mycoplasma pneumoniae*, Atypical pneumonia, P1, A, B, C, P90, P40, HMW1, HMW3, P30, Mutant I-2, Mutant II-3, Mutant III-4, Mutant IV-22, Bacteria, Prokaryotes, Adhesins, Cytadherence, Cytadherence-accessory proteins, Genetics, Gene regulation, Promoter, RNA, Transcription, Translational coupling, Operon, Gene cluster, Protein stability, Complementation, Chloramphenicol acetyltransferase, Reverse transcriptase-polymerase chain reaction, Primer extension, Western/Immuno-blot, Reporter fusion, Hemadsorption, Immuno-fluorescence microscopy.

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DEDICATION

This dissertation is dedicated to my family, Bob, Ruth, and Brett. Without their love, support, and understanding, all my work would have been insignificant if not impossible.

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INTRODUCTION

Cytadherence in the human pathogen *Mycoplasma pneumoniae* is a critical virulence factor in the infectious lifestyle of this bacterium. Previous work has shown a host of accessory proteins are important in allowing adhesin proteins to localize correctly in the cell and function properly. The genes encoding the major adhesin P1 and many of these cytadherence-accessory proteins are located in three operon-like structures on the *M. pneumoniae* chromosome. While the biochemical, cell biological, and immunological properties of these proteins are better known, genetic analysis of these operons has been hampered by the difficulty of working with *M. pneumoniae* and the lack of experimental tools. Recent developments in techniques for studying *M. pneumoniae* genetics and gene expression, some of which are presented in this work, have enabled a better understanding of the organization and expression of these operons relative to the function of their products.

After a review in Chapter 1 of the previous literature pertinent to this work, Chapter 2 deals with the transcription of the *hmw* gene cluster. This large operon-like group of genes has four transcriptional promoters distributed within it, and these promoters have identical eight nucleotide sequences directly upstream of the +1 site in a position analogous to the -10 Pribnow Box (2). The genes are transcribed in overlapping mRNAs, however the promoters do not drive the

expression of all downstream genes equally. This is shown using mutant complementation and, for the first time in *M. pneumoniae*, chloramphenicol acetyltransferase (*cat*) reporter fusions. The nature of this overlapping transcription may have important implications for the regulation of genes in the gene cluster in this organism, which lacks much in the way of other apparent regulatory mechanisms. Finally, it is shown that the eight-nucleotide sequence (TTAAAATT) is found upstream of open reading frames at a frequency much higher than expected, and a comparison of the promoter regions in the *hmw* gene cluster and all other previously experimentally determined *M. pneumoniae* promoters show that while a -10 region is loosely conserved, no similarities in the -35 region could be detected.

Chapter 3 discusses the requirement of the accessory proteins B and C for cytadherence and the nature of MPN142, the gene encoding them. MPN142 is part of the so-called *p1* operon (1), which contains the gene for the major adhesin P1 (MPN141) and a third gene of uncertain function. The genetic defect in a spontaneous cytadherence negative mutant (III-4) is mapped to the MPN142 gene, and the defect is complemented by selecting for a spontaneous cytadherence positive revertant and by the delivery of recombinant MPN142 in an expressible context.

Continuing in the *p1* operon, Chapter 4 shows how the gene for the P1 adhesin and the downstream gene encoding B/C are linked at the levels of transcription, translation, and even protein stability. This, along with previous data, indicates that the proteins are closely linked in function and likely act as

subunits of the actual adhesin complex. Reverse transcriptase polymerase chain reaction (RT-PCR) experiments using wild-type mRNA show that the three genes (MPN140-142) are expressed as a transcriptional unit and likely constitute an operon. Recombinant MPN141 (P1) and MPN142 (B/C) are delivered to a spontaneous cytadherence negative mutant (IV-22) which lacks the proteins P1, B, and C, restoring the levels of these proteins. I show that the lack of B and C in mutant IV-22 is due both to a polar effect resulting from the MPN141 mutation in MPN141, which disrupts translational coupling, and the dependence of B and C stability on the presence of P1.

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CHAPTER 1

REVIEW OF THE LITERATURE

Mycoplasma History and General Characteristics

The bacteria of the genus *Mycoplasma* (trivial name: mycoplasmas) and their close relatives are largely characterized by lack of a cell wall. In spite of this deficiency, the shapes of these cells often conform to one of several possibilities with varying degrees of intricacy. For example, the members of the genus *Spiroplasma* assume an elongated helical shape without the aid of a rigid structural cell envelope. These cell shapes presumably contribute to the ability of mycoplasmas to thrive in their respective environments. *Mycoplasma pneumoniae* cells possess an extended 'arm' protruding from a coccoid cell body, which is involved in the attachment of this pathogenic bacterium to the tissue of its human host, in movement along solid surfaces, and in cell division. *M. pneumoniae* cells are of small size and somewhat pleomorphic, but with a rough shape in longitudinal cross-section resembling that of a round-bottomed flask.

Mycoplasmas are extremely unusual among bacteria in that most require sterols for the stability of their cytoplasmic membrane. Sterols are acquired from the environment, usually as cholesterol from the animal host. Mycoplasmas also generally possess a relatively small genome reflecting their drastically reduced biosynthetic capabilities and parasitic lifestyle, with a low mol %G+C ranging from 18-40 %. This is

coupled with the use of an alternate genetic code where the codon UGA is preferred to encode the amino acid tryptophan instead of the usual opal stop.

In 1898 Nocard and Roux reported the cultivation of the causative agent of contagious bovine pleuropneumonia (CBPP), at the time a grave disease in agriculture and today a concern of cattle ranchers particularly in Africa and Southern Europe, and of customs officials elsewhere. The disease is caused by *Mycoplasma mycoides* subsp. *mycoides* SC (small-colony type), and the work of Nocard and Roux represented the first isolation of a mycoplasma. Its culture was difficult because of the complex growth requirements. These researchers succeeded by inoculating a semi-permeable pouch of sterile medium with pulmonary fluid from an infected animal and depositing this pouch intraperitoneally into a live rabbit. After fifteen to twenty days, the recovered pouch had an opacity that an uninoculated control lacked. This turbid broth could then be used to inoculate a second and third round and subsequently introduced into a healthy animal, causing disease. However this did not work if the material was heated, indicating a biological agent at work. Uninoculated media in the pouch, after removal from the rabbit, could be used to grow the organism *in vitro*, demonstrating cell-free culture and ruling out viral causes, although this was not fully appreciated at the time (119). The name *Mycoplasma*, from the Greek *mykes* (fungus) and *plasma* (formed), was proposed in the 1950's, replacing the term pleuropneumonia-like organisms (PPLO) referring to organisms similar to the causative agent of CBPP (41). It was later found that the fungus-like growth pattern of *M. mycodies* is unique to that species.

This confusion about mycoplasmas and virus would surface again 50 years later when Eaton and colleagues cultured the causative agent of human primary atypical

pneumonia (PAP) or 'walking pneumonia.' This agent could be grown in chicken embryos and passed through a filter that excluded normal bacteria, but could not be observed by the high magnification light microscopy of the day, and caused disease that could not be treated with the popular antimicrobials sulphonamides and penicillin (39). Eaton did consider the possibility the disease was caused by a mycoplasma, but the agent did not grow on the standard PPLO media of the time. These observations led to the conclusion that PAP had a viral etiology. Research at that time showed the cultured agent could induce disease in experimentally infected cotton rats and hamsters. In spite of controversy at the time about whether the researchers had truly isolated the causative agent of PAP (based largely on the unusual immunological response of patients with PAP), in retrospect their evidence along with that of colleagues and competitors appears to have been quite conclusive (109). In the early 1960's, there were reports linking Eaton's Agent to the PPLOs or mycoplasmas, well known then as parasites of cattle and rodents, using sensitivity to antimicrobial compounds (i.e. organic gold salt) (110). The ability to grow Eaton's Agent, now known as *Mycoplasma pneumoniae,* in cell free media allowed an explosion of research into what had overnight become the most medically important mycoplasma (from a human-centric perspective) and what was to become the most studied mycoplasma.

Recent advances in molecular biology and genomics have brought the genetically simple mycoplasmas, particularly *M. pneumoniae* and its close relative *Mycoplasma genitalium,* to a larger audience. The second published complete bacterial genome sequence was that of *M. genitalium,* which has the smallest genome of any free-living organism (50). The *M. pneumoniae* genome sequence was published soon

after and was the first genome sequence determined by primer walking of a cosmid library instead of the whole-genome shotgun method (61). Mycoplasma genomics and proteomics continue in efforts to understand the so-called minimal cell (66), catalog the entire protein content of a cell (132), and generally continue to take advantage of the small genome of these organisms to understand broad biological concepts.

Mycoplasma Taxonomy and Phylogeny

The medical and agricultural importance of members of the genus *Mycoplasma* and related genera has led to the extensive cataloging of many of these organisms by culture, serology, and small subunit ribosomal RNA gene and whole genome sequencing. A recent focus in the sub-discipline of molecular phylogenetics has both clarified and confused certain aspects of the organization of the class *Mollicutes*, and while a truce of sorts has been reached, the area is still somewhat of a moving target (81).

The name *mollicutes* is derived from the Latin *mollis* (soft) and *cutes* (skin), and all of these bacteria do lack a cell wall and the genetic capability to synthesize peptidoglycan. While the trivial name 'mycoplasmas' has commonly denoted all members of this class, this usage is somewhat imprecise and will not be used as such here. Despite the lack of a cell wall, *Mycoplasma* and relatives have been classified into the phylum *Firmicutes* consisting of low G+C Gram-positive bacteria such as *Clostridium*, based on 16S rRNA gene analysis. The cultured members of *Mollicutes* are currently arranged into four orders: *Acholeplasmatales*, *Anaeroplasmatales*, *Entomoplasmatales*, and *Mycoplasmatales*. The order *Mycoplasmatales* contains a

single family, *Mycoplasmataceae,* which contains two genera: *Mycoplasma* and *Ureaplasma*. Historically, the description of a bacterium lacking a cell wall was sufficient to classify it to the genus *Mycoplasma* and as such it is the oldest and largest genus of the class with about half of the class' species (107 validly described) each usually limited to a specific host and with many hosts harboring more than one species, some pathogenic and some commensal. In later studies, many of these species were found to be phylogenetically distributed among at least three separate orders. In fact, the type species, *Mycoplasma mycoides* would rightly be classified with the genus *Spiroplasma* in the order *Entomoplasmatales*. This and other discrepancies will likely remain unresolved because of the extreme confusion that change could engender among the medical and agricultural communities. The medically important genus *Ureaplasma* consists of seven described species that appear to be largely *Mycoplasma* with the addition of a urease activity. The bulk of the species in the genus *Mycoplasma* are divided into two non-taxonomic groups based on 16S rRNA gene sequences, hominis and pneumoniae. The hominis group contains the phylogenetic clusters of *Mycoplasma bovis*, *M. pulmonis,* and *M. hominis* among others. The pneumoniae group contains the clusters of *M. muris*, *M. fastidiosum, Ureaplasma urealyticum*, the uncultured Haemotrophic mollicutes, haemoplasmas (formally *Haemobartonella*), and the *M. pneumoniae* cluster. This cluster contains the species (and the usual or likely host) *M. alvi* (bovine)*, M. gallisepticum* (avian)*, M. genitalium* (human)*, M. imitans* (avian)*, M. pirum* (uncertain/human)*, M. testudinis* (tortoises), and *M. pneumoniae* (human). Most if not all of these species share some otherwise unique characteristics

including an attachment organelle, homologs of the *M. pneumoniae* cytadherence-accessory proteins, and specialized modifications of the cell-division apparatus.

A detailed analysis of the 16S rRNA genes from the order *Mollicutes* by Maniloff has given rise to a view of the evolution of these bacteria that includes an estimate of the time-scale for the emergence of some groups or features (108). This analysis suggests that about 600 million years ago (MYA), late in the Proterozoic era, *Mollicutes* branched away from the low G+C Gram-positive ancestor of the streptococci, losing their cell wall. At this time on Earth, molecular oxygen was present in the atmosphere at 1%, and the fossil record shows that multicellular marine animals had recently spread in the Cambrian explosion. One hundred million years later the requirement for sterols in the cytoplasmic membrane evolved along with the change to the alternate genetic code. Also, the ancestor of the the genera *Spiroplasma* and *Entomoplasma* (primarily plant and insect pathogens) and *Mycoplasma* emerged at this time and would itself diverge into the *Spiroplasma-Entomoplasma* and *Mycoplasma* lineages approximately 100 million years after that. This diversity coincided with the origin of land plants 500 MYA. It appears that the calculated rate of evolution for the *Mycoplasma* group increased several fold about 190 MYA, soon after the appearance of vertebrates, while the *Spiroplasama-Entomoplasma* ancestor continued to evolve at the previously shared slower rate until about 100 MYA, when angiosperms and their associated pollinating insects appeared. Then the evolution rate of these bacteria appears to have also increased significantly. This is an attractive hypothesis, but while it tracks the emergence of several of the unusual characteristics of *Mycoplasma* and related organisms, it does not address the selective pressures driving their evolution, except

perhaps the widespread close association of a parasite with a specific host. The advantages of a reduced genome, cell wall-less structure, and alternate genetic code remain murky.

Mycoplasma pneumoniae Disease and Epidemiology

M. pneumoniae is a common agent of community acquired respiratory infections, however the only certainty regarding *M. pneumoniae* disease is that the cost and difficulty of culture and diagnosis combined with its paradoxically chronic and self-limiting nature, make the reliable quantification of incidence impossible. Combinations of serology, culture, molecular diagnosis, and other methods have been used in several studies to estimate the incidence in the population, and the following descriptions can only be a summary of these imprecise results.

Atypical pneumonia: Three percent of *M. pneumoniae* infections result in pneumonia while the vast majority (approximately 77%) are less severe (e.g. tracheobronchitis, pharyngitis, etc.) and 20% result in an asymptomatic infection (24). Also, a variety of other pulmonary conditions such as pleuritis, asthma, and acute respiratory distress syndrome (ARDS) are thought to be occasionally caused or exacerbated by *M. pneumoniae* infection. Mycoplasma-associated pneumonias differ from those with various viral and bacterial etiologies in the milder symptoms and the protracted course of the disease. Typical pneumonias are characterized by an abrupt onset, often with a productive cough, fever, chest pain and neck stiffness. Atypical,

mycoplasma-like pneumonias, generally have a chronic onset with flulike symptoms including aches, chills, and a dry cough.

This distinct pneumonia was first recognized in the late 1930s and was later termed atypical or primary atypical pneumonia (PAP). In military circles, where the contagious nature of the disease amongst troops quartered in barracks spurred much of the early research, the name 'walking' pneumonia became popular, reflecting the milder symptoms compared to classical bacterial pneumonia. *M. pneumoniae* causes most cases of atypical pneumonia, but other agents have since been recognized including *Chlamydia pneumoniae*, *Legionella pneumophila*, and other bacterial as well as viral and fungal sources.

While the less severe upper respiratory tract *M. pneumoniae* infections are more common, much more is known about mycoplasma pneumonia. This is a result of increased clinical interest in the more severe disease combined with the fact that persons suffering from the more common diseases are less likely to consult a physician, so less data are collected. *M. pneumoniae* infections generally have an incubation period of 2-3 weeks after exposure before the onset of the acute phase of the disease, which lasts for more than two weeks to as much as a month. Early in disease, fever (96-100% incidence) and sore throat (53-71%) develop and resolve within 1-2 weeks. Throughout the disease and into convalescence, dry cough (93-100%), malaise (74-89%), and headache (60-84%) exist. Other symptoms include chills (58-71%) and chest discomfort (42-69%). These symptoms all have a slow onset of several days to a week and vary in severity and occurrence. The disease is usually mild and self-limiting, however mortality rates of up to 1.4% are reported (78).

Non-respiratory disease, sequelae and the chronic nature of infection:

Early on, PAP was associated with the frequent development of so-called cold hemagglutinins, which react with host tissue at 4°C and were found to be IgM antibodies against the I antigen of erythrocytes (80). The cold hemagglutinins are one of a range of observed anti-host immune responses to *M. pneumoniae* infections. The disease is self-limiting and successfully treated with appropriate antibiotics (24). However, puzzling evidence exists that the organism is sometimes not cleared from the host by the immune response or drugs, and bacteria can be cultured from these convalescent patients for as much as 20 days after treatment begins (140). Proposed explanations include the bacteria entering a dormant phase, their sequestration to an immune-privileged or drug-inaccessible site and/or intracellular location, and the ability to withstand bacteriostatic antimicrobials while not causing overt respiratory disease (13).

These observations, the anti-host immune response and a persistent subacute infection, impact our understanding of the wide variety of non-respiratory sequelae seen alongside *M. pneumoniae* infection. Through epidemiology, serology, culture, and molecular techniques (e.g. PCR), *M. pneumoniae* has been linked to disease and inflammation of tissues as diverse as dermal, cardiac, hepatic, renal, synovial joints, blood, and central nervous. Such connections are difficult to prove and must be considered tenuous without stronger evidence, however the possibility is intriguing and logical considering similar possible associations between organisms such as *Chlamydia pneumoniae* and conditions like heart disease. While reliable numbers are not available, these extrapulmonary complications seem far from rare. Existing studies have mostly relied on serologically diagnosed *M. pneumoniae* infections in hospitalized

patients. These show that 6-7% of cases have neurological complications, 14% have joint pain, 1-8.5% cardiovascular related complications, and 25% dermatologic abnormalities in addition to rarer disease in other organ systems (23, 23, 151). Their nature is thought to be either the result of an inappropriate autoimmune response on the part of the host or from a poorly understood traversal of the bacteria from the respiratory tract to other tissue. There is inconclusive evidence (14, 17, 72, 118, 150, 156) supporting both possibilities, and although the autoimmune etiology is favored, some combination of events may be responsible and the cause of one condition (e.g. arthritis) may be distinct from another (e.g. meningitis) (45).

Two significant diseases with likely *M. pneumoniae* etiologies (amongst other biological and non-biological causes) are Guillain-Barré Syndrome (GBS) and severe chronic allergic asthma. GBS is an acute peripheral neuropathy often thought to be the result of an improper immune response to several possible infectious agents (53). The similarity of some *M. pneumoniae* proteins to host proteins may be the source of the inappropriate immune response (molecular mimicry). Possible host targets include galactocerebroside (GalC) and the GM1 moiety (150). *M. pneumoniae* infection has been linked to 5-6% of GBS cases, ranking as the third or fourth most common known cause (71, 120). The picture for the development and/or exacerbation of allergic asthma is currently more complicated. Interplay of genetic predisposition, age at the time of infection, and course of disease (treated with antibiotic, naturally cleared, etc.) sometimes give rise to the increased Th2/IgE response associated with asthma. One study showed 10% of children with *M. pneumoniae* respiratory disease later developed asthma symptoms (115) and another report found 45% of studied adult patients with

chronic stable asthma were PCR positive for *M. pneumoniae* (42). In a more mundane fashion, it seems *M. pneumoniae* can also cause a urogenital infection (52) just as the closely related *M. genitalium* can cause a respiratory infection (10) although these organisms are most commonly found as parasites of their favored epithelial surfaces.

Immunity: In order to infect a naïve host, *M. pneumoniae* must first bypass the nonspecific defenses of the respiratory system. Mucus presents a formidable barrier to colonization. It is thought that cytadherence (described in detail elsewhere) and gliding motility allow *M. pneumoniae* to overcome normal mucociliary clearance and progress deeper into the lung (98). *M. pneumoniae* generates a strong specific immune response in healthy individuals. This response can lead to clearing the organism and a self-limiting disease, or chronic autoimmune disease in different cases by mechanisms that are not well understood (19). *M. pneumoniae* can also evade the immune system and establish a persistent infection (130).

Specific defenses against *M. pneumoniae* infection include an increase in reactive antibodies of the IgM, IgG, and particularly of the secretory IgA classes (64). *M. pneumoniae* is susceptible to killing by complement activated by the classical pathway (22). Infected persons generate antibodies against bacterial lipids (129), polysaccharides (1), and several surface proteins necessary for virulence, especially the major adhesin P1. Interestingly, patients develop a strong response to the P1 protein, however these antibodies are normally not directed against the receptor binding regions of the protein. Antibodies against these regions may be selected against by a healthy immune system because of similarity to self proteins (i.e. molecular mimicry) (72, 76).

IgM antibodies appear first, within two weeks after infection. The IgG and then IgA classes follow three to eight weeks post infection (16, 18, 44, 64).

Pathogenic mycoplasmas generally induce strong cytokine responses (130). *M. pneumoniae* infection induces IL-1β, IL-6, TNF-α, and INF-γ among others (90) and generates inflammation and the recruitment of professional phagocytic cells. This response is important for combating the infection, but it is also a major contributor to the disease itself.

Diagnosis: PAP is often diagnosed solely by the symptoms and perhaps the results of a chest X-ray, then treated indiscriminately with antibiotics. As the disease thus treated commonly clears and differential diagnosis is difficult and not readily available, it is largely not done. This has led to a lack of understanding of the extent and importance of *M. pneumoniae* disease.

Because a virus was initially suspected as the cause of PAP, serological testing has been very important in the diagnosis of *M. pneumoniae* infections. Complement fixation (CF) assays in particular have been used historically, with a four-fold increase in antibody titer showing a recent infection. CF is not widely used now, but several other serological tests exist (158). Serology is often not useful because of the requirement for acute in addition to convalescent sera and the variability or lack of immune responses between patients. Enzyme immunoassays (EIA) are used to detect IgM in the serum of patients with acute disease, but not all these develop a strong IgM response, leading to false negatives (46). These results may be explained in part by the observation that the immune response to re-infection differs from that to primary infection (137).

Culture has not been routinely used except in epidemiological studies because of the expensive and complex media and the time requirements (several weeks are often needed to perform primary culture). Few diagnostic labs have the expertise to culture *M. pneumoniae* reliably from clinical samples.

Genetic identification using PCR-based methods is quickly growing in popularity and reliability because of the ease and specificity combined with a rapid turnaround time. Strain identification in particular has benefited from these techniques. Random amplified polymorphic DNA (RAPD), amplified-fragment length polymorphism (AFLP), and amplification of the 16S ribosomal RNA gene or the gene encoding the major adhesin P1 with or without sequencing the PCR product have all been used successfully, and these techniques continue to be refined and expanded. Clinical samples from nasopharyngeal aspirates or pharyngeal swabs have been found to be convenient and accurate for the detection of *M. pneumoniae*.

PCR-based methods show a greater sensitivity than serological or culture techniques. The resulting increase of visible incidence may be real, but the possibility exists of dormant or dead organisms or those in an asymptomatic carrier state being detected. Therefore, a combined method of PCR and serology is often used (46). Overall, a better understanding of the role of *M. pneumoniae* in human disease depends on increased surveillance and improved diagnostic tools.

Treatment: As suitable diagnostic tests are not available, *M. pneumoniae* disease is most often treated empirically. Even severe respiratory disease in otherwise healthy patients is often self-limiting, and in these cases, untreated symptoms such as

fever, headache, and malaise clear in about 10 days. Antibiotic treatment is thought to shorten this time. Persons with the more common milder respiratory disease often forego medical intervention altogether. In the case of chronic or extrapulmonary disease, antibiotics become more important.

The bacteria seem to respond well to correctly selected antibiotics for the most part, and while resistance is seen *in vitro*, for atypical pneumonia (i.e. not streptococcal) it is not currently viewed as an imminent problem (158). The cell wall-less mycoplasmas are of course resistant to β-lactams and other antimicrobials that target the cell wall. All mycoplasmas are also resistant to polymyxins, sulfonamides, trimethoprim, nalidixic acid, and rifampin apparently due largely to differences in the mycoplasmal versions of the targets of these drugs (15). The important human pathogens *M. pneumoniae* and *U. urealyticum,* but not *M. hominis,* are also resistant to the newer drug linezolid (85).

M. pneumoniae is sensitive to tetracyclines, and although the *tetM* resistance marker on Tn*916* has been seen in other *Mycoplasma* and *Ureaplasma* species, this has not been found in *M. pneumoniae* (37). Tetracycline and doxycycline are frequently used to treat suspected *M. pneumoniae* infections in adults. Mycoplasmas are generally very sensitive to macrolides, and erythromycin, clarithomycin, and azithromycin are used particularly in children, for whom tetracyclines are not recommended. Then newer drugs clarithomycin and azithromycin are preferred because of their greater tolerability and favorable dosing requirements (158).

Mycoplasmas are notorious for their ability to survive in the presence of bacteriostatic antimicrobials for long periods of time (e.g. in cell culture). In the normal

course of disease, it is thought that treatment with antimicrobials simply allows increased clearing and eradication of the bacteria by the host immune system. This is supported by the sometimes chronic nature of even treated disease and the great difficulty of treating PAP in immunocompromised patients (158). Recent clinical studies showing the efficacy of some quinolones have been prompted by the hope they would prove bacteriocidal *in vivo* (15, 46, 158).

Epidemiology: *M. pneumoniae* infections are thought to occur in all locales and at all times of year, however increased incidence is seen in temperate areas and during late summer and early fall. Epidemics of disease occur on approximately a four-year cycle. Because of the community acquired nature of the disease it is difficult to separate effects due to the nature of the bacterium from those deriving from the behavior of the susceptible population. *M. pneumoniae* disease is often thought of as significant only in children and young adults, but it is now recognized that all individuals who spend extended periods of time enclosed with others who may harbor the bacterium are at risk (46, 152).

Disease incidence is difficult to gauge using data from previous studies, as many different diagnostic methods were used and were performed in different countries and populations. However it seems that non-epidemic years have an incidence around 2% of indication of the organism's presence (not necessarily disease) and rising to between 10-35% during epidemics (46). Children age 3-14 were most commonly found positive, and another incidence peak in adults between 25 and 40 years of age (105). *M. pneumoniae* is associated with 3-18% of all pneumonias requiring hospitalization and is

of serious medical and economic importance (107). Mild respiratory infections by *M. pneumoniae* are even more common than full-blown pneumonias, but their frequency is difficult to measure and seem to be only a small fraction of total upper respiratory disease (152). It is hoped that recent advances in the speed and sensitivity of detection alongside increased appreciation for *M. pneumoniae's* significance will spur more research into the extent of these diseases.

Pathogenesis: *M. pneumoniae* must colonize host epithelium to initiate respiratory disease, and mutants unable to bind to host tissue do not cause significant disease in animal models (96). The bacteria are spread by droplet inhalation from infected people (i.e. coughing), then must bypass innate host defenses such as mucus, ciliary clearance, and lactoferrin. As a common agent of community acquired pneumonias (CAPs), *M. pneumoniae* infection is often the result of prolonged, repeated exposure in confined spaces (48, 144). This is exemplified by the incidence of PAP in boarding schools, dormitories, and barracks. The nature of this transmission suggests that *M. pneumoniae* (and other agents of CAP) is not particularly good at overcoming respiratory defenses, but succeeds by inducing symptoms that encourage multiple exposures within a community. However, contrary to this is the observation that about 50% of experimentally infected humans contract disease upon a single exposure (27, 139), but the dose received in these experiments is likely much greater than that of a natural exposure.

Several experimental systems have been and continue to be developed in order to study *M. pneumoniae* pathogenesis. Humans are the only known natural host for this

bacterium, and the most appropriate subjects. However, obvious ethical and financial issues limit the experiments that are possible. Roughly in order of increasing cost and applicability, useful models for *M. pneumoniae* pathogenesis (especially attachment) include: inert surfaces (i.e. glass and plastic) (154), erythrocytes (141), transformed human cell lines, chick embryo (40), animal organ culture, rodents (38), chimpanzees (8), differentiated human tissue culture (28), and human volunteers (26). These are in addition to studying related animal mycoplasmas in their natural hosts (i.e. *M. gallisepticum* in poultry). Through careful use of and corroboration between appropriate models, researchers have gained understanding of the nature and mechanisms of *M. pneumoniae* disease.

The ability to form a direct and intimate attachment to host tissue (cytadherence) is of primary importance in *M. pneumoniae* pathogenesis. Mutants in adhesin proteins or the so-called cytadherence-accessory proteins show reduced attachment and no pathology in experimentally infected hamsters (96). The bacteria bind to neuraminidase-sensitive sialoglycoproteins and sulfated glycolipids present on respiratory epithelium and other host cells as well (128). Attachment is not limited to moieties on the epithelial surface, as some mycoplasma proteins bind to matrix proteins such as fibronectin (29) and to mucin (2). In addition to attachment, *M. pneumoniae* exhibits gliding motility, or movement along solid surfaces without the aid of flagella (89). In the course of disease, the bacteria must travel down the respiratory tract and pierce the mucus layer before entrance to the periciliary fluid and attaching to the epithelial surface. Gliding motility is likely crucial for passage to and persistence in deep lung tissue.

No conventional bacterial toxins have been recognized in *M. pneumoniae* (61). Reactive oxygen species (ROS) including hydrogen peroxide have been identified as responsible for the hemolytic activity of colonies on agar (25, 142). *M. pneumoniae* produces these ROS in the course of normal metabolism. While they lack the Krebs cycle and utilize glucose solely by the Embden-Meyerhof-Parnas pathway, *M. pneumoniae* grow extremely poorly if at all in anaerobic conditions (personal observation). As the bacteria form an intimate association with the host tissue, molecular oxygen, used as an electron acceptor, though not via oxidative phosphorylation, forms ROS that diffuse to the host epithelium, where they may contribute to host tissue damage and ciliostasis. *M. pneumoniae* also elicits an inflammatory response at the site of infection, recruiting macrophages and neutrophils, causing tissue damage. The damage to host tissue disrupts normal mucociliary clearance and presumably releases nutrients from the host cells, perhaps contributing to nutrient acquisition.

It has long been dogma that surface parasitism by *M. pneumoniae* is necessary and sufficient to cause serious respiratory disease in otherwise healthy individuals. However, because of the frequency of extrapulmonary and/or chronic disease, researchers have sought mechanisms by which the bacteria could traverse to distant tissues and persist in the presence of an immune response and antibiotic treatment. Other mycoplasma species seem to be able to exist inside host cells (106, 153), and it seems reasonable that *M. pneumoniae* might have an intracellular niche in some cases or stages of disease. The difficulty in culturing and directly observing mycoplasmas limits experiments using applicable models or even clinical

samples. To date, while intriguing *in vitro* data exist supporting this hypothesis (11, 112, 165), more research is necessary to define the importance of an intracellular role for *M. pneumoniae*.

M. pneumoniae Molecular Biology and Genetics

The *M. pneumoniae* genome consists of a single chromosome of 816,394 base pairs. Interestingly this was the first of a select number of organisms whose genome was sequenced not by the common random-shotgun method but by primer walking a cosmid library. The original and subsequent annotations of the genome predict 42 RNA genes and 688 open reading frames, although some of these are clearly psuedogenes. Limited mechanisms for DNA repair have been found in *M. pneumoniae* but include the components for excision repair and a portion of the SOS pathway. It is thought that the absence of other mechanisms may be involved in the reduced genetic stability (31, 61).

A striking feature of the genetics of *M. pneumoniae* is the use of an alternate genetic code (68). The use of UGA to encode tryptophan rather than the normal opal stop codon has severely limited the ability to express mycoplasmal genes in other more tractable organisms. Opal suppressor strains of *Escherichia coli* (138) or *Bacillus subtilis* (84), which occasionally translate UGA as tryptophan, have been reported, but such systems offer leaky translation at best and are unsuitable for the expression of genes with several UGA codons.

The reduced genome of *M. pneumoniae* includes a lack of identifiable regulatory controls. Alternate sigma factors, two-component systems, and recognized activator or repressor proteins have not been found in spite of the availability of genome sequences

for *M. pneumoniae* and many other mycoplasmas. While changes in the transcription and protein profiles at different temperatures have been reported, it is not understood if or how *M. pneumoniae* might regulate its gene expression (61, 117). Microarray studies analyzing the total mRNA complement or transcriptome of *M. pneumoniae* under normal (37ºC or 32ºC) and heat-shock (32ºC quickly raised to 43ºC) conditions found 47 genes to be upregulated and 30 genes with decreased levels of message. The upregulated genes included those for the typical heat-shock proteins DnaK, GroEL, and LonA (162).

The technical difficulties of working with *M. pneumoniae* have hindered the understanding of factors important for the transcription and translation of mycoplasmal genes. A reliable method of predicting Shine-Dalgarno-like ribosome binding sites (RBS) has not been identified, and when introducing recombinant genes, generally an extended region of upstream sequence is blindly used in the hope that it will direct translation (54). More is known about promoters of transcription of *M. pneumoniae* and the closely related *M. genitalium*. Likely Pribnow sequences have been identified in the -10 position upstream of the transcriptional starts of many genes. These are adenine-rich sequences (usually three out of four nucleotides are A) flanked by thymines. No pattern has been recognized in the -35 position relative to the known transcriptional start, and such a sequence may not be necessary for *M. pneumoniae* DNA-dependent RNA polymerase to initiate transcription (160, 161). Promoter trap experiments in non-orthologous systems (e.g. *E. coli*) have attempted to identify regions important for transcription of *M. pneumoniae* or *M. genitalium* genes (32), but these were of limited use. Seven sequences yielding positive results were found in *E. coli*, and when mycoplasma RNA was analyzed by primer extension, only three of these sequences

produced a product. Of these three, just one was in a comparable location to the *E. coli* result and even this was skewed from the +1 site by one nucleotide. Many primer extension studies in *M. pneumoniae* have shown that transcripts often have multiple +1 sites (usually within one nucleotide of each other) (70, 97, 160, 161). While a similar mechanism is used by other bacteria as a method of regulation, it does not appear that this is the case for *M. pnumoniae*, and the significance of this heterogeneous initiation is unknown (161).

Mycoplasmas have no natural plasmids, and *M. pneumoniae* has no known phage. *M. pneumoniae* has an identifiable *recA* gene in addition to other genes involved in homologous recombination and appears to undergo or have undergone antigenic variation by DNA rearrangement in nature (61, 87). However, attempts at homologous recombination and targeted disruption in the standard laboratory *M. pneumoniae* strain M129 have remained unsuccessful. Curiously, *M. genitalium,* with a much smaller genome, can undergo homologous recombination, but the importance of this difference from *M. pneumoniae* is not well understood (33). Nonetheless, great advances have been made using transposons from other organisms in suicide vectors delivered by electroporation. These integrate into the chromosome at single, random-like loci. Derivatives of the Tn*4001* transposon isolated from *Staphylococcus aureus* have been successfully used to disrupt genes (60, 91). These can also be used to deliver DNA fragments in an expressable context, using either the P_{IN} or P_{OUT} promoters of IS256 or promoters present in the DNA fragment cloned into the IS element (55). The insertion of a variant of Tn*4001* into the chromosome appears to be a single event,

but subsequent variants bearing different antibiotic resistance markers can be successfully added with a greatly reduced efficiency (see Chapter 4).

The sequenced genomes of two closely related species have been of interest in understanding the so-called minimal genome for life. Transposon-based experiments have been used to inactivate random genes singly to identify essential genes in *M. pneumoniae* and *M. genitalium*. Although this study was fundamentally flawed by the limitations of available technology (e.g. insertions in an 'essential' gene may have been overcome by the presence of a paralog), combined with comparisons of the genomes of other organisms, it remains the best published attempt at identifying a minimal genome, such as it is. An estimated 265 to 350 genes were thus predicted to be essential for *M. pneumoniae* and *M. genitalium* outside their host. Of these, about 100 were not assignable to specific functions at the time, by far the largest category of so-called essential genes. Unsurprisingly, genes involved in the synthesis and repair of nucleic acids and proteins and those of central energy metabolism are prominently required (65, 66).

Because of the relatively small genome and correspondingly limited protein complement, extensive efforts have been made to analyze the whole array of proteins or the proteome of *M. pneumoniae*. Two methods have been used for this task. Initially, a more traditional approach using large-format 2-D PAGE followed by automated spot picking and protein identification by tandem mass spectrometry was employed. Proteins from 305 ORFs were detected by this technique including five proteins for which no gene was called in the genome analysis (132, 157). This 2-D PAGE technique was also used to identify the protein components of the *M.*

pneumoniae cytoskeleton. In this sudy, about 100 spots were resolved and 41 of these were successfully identified. The functions of these proteins ranged from translation machinery, metabolism, the heat-shock response, cytadherence, and those of unknown function (131). These results are difficult to interpret, however. The heat-shock proteins DnaK and GroEL are among the most abundant proteins in *M. pneumoniae*. Furthermore, complexes involved in translation (i.e. ribosomes plus mRNA and elongation factor Tu) and metabolism (e.g. pyruvate dehydrogenase) are very large. These may be insoluble solely due to their abundance or size and not as a result of a specific interaction with the cytoskeleton. The proteins of unknown function are potential structrual/cytoskeletal components and warrant further study. A more ambitious approach to cataloging the proteome of *M. pneumonaie* was described recently (79). In this work, the whole cell lysate was subjected to trypsin digestion and analyzed by mass spectrometry. The resulting deluge of peaks was then mapped onto the coding potential of the *M. pneumoniae* genome by brute-force computational methods. This technique identified proteins for over 81% of the predicted ORFs of *M. pneumoniae* and 16 previously uncalled genes.

M. pneumoniae Cellular Biology

Despite the lack of a cell wall to provide a rigid basis for cell-shape, *M. pneumoniae* and closely related mycoplasmas possess a distinct typical morphology, although a wide range of variations on this shape can be seen in any population. In cross-section, *M. pneumoniae* cells appear as a round-bottomed flask, and when grown on a solid surface, they adopt an elongated form with the bulbous cell body between

tapered leading and trailing filaments (88). The average size of *M. pneumoniae* cells is around 0.2 μm in diameter and just over 2 μm in length (116). At the leading end of gliding motility the tapered portion of the cell has a distinct composition and appearance. This structure, known as the attachment organelle or tip, is important for gliding motility, cell division, and cytadherence (94). *M. pneumoniae* mutants lacking certain attachment organelle proteins, such as for example P30 or HMW1, exhibit altered morphology (see Cytadherence and the P1 Adhesin below). In the absence of P30, cells appear branched (133), and without HMW1, *M. pneumoniae* cannot form a proper attachment organelle (55).

Transmission electron microscopy (TEM) reveals an electron dense region at the center of the attachment organelle (20). The proteins of this core structure are thought to confer the shape of the attachment organelle. A sub-population of the *M. pneumoniae* proteome is insoluble in the non-ionic detergent Trition X-100 (TX-100). When cells grown on a solid surface are treated with TX-100, there remains a tangle of insoluble material that is reminiscent of the shape of the original bacterial cell. These observations, along with the fact that insolubility in TX-100 is a definitive feature of the eukaryotic cytoskeleton has led to the view that these proteins function as a prokaryotic cytoskeleton in *M. pneumoniae,* permitting the cells to form their characteristic shape without a cell wall (111).

Gliding motility is widespread in the prokaryotic world. However, none of the mechanisms of gliding which have been deciphered from other organisms have homologous proteins in *M. pneumoniae,* and the mechanism of its motility remains a mystery. Even the motility components of *M. mobile,* which is viewed as a model

organism for mycoplasmal motility because of its large size and rapid movement at room temperature, are not detected in the *M. pneumoniae* genome. Nonetheless, motility seems to be an important aspect of the *M. pneumoniae* lifestyle, and undoubtedly protein systems dedicated to this ability exist.

Little is known about the specifics of cell division in *M. pneumoniae*. More is known about the distantly related *M. capricolum*, which is capable of growth in a defined medium, allowing synchronization experiments. DNA replication in *M. capricolum* is linked with the cell cycle. Chromosome duplication is initiated once per cycle, allowing at maximum two copies of any gene to be present at any time, unlike rapidly growing organisms such as *E. coli* (135). Proceeding in concert with chromosome replication in *M. pneumoniae* is a duplication of the components of the attachment organelle and of the structure itself (114, 134). In *M. pneumoniae*, the core has been observed to exist as two distinct bundles, held together by a protein filament arranged in the manner of a barbershop pole. These two bundles suggest a semi-conservative mechanism for core and possibly organelle duplication (94). Gliding motility seems inextricably linked to cell division. Microscopic analysis suggests that after organelle duplication, normal motive forces pull the two daughter cells apart (21). *M. pneumoniae* also possesses some homologs of the cell division proteins identifed in systems such as *E. coli* and *B. subtilis* (those not involved with the cell wall) (61), and these proteins (e.g. FtsZ) are likely involved in cytokinesis. Several spontaneous mutants lacking attachment organelle proteins exist (see below), and these are impaired in motility and, while not lethal, in cell division as well. Without proper motility to assist or drive cytokinesis, the cells often

take on a fragile branched morphology indicating that motility may be important but not essential for cell division (133).

M. pneumoniae Biochemistry and Physiology

M. pneumoniae, like most other mycoplasmas, is a remarkably fastidious organism with exacting cultural requirements that have led to misunderstandings and artifacts in the past and continued experimental difficulties in the present. While a defined medium exists for some mycoplasmas (e.g. *M. mycodies*), culture of *M. pneumoniae* and its close relatives requires complex undefined media. Furthermore, variations in media components between manufacturers or even lots can greatly impact the ability of the bacteria to grow, especially during primary isolation.

Two media are commonly used to grow *M. pneumoniae*. Hayflick's modification of the PPLO medium (58) and SP-4 (155). Differences in growth and behavior are seen with these media but may be due to variation between batches and not the suitability of the basic formulations. Common components of these media include an amino acid rich base, sources of carbon, fatty acids and sterols provided by animal serum, and a complex yeast-based source of small molecules such as nucleotides, co-factors, and vitamins. The requirement for sterols and especially cholesterol is peculiar to certain animal mycoplasmas. Animal cells use cholesterol to provide strength and stability to their membranes and are uniquely able to synthesize cholesterol. Thus, *M. pneumoniae* in nature must obtain cholesterol directly from the host (113).

M. pneumoniae cells are small and cultures do not grow to a high density, hence turbity is not seen in mature cultures. However, the pH indicator phenol red is often

used in media to measure the accumulation of acidic products of fermentation and thus, indirectly, growth. *M. pneumoniae* colonies are barely visible to the naked eye on agar plates after 7-9 days of growth and must be observed microscopically or following overlay with sheep blood agar and a further 48-h incubation at 37°C to allow formation of hemolytic plaques due to hydrogen peroxide produced by the bacteria.

As cells with reduced biosynthetic capacity requiring a rich medium, *M. pneumoniae* must transport a wide variety of compounds for macromolecular synthesis. Based on a predicted membrane location and the presence of an ATP binding motif, *M. pneumoniae* seems to have 34 putative transport proteins. The specificity cannot be reliably determined by this method, but they seem to include transporters of oligopeptides. Nevertheless, the transporter complement of *M. pneumoniae* is smaller than initially hypothesized suggesting this minimal organism may use reduced substrate specificity to allow greater flexibility (61).

M. pneumoniae ferments glucose by the Embden-Meyerhof-Parnas pathway, and the genome sequence shows an incomplete pentose phosphate pathway. Acetate is a known fermentation end-product of *M. pneumoniae* glucose utilization, and genes to produce lactate and ethanol from reducing equivalents are seen in the genome. Other mycoplasmas can utilize arginine, and this characteristic is used in diagnosis and identification. *M. pneumoniae* reportedly lacks the ability to utilize arginine, but the genes for arginine utilization are present. *M. pneumoniae* seems to possess extensive but incomplete mechanisms to interconvert various substrates in nucleotide metabolism. Of particular note is the apparent lack of a kinase to convert nucleotide diphosphates to

nucleotide triphosphates as this activity would seem essential for the cycling of nucleotides (61).

Detailed studies of *M. pneumoniae* physiology have been limited by the lack of a defined medium and the general slow growth and low biomass. The insights we do have are largely based on experiments in related organisms and comparisons of the genome sequence. As the bioinformatic techniques to predict protein function improve, it is hoped more will become known about the specific requirement and reactions important for this mycoplasma.

Cytadherence and the P1 adhesin

Cytadherence, or the ability to attach to host tissue, is a crucial aspect of the pathogenesis of *M. pneumoniae* infections. Attachment is thought to initiate disease and play an important role in cytotoxicity. Several proteins have been linked to a direct role in receptor binding and attachment; however the presence of these proteins is not sufficient to enable cytadherence. The two best-studied adhesins, P30 and the major adhesin P1, are located at the polar attachment organelle. An array of proteins is important for the proper formation of this organelle but not directly involved in cytadherence. Without these cytadherence-accessory proteins, the adhesins are not localized correctly or fail to function in spite of apparently correct localization. The interplay of these proteins is complex, and while many are known to be vital, their exact function remains uncharacterized. Other proteins are hypothesized to have a role in cytadherence based on circumstantial data, but their requirement remains unproven (94).

Major adhesin P1: *M. pneumoniae* cells treated with the protease trypsin temporarily lose cytadherence while remaining viable. SDS-PAGE analysis of these cells revealed the loss of a high molecular weight protein amongst others. This 170-kDa protein was named P1 (63). It was discovered later that P1 was densely clustered at one pole of the cell, the attachment organelle (43, 62) and antibodies against P1 were capable of specifically blocking attachment (62, 95).

The P1 protein was purified and subjected to Edman degradation and sequencing (75), yielding a primary sequence for 12 residues beginning with an asparagine. When the sequence of the *p1* gene was determined it was found that the start codon was some 177 nucleotides upstream of the region coding the N-terminus of the mature protein at residue 60 (67). The deduced amino acid sequence of the pre-P1 protein begins with a region strongly resembling and predicted to be a normal Sec-dependent Gram-positive style signal peptide leader with several possible cleavage sites predicted between residues 20 and 40. It is likely that this signal peptide is processed and cleaved in a regular fashion, which would require that the remainder of the pre-protein be removed in a separate step involved in the transformation of P1 into a mature and functional form (93).

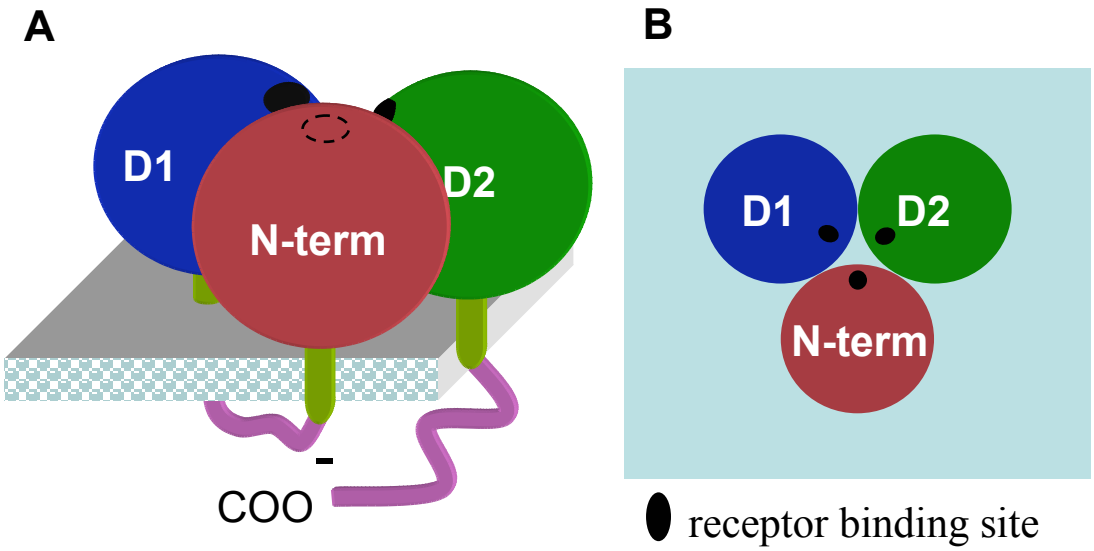
P1 is clearly an integral membrane protein (67, 128). It has a predicted secretion signal peptide and transmembrane helices in the mature protein, and is surface exposed and present in the membrane fraction of *M. pneumoniae* cells. Through epitope mapping using attachment inhibiting and non-inhibiting P1-specific monoclonal antibodies and secondary structure predictions, a model for the topology of P1 in the cell membrane has been proposed (51, 128). This model predicts the majority of the

protein, including the N-terminus, to be exposed on the cell surface as three domains that must be coordinated to yield functional receptor-binding activity (Fig. 1.1). The TX-100 insoluble portion of the *M. pneumoniae* proteome is thought to be involved in a cytoskeletal structure important for the morphology and function of the bacteria (see section on *M. pneumoniae* Cellular Biology). The P1 protein is associated with this cell fraction, and in fact the cytoskeleton was first viewed as possibly important based largely on the presence of the P1 adhesin within it (83). Different reports have shown 10-90% of P1 present in the TX-100-insoluble fraction. It seems that P1, like some other cytoskeletal proteins, is only weakly associated with the insoluble fraction and can be completely removed by repeated washing. This may account for the reported variability and provide insight into the nature of the cytoskelton (7, 159). Along with P1, several other proteins associated with cytadherence are located at the attachment organelle (see below). A study using paraformaldehyde to cross-link the proteins of wild-type *M. pneumoniae* found that many of these co-located proteins could be immunoaffinity-purified, once cross-linked, using P1-specific antisera (104). However, other proteins not generally thought to be associated with the attachment organelle (DnaK and pyruvate dehydrogenase subunit E1α) were also purified. This result confuses the significance of the study.

Copies of portions of the *p1* gene are spread throughout the chromosome (61, 146). The P1 gene from clinical isolates is historically found in two types (group I and group II) (148) although variations on these have been reported more recently (87). Within these types are at least eight subtypes (35). The potential for all the variation

FIG 1.1. Predicted structure of the P1 adhesin of *M. pneumoniae*.

The P1 adhesin is predicted to possess three major extracellular domains each with a portion of the receptor binding site. The N-terminal (N-term), and domains 1 and 2 (D1 and D2) must be coordinated in space to present a functional adhesin. (A) perspective representation of the adhesin. (B) Top-down view of the adhesin. Adapted from Razin and Jacobs (128).


A
D1
D2
N-term
COO
-
B
D1
D2
N-term
receptor binding site

found in *p1* can be attributed to the partial *p1* repeats found in the sequenced strain M129 (a type I strain), (36, 61, 87, 149). In nature, it seems that these gene fragments may come together and rearrange to create new *p1* alleles possibly to vary the epitopes of the protein in order to evade the host's immune response. While it is obvious how such variation could assist immune evasion, it is unclear if or how it may play a role in the basic process of cytadherence and receptor recognition.

The P1-specific immune response is complicated. P1 is a large immunogenic protein (73), and antibodies raised against P1 in animals block cytadherence. This led to great interest in P1 as a vaccine candidate. Guinea pigs immunized with P1 develop antibody and have fewer detected infectious organisms than non-immunized animals, but are not protected against disease. Also, the lesions caused by disease are more extensive in the immunized and previously infected animals compared to naïve animals (74, 77). In patients and animals having high levels of P1-specific antibodies, less than 3% produce antibodies capable of strongly inhibiting the attachment of *M. pneumoniae*. These non-inhibiting antibodies fail to target epitopes in regions of the P1 protein associated with receptor binding (76). Some of these regions share sequence similarity with human proteins such as glyceraldehyde-3-phosphate dehydrogenase and 2-phospho-D-glycerate hydrolyase, and it is thought that any antibodies capable of inhibiting attachment would also react with host tissue and would be excluded in a healthy immune response (72). This molecular mimicry would help explain the frequent failure of previous disease to prevent subsequent infection in the long term, the inefficacy of P1-base vaccines in animals, and the occurrence of disease in other tissue related to autoimmunity with *M. pneumoniae* infection.

The gene encoding P1 is found as the second gene in a proposed three-gene operon (Fig. 1.2). The first gene of the operon, ORF4 (see below), is directly downstream of the only known promoter. The *p1* gene begins 12 nucleotides after ORF4, and just five nucleotides separate *p1* from the downstream gene ORF6 (see below). It is thought that the translation of *p1* and ORF6, but not ORF4, is linked, but all three are transcribed together (see Chapter 4). There is also a predicted stem-loop terminator downstream of ORF6, the third and final gene of the operon (70).

Several spontaneous cytadherence-negative mutants have been isolated (96). One of these, mutant IV-22, fails to produce the P1 protein due to a frameshift mutation in a poly-A stretch at the 5' end of the gene (147). This mutant also lacks three proteins A, B, and C (see below) identified by 2-D PAGE analysis (56). As no strain lacking only P1 has been identified, it has heretofore been difficult to determine which aspects of *M. pneumoniae* biology are due solely to P1.

A, B, & C: Chemical mutagenesis resulted in the identification of three proteins that were always present or absent in a coordinated manner which correlated with cytadherence (56, 57). A similar pattern was also observed in spontaneously arising cytadherence mutants (96). These are the proteins A, B, and C (72, 85, and 37-kDa, respectively). In independent studies, antibodies were raised against peptides encoded by regions of the gene downstream of the *p1* gene, ORF6, and these antibodies detected proteins of either 40 or 90-kDa (143). In a reannotation of the *M. pneumoniae* genome, all ORFs were given consecutive numbers, and ORF6 became MPN142 (31).

The MPN142 gene product undergoes a post-translational cleavage to yield the two proteins named P40 and P90

FIG 1.2. Organization of the *p1* operon of *M. pneumoniae.*

Map of the *p1* operon of *M. pneumoniae*. Arrow indicates transcriptional promoter upstream of MPN140 (ORF4). Twelve nt separate MPN140 and MPN141 (*p1*), and five separate MPN141 and MPN142 (ORF6). Predicted stem-loop terminator begins 17 nt downstream of MPN142.


MPN140
MPN141
MPN142
1kb

(100), which appear to correspond to C and B, respectively (Chapter 3). Both elicit a strong immune response in experimental infections and are integral membrane proteins located at the attachment organelle, with a normal signal peptide predicted at the N-terminus of the MPN142 gene product (49, 70). These proteins have been reported missing in several non-cytadherent mutant strains (56, 57, 96, 99). Because of the similarity in their sizes, expression patterns, and implied function in cytadherence, proteins B and C have been linked to P90 and P40, respectively. Particularly telling is the observation that antisera raised against regions of P90 or P40 react with bands of the correct size present in the wild-type but absent in an A/B/C mutant. The gene for the A protein remains unknown in spite of searches including the sequencing of the *M. pneumoniae* genome, and the spot may be the result of an experimental artifact.

Cross-linking studies using 3,3'-dithiobis(sulfosuccinimidylpropionate) (DTSSP) showed that P40 and P90 are found in proximity to P1 (within 12 Å) (101), and there is weak sequence similarity between the C-terminus of P1 and deduced amino acid sequence from the 3' end of MPN142, which encodes the protein P90 (70). Also the MPN142 homologs in the closely related *M. genitalium* (69) and *M. gallisepticum* (122) are present as uncleaved (i.e. single) proteins. Taken together, these observations support the hypothesis that P1, B, and C may be present as a protein complex that is responsible for the major adhesin activity associated with P1.

Two independent spontaneous mutants lacking the MPN142 gene products have been isolated. The III-4 (96) and M5 (99) mutants are non-cytadherent and have a branched morphology. P1 is present at one or two foci, perhaps at the branch that is the true attachment organelle (134, 136). However, older data using immuno-electron

microscopy show that in the III-4 mutant, P1 is not localized to the attachment organelle (9). The nature of the mutation in these mutants and whether the lack of MPN142 gene products definitively affects cytadherence is examined in Chapter 3 for mutant III-4.

Protein P30: Another protein associated with a direct role in cytadherence is the 30-kDa membrane protein P30. This protein is located at the attachment organelle in wild-type cells (12), and mutants lacking P30 have reduced cytadherence (96). Furthermore, antibodies against P30 block attachment (12). P30 has an unremarkable predicted signal sequence and a single transmembrane helix in the mature protein. Carboxypeptidase Y treatment of whole cells indicates that the C-terminus of P30 is exposed, and the N-terminus would then be cytosolic (103). The P30 sequence shows a series of three proline-rich sequences repeated multiple times in the C-terminus of the protein. The function of these repeats is unknown (30).

Spontaneous mutations in *p30* include the II-3 null mutant and the II-7 mutant, which has a 144-nucleotide in-frame deletion in the proline-repeat region. This shortened P30 is stable, and the phenotype (partial cytadherence and motility) of mutant II-7 is closer to wild-type than mutant II-3, indicating the shortened P30 it is partially functional (133).

High Molecular Weight Proteins: Three high molecular weight proteins (HMWs) have been linked to cytadherence (96). These proteins: HMW1, HMW2, and HMW3, each have different locations in the cell and function in cytadherence, but their responsibilities are intertwined in complex ways.

HMW2 is a large coiled-coil protein present inside the *M. pneumoniae* cell in tight association with the TX-100-insoluble fraction. HMW1 and HMW3 are not detected or are present at very low levels in both spontaneous and transposon-mediated mutants of *hmw2* (59, 96). This is explained by the fact that in the absence of HMW2, HMW1 and HMW3 are degraded over the course of 1-8 hours (124). The cells also lack a wild-type morphology and the ability to cluster P1 to the attachment organelle, rendering them non-cytadherent. The exact nature of this requirement of HMW2 for stability is not understood, but it is known that certain regions of HMW1 are necessary for this turnover to occur (55). The normal association of HMW1 with the cytoskeleton is impaired in the absence of HMW2 and without proper incorporation into the TX-100-insoluble fraction, the protein is unstable and HMW1 degrades (4).

HMW2 has been localized to the attachment organelle using a green fluorescent protein (GFP) fusion (6) and is thought to be a component of the electron-dense core (7). Surprisingly, an allele of *hmw2* with 80% of the middle of the gene deleted in-frame is capable of restoring many phenotypes associated with cytadherence in a *hmw2* mutant. These include an elongated cell shape, partial hemadsorption, and stabilization of most cytadherence-accessory proteins present at reduced levels in the mutant save HMW1. In this partially complemented strain, P1 is not clustered exclusively at the attachment organelle, however it still possesses the ability to attach to surfaces, which is lacking in the uncomplemented mutant (5). The 3' end of *hmw2* encodes a smaller additional protein with a molecular weight of 28-kDa. This protein, P28, shares the amino acid sequence of the C-terminus of HMW2 and is thought to be the result of an

internal translation initiation (47). P28 is thought to be important in cytadherence in many of the ways HMW2 is and they likely have similar functions (5).

HMW1 is located on the surface of the mycoplasma cell at the attachment organelle but lacks any typical signal for secretion to the outside (4, 134). HMW1 is essential for cytadherence, P1 clustering, and normal morphology, but the mechanisms by which it functions are not well understood, and very low levels of HMW1 may be sufficient for function (5, 55). HMW1 is divided into three major domains. The N-terminal domain is a section of predicted β-strand structure, the middle domain is an acidic and proline-rich (APR) sequence, and the C-terminal domain is of uncharacterized structure, but contains two smaller regions of predicted coiled-coil structure. Within the first domain (Domain I) is a motif enriched in aromatic and glycine residues, or the EAGR box. This motif is seen only in HMW1 and P200, both proteins associated with the cytoskeleton, and a protein of unknown function (MPN119) (4). As noted above, the stability of HMW1 depends on the presence of HMW2. Likewise, in the absence of HMW1, HMW2 is present at reduced levels (7, 163). This reciprocal stability requirement complicates any interpretation of roles of the individual proteins.

The protein HMW3 is located inside the cell, also at the attachment organelle in association with the TX-100-insoluble cytoskeleton. The protein is present at the bulbous tip of the attachment organelle and appears to form a linear structure in association with the electron-dense core (145). The stability of HMW3 is dependent on the presence of HMW1 and HMW2, however these proteins rely only on each other, as they are present at wild-type levels in the absence of HMW3 (164). Like HMW1, HMW3 contains an APR domain (121). HMW3 is important for cytadherence, and in its

absence cells lack a wild-type morphology and the ability to localize P1. Furthermore, the electron-dense core is abnormal in the absence of HMW3 and is sometimes seen as two bundles joined at the terminal bulb of the attachment organelle and spreading out in a V-shaped arrangement (164).

Several spontaneous and transposon-generated mutants in the genes for the HMW proteins have been studied extensively. Mutant I-2 has a frameshift addition in a poly-A track in the gene for HMW2 (47, 96). The M6 mutant has a frameshift in *hmw1* and a truncated *p30* (102) and wild-type recombinant *p30* can be restored to examine effects of the loss of HMW1 (55). Recently, a transposon-insertion mutant in *hmw3* was isolated and this has allowed an examination of the role of HMW3 in cytadherence (164).

Suspected Cytadherence Accessory Proteins: Several genes are located in proximity to those for known cytadherence proteins. Similar organizations are found in the same locations in the genomes of organisms closely related to *M. pneumoniae* (50, 123). There are also a great number of proteins of unknown function which are nonetheless associated with the *M. pneumoniae* cytoskeleton, which is viewed as essential for cytadherence (125, 131). These circumstantial factors suggest that additional uncharacterized proteins may also be associated with cytadherence. Because directed knock-out mutagenesis in *M. pneumoniae* is not possible, more direct evidence requires serendipitous isolation of mutants for these and evaluation for a mutant phenotype.

The gene encoding the 65-kDa protein P65 is located directly upstream of *hmw2* and is the first gene of that operon. P65 is associated with the mycoplasma cytoskeleton and has an APR domain like those found in HMW1 and HMW3 but is more similar to that of HMW3 (126). P65 is a peripheral membrane protein located on the surface of the cell, but like HMW1, lacks apparent secretion signals (125). P65 is localized to the attachment organelle in wild-type *M. pneumoniae* but not in the mutants I-2, M6 + *p30*, or the *hmw3* mutant (82, 134, 136, 164). When HMW3 or P30 are reduced or absent, P65 is present at reduced levels as well (82, 164). This indicates that HMW3 and P30 are required for P65 stability, however HMW1 and/or HMW2 may also be involved.

A 200-kDa protein was identified by its association with the TX-100-insoluble fraction in *M. pneumoniae* (125). This protein, P200, was remarkable for the presence of APR domains like HMW1, HMW3, and P65 (127). Like HMW1 and the protein encoded by gene MPN119, P200 has the EAGR box motif, albeit in multiple copies (4). P200 is localized to the attachment organelle in wild-type cells, but not in the IV-22 mutant (J.L. Jordan, *et al,*. unpublished results). In spite of all these data, the functions of P65 and P200, and the nature of any possible role in cytadherence remain unknown.

The genes for several cytadherence associated proteins are located in one of three loci on the *M. pneumoniae* chromosome (92). The first gene of the so-called *p1* operon, ORF4, encodes a protein whose function is unknown, but a computational analysis linked ORF4 to a phosphoesterase family of proteins (3), and it is expressed in wild-type *M. pneumoniae* (79). ORF4 was originally described as having a predicted molecular weight of 30-kDa (70). Subsequent analysis identified an in-frame ATG

codon upstream of the previously annotated start of ORF4, closer to the promoter (61). It is more likely that this is the real start of the gene, which would then encode a protein with a predicted molecular weight of 37-kDa. The cytadherence regulatory locus or cytadherence related locus (*crl*) contains *hmw2* and *p65* in addition to two genes, *p41* and *p24*. Bands with molecular weights of 47- and 24-kDa were detected by immunoblot analysis using antisera raised against P41 and P24 fusion proteins, respectively, in wild-type *M. pneumoniae* and found to be present at reduced levels in the *hmw2* mutant I-2. These proteins have no known homologs save in other closely related mycoplasmas. However, P41 is predicted to have coiled-coil regions (47, 97). Protein fusions to derivatives of green fluorescent protein (GFP) localized P41 and P24 to the base of the attachment organelle (86). Also the large operon that contains the genes *hmw3, hmw1*, and *p30* and the gene for the ribosomal protein RpsD has several ORFs encoding proteins for which no role is known but are suspected to be involved in cytadherence because of their neighboring genes. The expression of this so-called HMW gene cluster is coordinated as a series of overlapping transcripts with independent transcriptional start signals which may control the relative levels of these proteins (see Chapter 2) (34, 160).

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CHAPTER 2

TRANSCRIPTIONAL ANALYSIS OF THE HMW GENE CLUSTER OF MYCOPLASMA PNEUMONIAE[1]

[1] Waldo RH 3rd, Popham PL, Romero-Arroyo CE, Mothershed EA, Lee KK, Krause DC. 1999. J Bacteriol. 181(16):4978-85.

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Abstract

Mycoplasma pneumoniae adherence to host cells is a multifactorial process that requires the cytadhesin P1 and additional accessory proteins. The *hmw* gene cluster consists of the genes *p30*, *hmw3*, and *hmw1*, the products of which are known to be essential for cytadherence, the *rpsD* gene, and six open reading frames of unknown function. Putative transcriptional terminators flank this locus, raising the possibility that these genes are expressed as a single transcriptional unit. However, S1 nuclease protection and primer extension experiments identified probable transcriptional start sites upstream of the *p32*, *p21*, *p50*, and *rpsD* genes. Each was preceded at the appropriate spacing by the -10-like sequence TTAAAATT, but the 35 regions were not conserved. Analysis of the *M. pneumoniae* genome sequence indicated that this promoter-like sequence is found upstream of only a limited number of open reading frames, including the genes for P65 and P200, which are structurally related to HMW1 and HMW3. Promoter deletion studies demonstrated that the promoter-like region upstream of *p21* was necessary for the expression of *p30* and an *hmw3-cat* fusion in *M. pneumoniae*, while deletion of the promoter-like region upstream of *p32* had no apparent effect. Analysis by reverse transcription-PCR confirmed transcriptional linkage of all the open reading frames in the *hmw* gene cluster. Taken together, these findings suggest that the genes of this locus constitute an operon expressed from overlapping transcripts.

Introduction

The cell wall-less prokaryote *Mycoplasma pneumoniae* causes atypical pneumonia and tracheobronchitis in older children and young adults. *Mycoplasma* colonization of host respiratory epithelium (cytadherence) is mediated largely by a differentiated polar attachment organelle. This tip structure is a membrane-bound extension of the mycoplasma cell and contains an electron-dense core that enlarges to form a terminal button at the tip of the cell (3, 18, 19). The adhesin protein P1 is primarily found densely clustered on the surface of the attachment organelle (27). However, additional proteins, including the high-molecular-weight proteins HMW1, HMW2, and HMW3 are required for cytadherence (18, 20).

HMW1, HMW2, and HMW3 are encoded by two unlinked genetic loci in the *M. pneumoniae* chromosome (6, 15, 21). The *hmw2* gene is part of the P65 operon, also known as the cytadherence regulatory locus (*crl* [14]). Spontaneous frameshifts in poly(A) stretches in *hmw2* (7) or transposon insertional inactivation of *hmw2* (21) results in accelerated turnover of HMW1, HMW3, and P65 (24). It is not clear, however, whether this accelerated proteolysis reflects strictly a housekeeping activity or possibly a regulatory mechanism functioning improperly in the mutant. The *hmw1* and *hmw3* genes are located approximately 160-kbp from the P65 operon, in what is designated the *hmw* gene cluster (6, 18). This locus also includes the gene for P30, a putative cytadhesin that is required for normal cell development (2, 4, 28), and six open reading frames (ORFs) of unknown function (6) (Fig. 2.1). Like P30, HMW1 and HMW3 have important roles in the architecture and assembly of the attachment organelle. HMW1 is found along the leading and trailing extensions of the mycoplasma cell and is essential

for proper development of the tip structure (12, 31), while HMW3 is a major component of the terminal button of the electron-dense core (32).

Duplication of the attachment organelle is believed to precede cell division in *M. pneumoniae* (3). The tip structure is thought to function in chromosome partitioning and, therefore, one might expect the synthesis of attachment organelle components to be regulated in a manner that is coordinated with cell division (19). However, transcriptional control is poorly understood in mycoplasmas and, based on the presence of a single sigma factor and the lack of predicted two-component or other typical transcriptional regulatory systems, mechanisms for controlling *M. pneumoniae* gene expression may be limited (15). In the current study we have undertaken a detailed analysis of transcription of the *hmw* gene cluster of *M. pneumoniae*. S1 nuclease protection and primer extension were used to define two probable transcription start sites near the beginning of the gene cluster, as well as two additional sites much farther downstream, indicating likely overlapping transcripts. The nucleotide sequences upstream of each putative transcriptional start site exhibited homology to the consensus prokaryotic Pribnow-Schaller box (25). The frequency and distribution patterns for this promoter-like sequence in the *M. pneumoniae* genome were likewise consistent with promoter function. Analysis of reporter gene expression after deletion of each putative promoter near the 5' end of the locus identified a region that is essential for the expression of *p30* and *hmw3* in *M. pneumoniae*. Finally, data from reverse transcription-PCR (RT-PCR) analysis were consistent with coexpression of the genes in the *hmw* locus.

Materials and Methods

Bacterial strains and culture conditions.

M. pneumoniae strains used in this study included wild-type strain M129 (broth passage 17) and two noncytadhering mutants derived from M129, I-2 and II-3 (2, 20). The relevant phenotypes of each are summarized in Table 2.1. Mycoplasmas were cultured at 37°C in Hayflick medium (13) until the mid-logarithmic phase and then harvested as described previously (10). Cultures were plated on PPLO agar (23), incubated at 37°C for 6 to 9 days, and visualized by hemolytic plaques for the isolation of individual colonies or the enumeration of CFUs (20). Gentamicin was included at a concentration of 18 μg/ml for selection and culturing of mycoplasma transformants. *Escherichia coli* Sure (Stratagene, La Jolla, Calif.) grown in Luria broth was prepared as competent cells for transformation and used for plasmid preparation by standard techniques (29). Plasmid DNA was purified by using pZ523 columns (5'3', Inc., Boulder, Colo.) according to the manufacturer's protocol.

RNA preparation.

All solutions and plasticware were rendered RNase-free by diethyl pyrocarbonate treatment (29). RNA was extracted from mid-logarithmic-phase cultures of *M. pneumoniae* as described previously (24), except that nucleic acids were incubated at 68°C for 10 min to enhance resuspension prior to treatment with RNase-free DNase I (30 U; Boehringer Mannheim, Indianapolis, Ind.) for 1 h at 37°C in 0.25 ml of 100 mM sodium acetate-5 mM MgSO4 (pH 5.0) containing 39 U of RNasin (Promega, Madison, Wis.).

S1 nuclease protection and primer extension analyses.

S1 nuclease protection analysis was conducted to localize the 5' end of RNA transcripts extending into the *hmw3* gene. The double-stranded DNA templates used for nuclease protection spanned from either the *Eco*RI site or the *Kpn*I site upstream of the *hmw* gene cluster to the first *Bam*HI site in the *hmw3* gene (Fig. 2.1) and were labeled at the 5' end with [γ-32P]ATP. Hybridization and nuclease protection were carried out by using standard techniques (29) at hybridization temperatures based upon the G+C content of the predicted DNA-RNA hybrids.

Primer extension analysis was utilized to identify precisely the 5' end of RNA transcripts for the *hmw* gene cluster. Mycoplasma total RNA was reverse transcribed by using the avian myeloblastosis virus (AMV) Reverse Transcriptase Primer Extension System (Promega) according to the manufacturer's protocol. Primers corresponded to regions within 150 nucleotides (nt) of the expected transcription initiation sites (Table 2.2). Oligonucleotide primers (Life Technologies, Grand Island, N.Y.) were end labeled by incubating 10 pmol of primer with 10 U of T4 polynucleotide kinase, 30 μCi of [-32P]ATP (3,000 Ci/mmol; NEN Research Products, Boston, Mass.), 50 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 5 mM dithiothreitol, and 0.1 mM spermidine at 37°C for 10 min. Samples were heated to 90°C for 2 min to inactivate the T4 polynucleotide kinase, and nuclease-free water was then added to bring the final primer concentration to 100 fmol/μl. Radiolabeled primer (100 fmol) was annealed with 35 μg of total mycoplasma RNA in 50 mM Tris-HCl (pH 8.3), 50 mM KCl, 10 mM MgCl2, 10 mM dithiothreitol, a 1 mM concentration of each deoxynucleoside triphosphate (dNTP), and 0.5 mM spermidine at 58°C for 20 min. Annealed samples were cooled at room

temperature for 10 min, and primers were extended by RT for 30 min at 41.5°C in 50 mM Tris-HCl (pH 8.3), 50 mM KCl, 10 mM MgCl2, 10 mM dithiothreitol, 1 mM each dNTP, 2.8 mM sodium pyrophosphate, 0.5 mM spermidine, and 1 U of AMV reverse transcriptase. After extension, an equal volume of loading dye (98% formamide, 10 mM EDTA, 0.1% xylene cyanol, 0.1% bromophenol blue) was added to each sample.

Extension products were analyzed by polyacrylamide gel electrophoresis (PAGE). Approximately 20% (6 to 8 μl) of each primer extension sample was loaded onto a 6% polyacrylamide sequencing gel containing 8 M urea, 89 mM Tris base, 110 mM boric acid, and 2 mM EDTA. Cloned mycoplasma DNA corresponding to the region being analyzed by primer extension was used as a template for DNA sequencing reactions with the same primers used for primer extension analysis. The *fmole* DNA Sequencing System (Promega) was used for all sequencing reactions according to the manufacturer's protocol. Sequencing gels were dried and exposed to film overnight.

Promoter deletion analysis.

Two putative promoter regions upstream of the *p30* gene were evaluated in *M. pneumoniae*. First, a promoterless copy of a *Staphylococcus aureus cat* gene (11) was amplified by PCR so as to engineer flanking *Bam*HI sites and an appropriate upstream stop codon. The PCR product was digested with *Bam*HI and cloned into the *Bam*HI site of pGEM7Zf(+) (Promega) to create pKV170. The sequence of the *cat* insert in pKV170 was confirmed by automated DNA sequencing (Molecular Genetics Instrumentation Facility, University of Georgia, Athens, Ga.). Plasmid pKV112 (28), which contains the 3.215-kb region of the HMW gene cluster spanning *Xba*I to *Bam*HI

(Fig. 2.1), was digested with *Bam*HI, and the *cat* fragment was excised from pKV170 with *Bam*HI and cloned into the corresponding site of pKV112 to yield pKV181. Orientation of the insert in the same direction as *hmw3* was confirmed by restriction endonuclease digestion and sequencing. For deletion of the first putative promoter region, plasmid pKV181 was transformed into *E. coli* GM33, a *damA* strain (kindly provided by K. Dybvig, University of Alabama at Birmingham), so that a *Bcl*I site near the 5' end of ORF *p32* was no longer methylated and was cleavable by that restriction endonuclease (see Fig.2.5). After digestion with *Bcl*I and *Csp*45I to remove a 233-bp fragment, the DNA was blunt ended with Klenow fragment of DNA polymerase and religated to yield pKV187 (29). The promoter region upstream of ORF *p21* was deleted by digestion of pKV181 with *Mlu*I and *Spe*I to remove a 292-bp fragment, making the ends blunt with Klenow, and religating it to yield pKV184. Promoter deletions in each case were confirmed by DNA sequencing. The *hmw3-cat* fusions from pKV181, pKV184, and pKV187 were excised with *Eco*RV and *Bgl*II and blunt ended with the Klenow fragment. These were then ligated into the *Sma*I site of pISM2062 (17) to yield pKV193, pKV197, and pKV198 respectively (see Fig. 2.5).

Promoter function for each deletion mutant was assessed on the basis of chloramphenicol acetyltransferase (CAT) reporter activity in wild-type and mutant II-3 backgrounds and on the basis of production of recombinant P30 in the mutant II-3 background, as measured by Western blotting and complementation of the wild-type hemadsorption phenotype. Mycoplasma transformants were isolated and expanded as described previously (14). The presence of the transposon and two *p30* alleles (resident and recombinant) was demonstrated for each transformant by Southern blot

hybridization (29). The activity of the CAT reporter was measured as the MIC and by using the FAST CAT Green (deoxy) CAT Assay Kit (Molecular Probes, Eugene, Oreg.), which was carried out as described previously (11). P30 production was assessed by discontinuous sodium dodecyl sulfate-PAGE (12% polyacrylamide separating gel [10]), followed by Western immunoblotting with P30-specific antibodies (28). Hemadsorption assays were conducted as described previously (20).

RT-PCR.

Northern blot analysis of *hmw1*- and *hmw3*-specific mRNA failed to yield a clear indication of transcript size or to establish the cotranscription of these genes (data not shown). Therefore, RT-PCR was used to evaluate transcriptional linkage in the *hmw* gene cluster and to identify the likely 5' and 3' ends of the transcripts. Primer pairs for RT-PCR were chosen to enable synthesis of a PCR product that was 400 to 1,500 nt in size that would span each intergenic region (Table 2.3). Mycoplasma RNA was reverse transcribed by using the AMV Reverse Transcriptase Primer Extension System as described above, except that no radiolabel was necessary. After the extension step, 180 μl of 10 mM Tris-10 mM EDTA (pH 7.5) was added to each sample to stop T4 polynucleotide kinase activity (as modified from Ausubel et al. [1]). Nucleic acids were extracted with phenol, phenol-chloroform, and chloroform and then precipitated with ethanol and suspended in 20 μl. Then, 10-μl volumes were added to Easystart 50 reaction tubes (Molecular Bio-Products, San Diego, Calif.) containing 2 mM MgCl2, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 0.2 mM dNTP, and wax to overlay the reaction. The PCR primers (2 μM) and *Taq* DNA polymerase (2.5 U; Promega) were added, and

samples were immediately heated to 95°C for 5 min. The PCR cycle consisted of 95°C for 1 min (denaturation), 55°C for 1 min (annealing), and 72°C for 1 min (extension), and this was repeated 30 times. PCR products were visualized after agarose gel electrophoresis and ethidium bromide staining.

Computer analysis.

Sequence analysis was performed by using the Wisconsin Package version 9.0 (Genetics Computer Group) through the Research Computing Resource at the University of Georgia. The FindPatterns program was used to locate possible promoter sequences in the *M. pneumoniae* genome, and a script written in the "Practical Extraction and Report Language" (PERL) was used to compare those matches with the annotated genome (15). A total of 696 genes, including predicted ORFs and RNA-encoding sequences, were considered.

Results

Identification of potential transcription initiation sites.

Previous studies identified two possible Rho factor-independent terminators downstream of the *hmw3* gene (6) (Fig. 2.1). The first immediately precedes *hmw1* and probably functions as an attenuator, given its weak ΔG (7.4 kcal) and nucleation with an AT pair (33). The second follows *rpsD*, with a calculated ΔG of 10.6 kcal and hairpin nucleation beginning with a GC pair. Examination of the sequence upstream of *hmw3* in the same manner revealed a possible transcriptional terminator upstream of ORF *p32* consisting of a stem and loop of 16 and 6 nt, respectively. Hairpin nucleation begins

with a GC pair, and the calculated ΔG is 10.2 kcal (nt 467491; 5'-TAAAAAAAGCACATCCCCCAAAAGGTGTGCTTTTTTAA). Based upon the location of these terminator-like sequences, the *hmw* operon is predicted to span from ORF *p32* through *rpsD*, constituting potentially a single transcriptional unit.

The likely start site for transcription of this gene cluster was evaluated relative to the *hmw3* gene by S1 nuclease protection analysis. Two faint bands with estimated lengths of 2.6 and 2.0 kb were detected in S1 nuclease protection studies by using a 5'-end-labeled, double-stranded DNA probe extending from the first *Bam*HI site in *hmw3* upstream to the *Kpn*I site preceding ORF *p32* (Fig. 2.1 and data not shown). These sizes are consistent with transcription initiation downstream of the predicted terminator preceding ORF *p32*. Furthermore, the presence of two bands suggests overlapping transcripts with different start sites.

The 5' ends of the *hmw3* transcripts identified by nuclease protection were localized more precisely by primer extension, as summarized in Fig. 2.1. The 5' end of one transcript corresponded to the adenine nucleotide 12 bp upstream from the putative ATG of ORF *p32* (Fig. 2.2A). The 5' end of a second transcript was identified as an adenine nucleotide 22 bp upstream from the putative ATG of ORF *p21* (Fig. 2.3A). Both 5' ends were confirmed by using a different oligonucleotide primer for primer extension (data not shown) and were consistent with findings obtained by S1 nuclease protection. Finally, the primer extension products obtained with RNA from noncytadhering mutants I-2 and II-3 were indistinguishable from those obtained with RNA from wild-type *M. pneumoniae* (Fig. 2.2B and 2.3B). This finding is consistent with previous findings that the *hmw3* and *hmw1* genes are transcribed at wild-type levels in mutant I-2 (24).

Six sites having the consensus -10-like (Pribnow-Schaller box) sequence TANANT were identified within the region spanning from the Ser-tRNA genes upstream of ORF *p32* to the *hmw3* gene (Fig. 2.1). A promoter-like sequence preceded the predicted start codon of ORF *p32* by 19 nt (Fig. 2.1) and the 5' end of one transcript by 6 nt (Fig. 2.2A). A second promoter-like sequence was identified 28 nt upstream of the predicted start of ORF *p21* (Fig. 2.1) and 6 nt upstream of the 5' end of the second *hmw3* transcript (Fig. 2.3A). No primer extension products were identified corresponding to the other four possible -10-like sequences upstream of *hmw3* that were tested (data not shown).

Two potential transcription start sites were also identified by primer extension downstream of the *hmw3* gene (Fig. 2.1). The first was located approximately 75 nt upstream of ORF *p50* and was confirmed by using a second oligonucleotide for primer extension. In each case, two primer extension products were identified; they differed in length by 3 nt and began 3 and 6 nt downstream of a -10-like consensus sequence (Fig. 2.4A). These primer extension products were likewise comparable in intensity regardless of whether wild-type or mutant I-2 RNA was used (Fig. 2.4B). Finally, the probable 5' end of the transcript for *rpsD* (Fig. 2.1) was identified only 4 nt upstream of this gene and 6 nt downstream from a consensus -10-like sequence (data not shown).

Each putative transcriptional start site was preceded at the appropriate spacing by the sequence (TTAAAATT), corresponding to a consensus-like -10 region. The sequence TAAAAT matches five of six residues in the consensus prokaryotic 70-dependent -10 site (TATAAT; Table 2.2). Furthermore, this putative -10 sequence is identical to that identified for the P65 operon of *M. pneumoniae* (21), but it differs from -10 sequences previously described in this species (Table 2.2). No pattern was evident

in the putative -35 region of the sequence. Nevertheless, we hypothesize that these -10-like sites function in *M. pneumoniae* in RNA polymerase recognition, and we refer to these sites as PromP32, PromP21, PromP50, and PromRpsD.

Promoter deletion analysis.

The recombinant wild-type *p30* allele restores P30 to normal levels in P30 mutants when introduced by transposon delivery (28). We reasoned that it should be possible to test the requirement for PromP32 and PromP21 in the expression of recombinant *p30* by engineering promoter deletion mutants and examining the consequences on P30 production (Fig. 2.5). P30 mutant II-3 cells transformed with recombinant *p30* lacking the PromP21 (pKV197) promoter produced very little P30 that was detectable by Western blotting. However, deletion of PromP32 (pKV198) had no effect on P30 levels (data not shown). Wild-type *M. pneumoniae* has a hemadsorption-positive phenotype, while mutant II-3 is hemadsorption negative. *M. pneumoniae* mutant II-3 transformed with pKV197 remained hemadsorption negative, but when transformed with pKV193 or pKV198, it was restored to a hemadsorption-positive phenotype (Fig. 2.5).

The effect of promoter deletion on expression of an *hmw3-cat* transcriptional fusion was examined in parallel studies. The *S. aureus cat* gene is expressed in *M. pneumoniae*, conferring resistance to chloramphenicol at a concentration of more than 50 μg/ml (11) compared to an MIC of 3.2 to 12.5 μg of chloramphenicol/ml for untransformed wild-type and mutant II-3 *M. pneumoniae*. Similar levels of chloramphenicol resistance were seen for transcriptional fusions with a promoterless *cat*

cloned into the *Bam*HI site of *hmw3* (MIC of 50 to 100 µg of chloramphenicol/ml). Deletion of PromP32 had no effect on chloramphenicol resistance, while deletion of PromP21 rendered the transformants chloramphenicol sensitive at levels comparable to the untransformed controls (Fig. 2.5). Chloramphenicol resistance in these promoter deletion transformants correlated with CAT activity as measured by the FAST CAT Green (deoxy) assay (data not shown).

Transcript size.

Northern blot hybridization analysis of *hmw1*- and *hmw3*-specific mRNA failed to establish the cotranscription of these genes, probably due to the large transcript size predicted (up to 13.5 kbp) (data not shown). Therefore, RT-PCR was used to evaluate transcriptional linkage in the *hmw* gene cluster and to identify the likely 5' and 3' ends of the transcripts (Table 2.3). Primer pairs for RT-PCR were chosen to enable synthesis of PCR products 400 to 1,500 bp in length that would span each intergenic region and to test whether putative terminators flanking this gene cluster actually coincided with the 5' and 3' ends of the operon. RT-PCR products of the predicted length were generated for all primer pairs spanning intergenic regions from ORF *p32* to *rpsD* (Fig. 2.6A; Table 2.1). Importantly, control reactions containing total RNA without reverse transcriptase enzyme or else containing reverse transcriptase but not RNA yielded no PCR products. As expected from the location of the predicted transcriptional terminators, no RT-PCR products were observed extending upstream from ORF *p32* or downstream from *rpsD* (Fig. 2.6B). In both cases the expected PCR product was observed when cloned DNA corresponding to each region was included in the control samples (Fig. 2.6B).

Discussion

The *hmw* gene cluster of *M. pneumoniae* spans nearly 13 kbp from ORF *p32* through *rpsD*, including the genes for the cytadherence proteins P30, HMW1, and HMW3, and is flanked by predicted stem-loop structures (Fig. 2.1). Washio et al. calculate, based upon free-energy changes around stop codons, that hairpin termination is evident, for example, in *E. coli*, but is not evident in several archeal genomes and is questionable in mycoplasma genomes (35). However, their calculations are averaged around stop codons over the entire genome and may not necessarily apply to any given ORF. Furthermore, our RT-PCR findings are consistent with a terminator function for the predicted stem-loops flanking the *hmw* operon (Table 2.3). The ORFs of this gene cluster are oriented in the same direction and, based upon the data presented here, are transcribed in four overlapping transcripts. Little is known regarding the potential regulation of transcription in mycoplasmas, and this gene cluster is a good candidate for such studies, largely because of the availability of several mutants lacking products of this locus, making it possible to use complementation techniques to explore genetic elements necessary for expression.

Analysis by S1 nuclease protection indicated that *hmw3* is expressed by overlapping transcripts beginning approximately 2.0 and 2.6 kbp upstream. Subsequent studies by primer extension identified the 5' end of transcripts immediately upstream of ORF *p32* and ORF *p21*. Each was preceded by a likely Pribnow-Schaller box (TAAAAT) 6 bp upstream of the 5' end of the mRNA (Table 2.2). The sequence corresponding to the putative -35 region was not conserved. The putative -10 sites in the *hmw* operon were identical to that of the P65 operon (21) but differed from predicted -10 sequences

for other *M. pneumoniae* genes (Table 2.2). We assessed the predicted and actual frequency of each putative -10 sequence in the *M. pneumoniae* genome, as well as the number of each found within 100 bases upstream of an ORF or RNA gene (Table 2.4). The ratio of the actual to the predicted number of ORFs preceded by the 8-base sequence identified here was even higher than that for the consensus σ^{70} promoter (Table 2.4). Perhaps more significant, however, was the finding that, among the genes downstream of a TTAAAATT promoter-like sequence was the gene encoding P200 (data not shown). This protein shares common deduced structural features with HMW1, HMW3, and P65, and all are associated with the mycoplasma cytoskeleton (26). The relative incidence of this 8-base sequence upstream of ORFs in the *M. pneumoniae* genome and its association with transcription of a family of structurally and functionally related proteins may reflect some form of regulation for this putative promoter.

Deletion studies were conducted to assess the requirement for each promoter-like region in the expression of *p30* and *hmw3*. Recombinant transposons were engineered that contained a *cat* transcriptional fusion in *hmw3* but that lacked specific putative promoters. These were transformed into wild-type *M. pneumoniae* and the cytadherence mutant II-3. Deletion of PromP32 had no effect on P30 production or CAT activity. However, deletion of PromP21 resulted in very little recombinant P30 and no chloramphenicol resistance (Fig. 2.5). Given the results from the nuclease protection studies, it is not clear why PromP32 was insufficient for the production of P30 or CAT. The deletion of PromP21 removed the last six codons from *p32* and the first 60 codons from *p21*. Therefore, if P30 translation were coupled to that of P21, this might account for the inability to produce P30 with deletion of PromP21. The failure to produce CAT

with the deletion of PromP21 cannot be attributed directly to translational coupling, but the translation of P21 or P30 may be essential for RNA stability or ribosome-binding site accessibility. Preliminary analysis by RT-PCR detected *cat*-specific transcripts despite the absence of CAT activity (data not shown), a finding consistent with this scenario. Nevertheless, the role of the overlapping transcripts encompassing *p30* and *hmw3* is unclear. They may reflect a means for optimizing the stoichiometry of the gene products or perhaps a mechanism for the regulation of expression, for example, during cell growth and development of the tip structure. Additional studies employing site-specific mutagenesis in each putative promoter, or the introduction of nonsense mutations within the genes for P21 and P30, may provide more insight.

Analysis by primer extension identified two additional likely transcriptional start sites within the *hmw* gene cluster, one just upstream of ORF *p50* and the second immediately upstream of *rpsD*. Both were preceded by a -10 sequence identical to that of PromP32 and PromP21 and appropriately spaced upstream from the +1 site of the mRNA. Like PromP32 and PromP21, PromP50 and PromRpsD exhibited no conserved -35 region. Given the sequence identity within the -10 regions identified here, we feel that it is highly probable that these represent *M. pneumoniae* promoter elements. The function of the overlapping transcripts generated is not known but may reflect the same requirements as with the putative promoters in the upstream region of this gene cluster.

The presence of a likely promoter immediately upstream of *rpsD* was surprising. The *rpsD* in closely related gram-positive organisms is subject to autogenous control by the gene product, ribosomal protein S4, which binds to a region in the untranslated leader of the monocistronic *rpsD* transcript (8, 9). However, the very short untranslated

leader observed in *M. pneumoniae* would seem to preclude this mechanism of regulation.

Attempts to determine the size of *hmw1*- or *hmw3*-specific mRNA by Northern blot hybridization have been unsuccessful. As an alternative means to assess transcript size, we evaluated whether each intergenic region in the *hmw* gene cluster could be spanned by RT-PCR. As expected, no RT-PCR products were observed with primers flanking the putative terminators on either side of the gene cluster. Furthermore, each intergenic region was bridged by RT-PCR, suggesting that these genes are cotranscribed as a unit. The cotranscription of *p30*, *hmw1*, and *hmw3* is consistent with coordinated function and suggests that the putative proteins encoded by ORFs *p32*, *p21*, *p43*, *p36*, *p50*, and *p33* may also participate in the assembly or structure of the attachment organelle (36). The predicted weak stem-loop structure upstream of *hmw1* may have a regulatory role and help account for the apparent overlapping transcript in this region of the gene cluster. However, because of the sensitivity of PCR, we cannot rule out the possibility that the RNA detected may be produced at trace rather than biologically significant levels. In the absence of likely transcription terminators, however, we predict that the 3' end of the transcript extends to the end of the gene cluster (Fig. 2.1). Finally, the *hmw* gene cluster is reminiscent of the superoperons described in *E. coli* containing genes for diverse cellular functions (34). Hence, interpretation of their probable cotranscription as an indication of coordinate function must be approached cautiously, especially since the *rpsD* gene, encoding a ribosomal protein, appears to be cotranscribed with the *hmw* genes. Additional studies are required to establish whether

the products of each unknown ORF are required in tip assembly, cell division, and/or cytadherence.

Mycoplasmas continue to be paradoxical. Their greatly reduced genome, lack of obvious transcriptional regulators, parasitic lifestyle, and small size belie a complex subcellular structure and regulation. The studies described here reveal a complex transcriptional organization when a much simpler pattern was expected. A better understanding of the role of overlapping transcripts and multiple promoter-like sites is likely to increase our appreciation for this unusual microorganism.

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FIG. 2.1. Map of the *hmw* gene cluster of *M. pneumoniae*.

The number of the first nucleotide relative to the published genome sequence of *M. pneumoniae* (15) is given to the left, and the scale in kilobase pairs is shown below the map. The genes for *p30*, *hmw3*, *hmw1*, serine tRNA (t), and *rpsD* are indicated, as are ORFs encoding predicted proteins of unknown function. Stem-loop structures predicted to function as terminators are designated by solid circles, while a possible attenuator is indicated by the open circle. The promoter-like sequences evaluated by primer extension are indicated, with those underlined by arrows yielding primer extension products. The predicted overlapping transcripts, based upon S1 nuclease protection, primer extension, and RT-PCR, are shown below the map. B, *Bam*HI; E, *Eco*RI; K, *Kpn*I; X, *Xba*I.

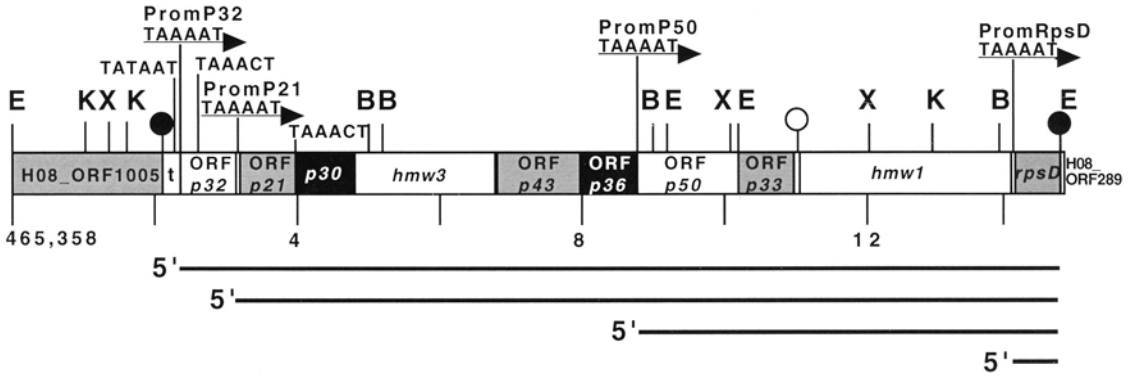
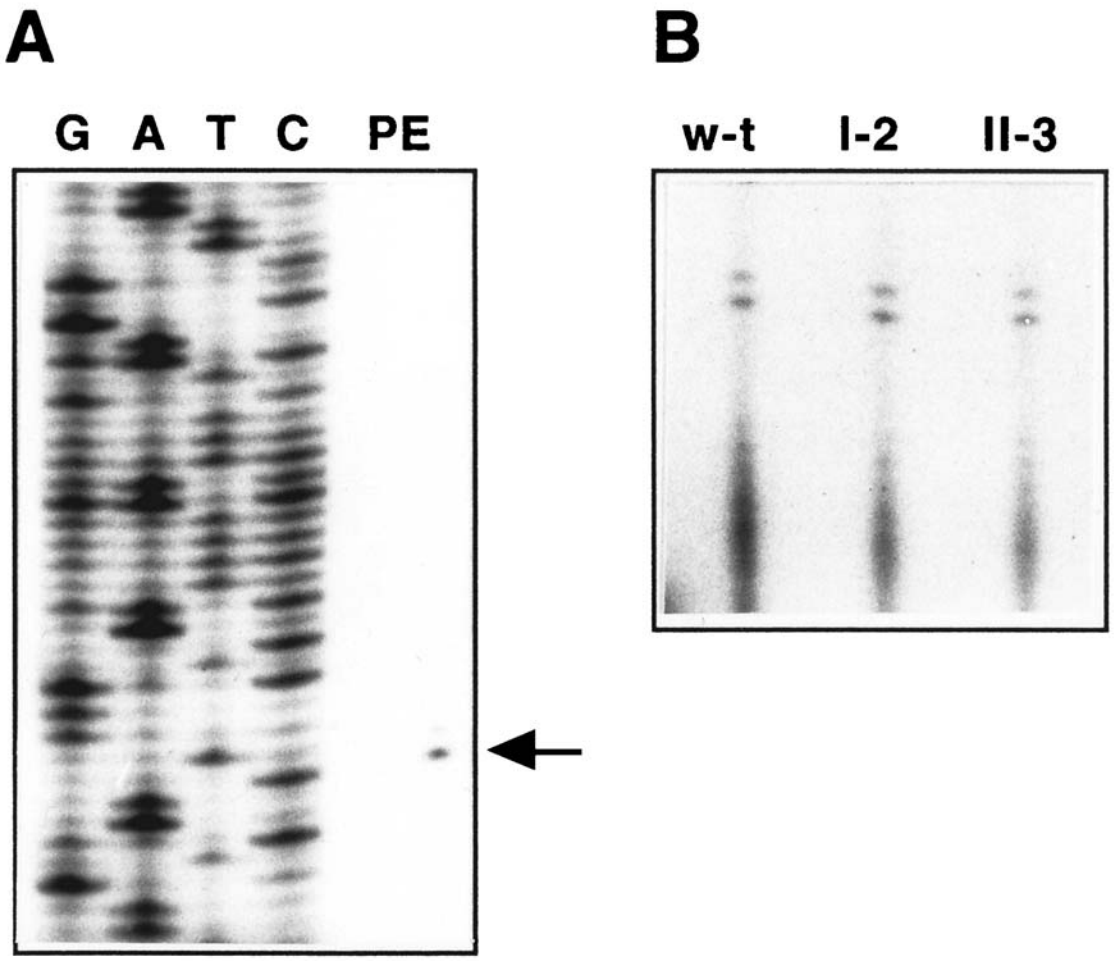

PromP32
TAAAAT
TATAAT
TAAACT
PromP21
TAAAAT
TAAACT
PromP50
TAAAAT
PromRpsD
TAAAAT
E
KX K
BB
BE
XE
X
K
B
E
H08_ORF1005
t
ORF p32
ORF p21
p30
hmw3
ORF p43
ORF p36
ORF p50
ORF p33
hmw1
rpsD
H08_ORF289
465,358
4
8
12
5'
5'
5'
5'

FIG. 2.2. Primer extension analysis upstream of ORF *p32*.

The 5' end of the oligonucleotide primer (5'-GACAAACTGTTGCTCGGAAAAATTGACCTG; sense strand) corresponds to nt 467836 of the *M. pneumoniae* genome (15).

(A) The primer extension product (PE) obtained with wild-type *M. pneumoniae* RNA as a template is indicated by the arrow. The sequencing ladder obtained with the same primer and cloned *hmw* gene cluster DNA as template is given, with the corresponding sequence (sense strand) shown below. The asterisk indicates the +1 site, and a likely -10 box is underlined.

(B) Primer extension products with equal amounts of RNA from wild-type *M. pneumoniae* (w-t) and cytadherence mutants I-2 and II-3. Control reactions lacking RNA template or reverse transcriptase yielded no primer extension product (data not shown).



AATTGCGCAATGTTTAATTTTAACTGGGT*CAACTGAA

FIG. 2.3. Primer extension analysis upstream of ORF *p21*.

(A) Primer extension analysis upstream of ORF *p21*. The 5' end of the primer (5'-CTTCCTTAGAGAGAAAAAACGGTTAAACACATCCATTG; sense strand) corresponds to nt 468897 of the *M. pneumoniae* genome (15). The primer extension product (PE) obtained with wild-type *M. pneumoniae* RNA as a template is indicated by the arrow. To the left is the sequencing ladder, as described in the legend of Fig. 2.2. The asterisk indicates the +1 site, while a likely -10 box is underlined.

(B) Primer extension products obtained with equal amounts of RNA from wild-type *M. pneumoniae* (w-t) and cytadherence mutants I-2 and II-3. Control reactions lacking RNA template or reverse transcriptase yielded no primer extension product (data not shown).

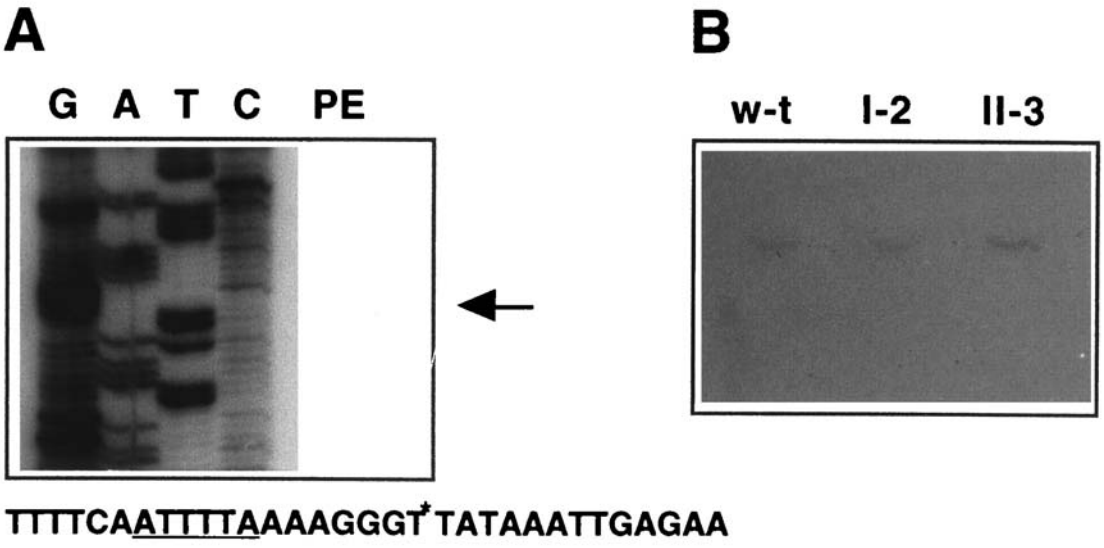
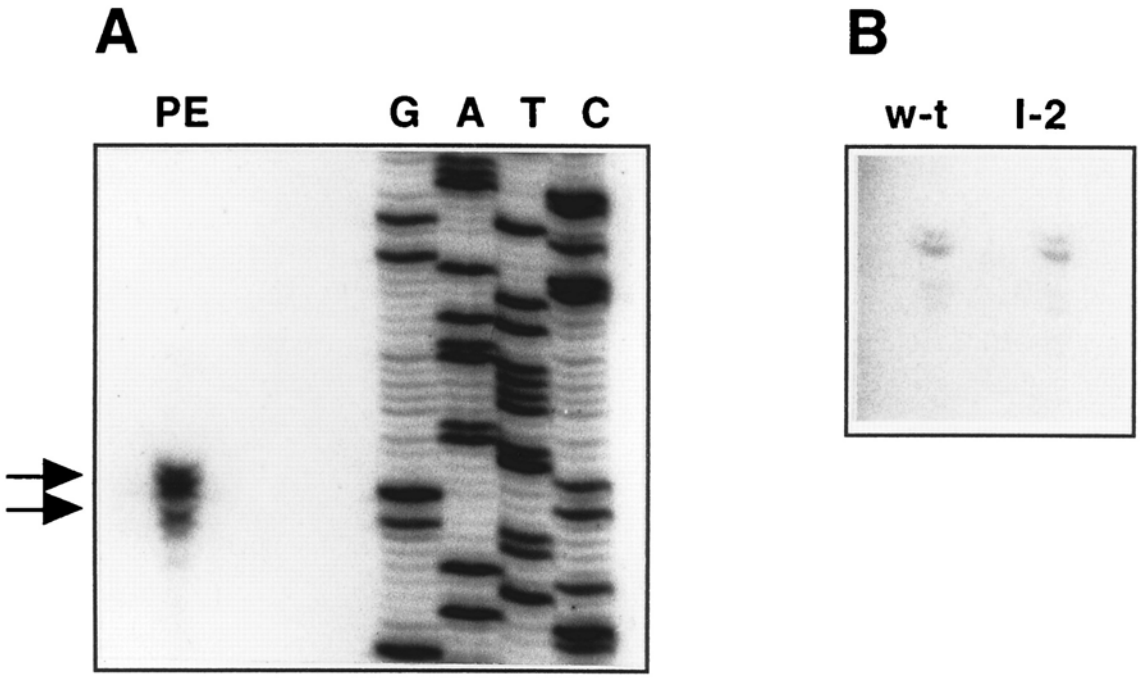

A
G A T C PE
B
w-t I-2 II-3
TTTTCAATTTTAAAAGGGT* TATAAATTGAGAA

FIG. 2.4. Primer extension analysis upstream of ORF *p50*.

(A) Primer extension analysis upstream of ORF *p50*. The 5' end of the primer (5'-CACGAAGGTCTTTAGCAAATG; sense strand) corresponds to nt 474144 of the *M. pneumoniae* genome (15). The primer extension products (PE) obtained with wild-type *M. pneumoniae* total RNA as a template are indicated by arrows. The sequencing ladder is shown to the left, as described in the legend of Fig. 2.2. The sequence (sense strand) is given below, with asterisks indicating the +1 nucleotides, and a likely -10 box is underlined.

(B) Primer extension products synthesized with equal amounts of RNA from wild-type *M. pneumoniae* (w-t) and cytadherence mutants I-2 and II-3. Control reactions lacking RNA template or reverse transcriptase yielded no primer extension product (data not shown).



AAACCGTCGACCTATAATTTTAATTC* GC* GTTACTACCG

FIG. 2.5. Analysis of *p30* and *hmw3-cat* expression with deletion of the putative promoter regions.

Construction of pKV193 in Tn*4001*mod (17) and deletion of PromP32 and PromP21 are described in detail in the text. The scale is in kilobase pairs. PromP32 and PromP21 are indicated above the restriction map, with arrows underlining the predicted -10 region. *M. pneumoniae* transformants for each construct were evaluated for P30 synthesis, hemadsorption (HA), and growth on chloramphenicol. B, *Bam*HI; Bc, *Bcl*I; Bg, *Bgl*II; C, *Csp*45I; M, *Mlu*I; R, *Eco*RV; S, *Spe*I; Sm, *Sma*I.

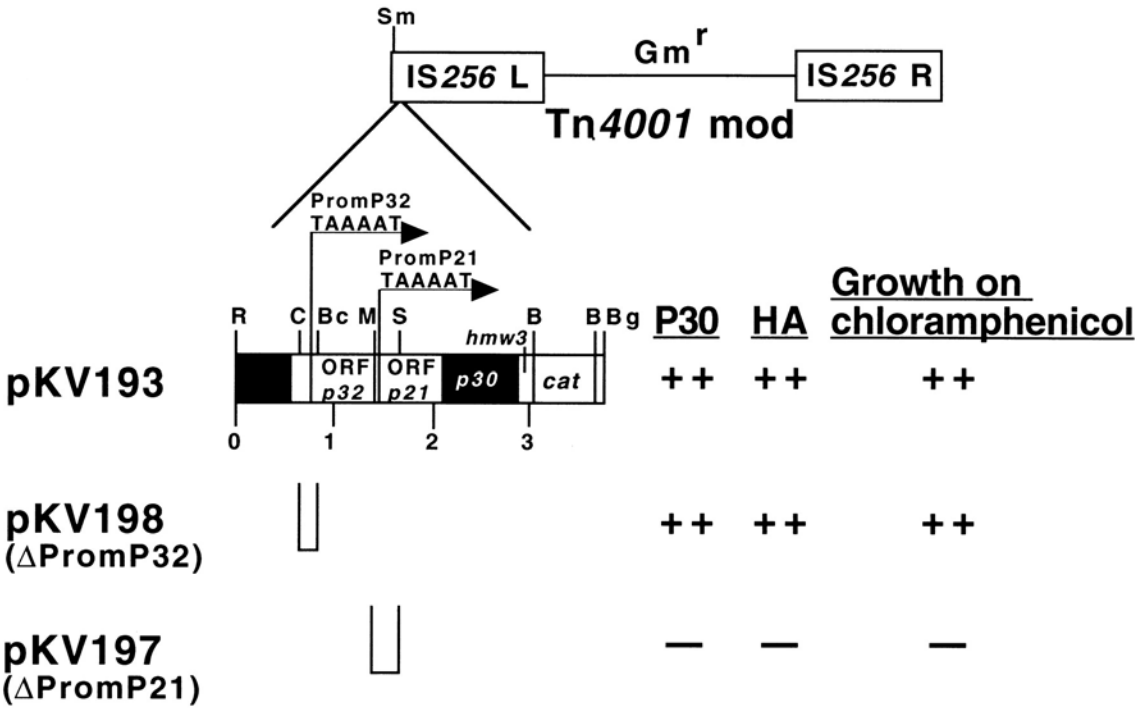

Sm
IS256 L
Gm r
IS256 R
Tn4001 mod
PromP32
TAAAAT
PromP21
TAAAAT
R C Bc M S hmw3 B BBg
ORF p32
ORF p21
p30
cat
0 1 2 3
P30
HA
Growth on chloramphenicol
pKV193
++ ++ ++
pKV198
(ΔPromP32)
++ ++ ++
pKV197
(ΔPromP21)
— — —

FIG. 2.6. Analysis of the *hmw* gene cluster by RT-PCR.

The primers used and the predicted sizes of the products are listed in Table 2.3.

(A) RT-PCR products from primers designed to span the intergenic regions between *p32-p21*, *p21-p30*, *p30-hmw3*, *hmw3-p43*, *p43-p36*, *p36-p50*, *p50-p33-hmw1*, and *hmw1-rpsD* (lanes b, c, d, e, f, g, h, and i, respectively).

(B) RT-PCR reactions for primers spanning the regions between H08_orf1005-*p32*, and *rpsD*-H08_orf289 (15) (Fig. 2.1) are shown in lanes b and c, respectively. PCR products for the indicated primers with genomic DNA as a template are in the lanes marked "DNA". The sizes of the DNA markers in lanes a are indicated in base pairs, and a plus (+) indicates that reverse transcriptase was included, while a minus (-) indicates that reverse transcriptase was omitted.

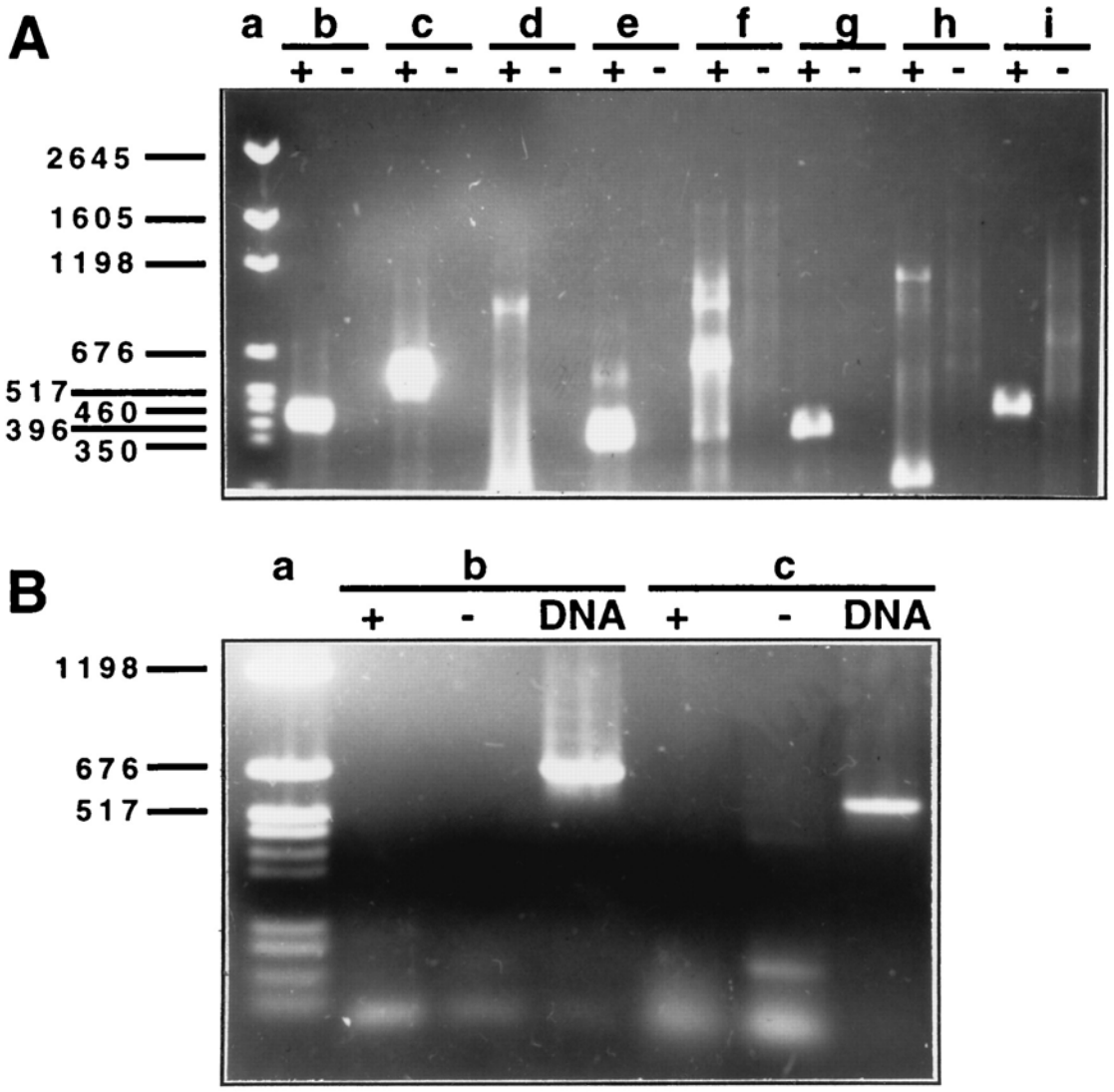

A
a
b
c
d
e
f
g
h
i
+ - + - + - + - + - + - + - + -
2645
1605
1198
676
517
460
396
350
B
a
b
c
+ - DNA + - DNA
1198
676
517

TABLE 2.1. Relevant phenotype of the *M. pneumoniae* strains used in this study.

Strain	Defect	Relevant phenotype[a]					
		P1	P30	HMW1	HMW2	HMW3	HA
Wild type	None	++	++	++	++	++	+
I-2	Frameshift in *hmw2*	++	++	+/-	-	+/-	-
II-3	Frameshift in *p30*	++	-	++	++	++	-

[a] ++, protein present at wild-type levels; +/-, protein present at greatly reduced levels; -, protein absent. Hemadsorption (HA) is indicated as either present (+) or absent (-).

TABLE 2.2. Nucleotide sequence of putative promoter regions from *M. pneumoniae.*

Putative promoter	Sequence[a]	Source or reference
PromP32	CATTTATTAGCAATTAAGCGCTTACAAA**TTAAAATT**GACCCAGTT	This study
PromP21	GTAAGTGGGGAATTAAAGCTTTGAAAAG**TTAAAATT**TTCCCAATA	This study
PromP50	TTAATTAATGCGATTTGGCAGCTGGATA**TTAAAATT**AAGCGCAAT	This study
PromRpsD	GGATTTTAATCTTGATTATTAAGATAAT**TTAAAATT**GTCACCACA	This study
PromP65	TTTATTCATTTGCATTTTTTTAGATAAA**TTAAAATT**AATGGTATA	21
PromORF4	ACGACAACAACAGTTGCTGTTTGATTCT**TTAAACTT**AAACAGAAT	22
Prom4.5S	AAATTAATTGATTAAAAGCAGTTAAGTG**TTAGAATT**TAGAGCCGT	30
Prom16S	CTTTAAACATAAATAAAAAAGTTTTTCTG**TATAAT**CTTCAGGCTG	16
Prommp1	AAGGATTGTCAAAAATATTTTTAAAGTGC**TAGAAT**AAAGC	5

[a] The conserved -10-like region is shown in bold-face, while the +1 nucleotide(s) for each transcript is underlined

TABLE 2.3. Summary of RT-PCR analysis of the *hmw* gene cluster.[a]

ORF spanned	nt	Sequence	PCR-predicted Size (bp)	RT-PCR		
				+RT	-RT	PCR
H08_orf1005-*p32*	(+)467241	5'-CCAGCTCAACCGCTTTAACA	595	-	-	++
	(-)467836	5'-GACAAACTGTTGCTCGGAAAAATTGACC				
p32-p21	(+)468446	5'-TCGGTTTACTTTGTTTGTC	451	++	-	++
	(-)468897	5'-CTTCCTTAGAGAAAAAACGGTTAAACACATCCATTG				
p21-p30	(+)468861	5'-CAATGGATGTGTTTAACCGTTTTTTCTCTAAGGAAG	620	++	-	++
	(-)469482	5'-CTCAATTCACTCTTAACTTCTGTC				
p30-hmw3	(+)469458	5'-GACAGAAGTTAAGAGTGAATTGAG	950	++	-	++
	(-)470409	5'-CAAAGCTAATTGGTTCATCACTGTC				
hmw3-p43	(+)472097	5'-GAGCAATTACACCAGTTTCAC	373	++	-	++
	(-)472470	5'-GTCAGTTCGCTTTCATTAGTTTG				
p43-p36	(+)473154	5'-CAGTGAGCCTTAAACACGAC	637	++	-	++
	(-)473791	5'-CACACAAAACGGCTTCTAAC				
p36-p50	(+)473772	5'-GTTAGAAGCCGTTTTGTGTG	372	++	-	++
	(-)474144	5'-CACGAAGTGCTTTAGCAAATG				
p50-p33-hmw1	(+)475493	5'-CAATGATTTAACACCCCCAG	1,067	++	-	++
	(-)476560	5'-CTGTTCGGGATTTTCCTCAGTG				
hmw1-rpsD	(+)479252	5'-GAGACTGAACCGCAAGCCA	422	++	-	++
	(-)479674	5'-GTTTTCCGCTTCTTCCCTTTC				
rpsD-H08_orf289	(+)479654	5'-GAAAGGGAAGAAGCGGAAAAC	675	-	-	++
	(-)480329	5'-CACACCACTTTCTTTGAGCAG				

[a] For RT-PCR, results are presented with (+RT) and without (-RT) reverse transcriptase present. -, No RT-PCR product detected; ++, RT-PCR product detected. For PCR alone, "++" indicates that PCR product was detected in control samples with the appropriate DNA template.

TABLE 2.4. Predicted and actual frequency of putative promoter sequences in the *M. pneumoniae* genome.

Putative Promoter sequence	Operon	Total no.		No. upstream of start site[b]		
		Predicted[a]	Found	Predicted	Found	Found/predicted
TTAAAATT	HMW[c]	106	93	4.6	23	5
TAAAAT	HMW[c]	1,188	609	51	97	2
TAAACT	P1[d]	794	935	34	50	1.5
TATAAT	σ^{70}[e]	1,188	117	51	47	0.9
TAGAAT	Other[f]	794	188	34	29	0.9

[a] Predictions are based on the *M. pneumoniae* genome size and the G+C content.
[b] Predicted within 100 bp upstream.
[c] 8-Base (and internal 6-base) 10-like sequence in the HMW operon.
[d] 6-Base 10-like sequence in the P1 operon.
[e] 6-Base consensus 10 sequence.
[f] 6-Base 10-like sequence of Prommp1, Prom4.5S.

CHAPTER 3

IDENTIFICATION AND COMPLEMENTATION OF A MUTATION ASSOCIATED WITH LOSS OF *MYCOPLASMA PNEUMONIAE* VIRULENCE-SPECIFIC PROTEINS B AND C[2]

[2] Waldo RH 3rd, Jordan JL, Krause DC. 2005. J Bacteriol. JB01256-04. In press. Reprinted here with permission of publisher.

Abstract

A mutation in gene MPN142 (*orf6*) was identified in the *Mycoplasma pneumoniae* cytadherence mutant III-4. MPN142 encodes virulence-specific proteins P90 and P40 (B and C, respectively). Analysis of MPN142 in a cytadhering revertant and complementation using a recombinant wild-type allele confirmed the role of this mutation in the cytadherence defect.

Introduction

The cell wall-less bacterium *Mycoplasma pneumoniae* is a significant cause of bronchitis and atypical pneumonia in humans. Early in the course of disease the bacteria establish an intimate association with the host respiratory epithelium (cytadherence), mediated largely by a differentiated polar structure, the attachment organelle. A number of proteins have been identified that are associated with the attachment organelle and involved in cytadherence, some directly but most by means other than a classical receptor-binding role. These accessory proteins are essential to the architecture of the attachment organelle, providing a scaffolding for the localization and/or maturation of other proteins such as the major adhesin, P1. This large transmembrane protein is densely clustered, although not exclusively, at the attachment organelle of wild-type *M. pneumoniae* cells (9).

Passage of *M. pneumoniae* in broth culture leads to the spontaneous loss of cytadherence at a high frequency (11). One such cytadherence mutant, designated III-4, lacks the three virulence-specific proteins A, B, and C (72, 85, and 37 kDa, respectively), originally described by comparing virulent and avirulent strains of *M.*

pneumoniae by two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) (6). Proteins B and C appear to correspond to the 90-kDa and 40-kDa products of MPN142 (*orf6*), respectively. The predicted product of MPN142 is 130 kDa, but antisera raised against recombinant protein fragments derived from regions at the 5' and 3' ends of MPN142 detected proteins of 40-kDa and 90-kDa, respectively, in wild-type *M. pneumoniae* (22), indicating that the gene product undergoes a processing event (14). P90 and P40 are integral membrane proteins located at the attachment organelle in wild-type *M. pneumoniae* (3) in proximity to P1 (15). In mutant III-4 and the apparently similar non-cytadherent strain M5 (13), which also lacks the 90- and 40-kDa proteins, P1 is located at a focus in one or more but not all branched structures (19, 20). Mutants III-4, M5, and an avirulent high broth-passage strain (6, 12, 16) lack the 90- and 40-kDa proteins detected with antibodies to the MPN142 gene products, suggesting that B and C correspond to the 90- and 40-kDa proteins, respectively. Despite the availability of the *M. pneumoniae* genome sequence, the identity of protein A or its gene remains unknown.

The genetic defect responsible for the cytadherence phenotype of mutant III-4 has not been previously determined. Here we identify a frameshift mutation in MPN142 of mutant III-4 and demonstrate that a spontaneously arising, cytadhering revertant of III-4 (III-4-R1) restores the MPN142 reading frame. Furthermore, a recombinant wild-type MPN142 was engineered and introduced into mutant III-4 restoring a wild-type phenotype.

Experiments

Using primers corresponding to the published genome sequence (7) and genomic DNA from mutant III-4 as template, regions of MPN142 and flanking sequences were amplified to yield five overlapping PCR fragments. These were cloned into pCR®II using the TA Cloning® Kit (Invitrogen, Carlsbad, Cal.) and sequenced for both strands (IBL, University of Georgia). A direct repeat of the sequence TAAA was identified at nucleotide 14 in the coding sequence of MPN142 in mutant III-4 but not in wild-type *M. pneumoniae,* causing a frame-shift (Fig. 3.1). This difference from the published sequence was confirmed by repeated amplification and sequencing. The resulting gene encodes a peptide of 98 residues, compared to 1218 residues for wild-type MPN142.

In previous studies the identification of cytadhering revertants of *M. pneumoniae* adherence mutants required enrichment by attachment to erythocytes (10) or inert surfaces (18). A cytadhering revertant of mutant III-4 (III-4-R1) was obtained by repeatedly enriching for attachment to plastic. The attachment of erythrocytes to *M. pneumoniae* colonies (hemadsorption) is a convenient model for cytadherence (21), and III-4-R1 was hemadsorption-positive (Fig. 3.2A) using the qualitative hemadsorption assay performed as described previously (4) except that sheep blood was used. The MPN142 gene of III-4-R1 was sequenced as described for III-4, revealing that the reading frame was restored by a single deletion in a stretch of five thymine bases starting at nucleotide 37 in the coding sequence of wild-type MPN142. The resulting deduced amino acid sequence differed from wild-type by the nine residues defined by the sites of the original and second-site mutations (Fig. 3.1). In addition to a

hemadsorption-positive phenotype, III-4-R1 produced wild-type levels of P90 and P40 (Fig. 3.4) and exhibited a wild-type cellular morphology and P1 localization pattern (Fig. 3.5A) when examined using methods described previously (1).

The genetic tools available for *M. pneumoniae* are limited. Neither allelic exchange nor suitable plasmid vectors have been described, but complementation is possible by recombinant transposon delivery (4). Further, complementation studies with mutant III-4 have been difficult because of the complexity of the operon that includes MPN142. The MPN142 gene lacks an obvious ribosome-binding site, and a frameshift mutation in the upstream MPN141 gene encoding P1 also results in the loss of proteins B and C (23), raising the possibility of translational coupling between P1 and the B/C precursor. Therefore, a construct was engineered with MPN141 and MPN142 in Tn*4001*mod (5) under the transcriptional control of the P_{out} promoter (2) of the transposon, and with certain restriction sites removed to facilitate future studies. Briefly, a 9.1-kb fragment beginning 345 bp upstream of MPN141 and extending through MPN142 was excised from pCOS_E07 (24) using *Pme*I and *Sph*I (Fig. 3.3a) and cloned into pGEM-7zf+ (Promega, Madison, Wis.) to yield pKV208. The construction of pKV258 from pKV208 is described in Figure 3.3.

In order to engineer a construct that would not introduce a second copy of wild-type MPN141 we created a deletion derivative of pKV258. Briefly, a plasmid containing a 5.7-kb *Eco*RI fragment bearing MPN141 in pUC19 (a gift from J. Baseman) was digested with *Bsa*BI and *Stu*I and religated. This resulted in an in-frame internal deletion of 95% of MPN141 (88 residues compared to 1627 in wild-type). The 1.1 kb *Eco*RI fragment bearing the altered MPN141 was exchanged with the 5.7 kb *Eco*RI

fragment of pKV258 containing MPN141 to yield pKV264, which contains the altered MPN141 and wild-type MPN142 (Fig. 3.3). We hypothesized that this altered MPN141 would allow the production of MPN142 at wild-type levels if translational coupling were required, while removing potential effects of higher MPN141 gene dosage when transformed into the *M. pneumoniae* mutant III-4 background.

Plasmids pKV258 and pKV264 were transformed into wild-type *M. pneumoniae* and mutant III-4. Transformants were isolated and immunoblot and hemadsorption analyses performed as previously described (4). Several transformants were examined for each plasmid/background combination to control for potential variability associated with the site of transposon insertion. Wild-type levels of P90 and P40 were observed in wild-type and mutant III-4 *M. pneumoniae* transformed with pKV258 or pKV264 when examined by sodium dodecyl sulfate-PAGE and immunoblotting with P90- or P40-specific antisera; as expected, P90 or P40 were not observed in mutant controls (Fig. 3.4). The transformants attached to plastic (data not shown), were hemadsorption-positive (Fig. 3.2B), and exhibited a wild-type P1 localization pattern (Fig. 3.5B). Mutant III-4 transformed with pKV258 had a pronounced extended cellular morphology compared to wild-type *M. pneumoniae* and mutant III-4 transformed with pKV264 (Fig. 3.5B). This phenotype was consistent in independent transformants, and we speculated it might be due to the increased gene dosage of MPN141 in mutant III-4 with pKV258. However, when an extra copy of MPN141 without MPN142 was added to wild-type *M. pneumoniae*, transformant cells appeared normal (data not shown), and the explanation and importance of this observation is not known.

Discussion

Several lines of evidence suggest that the virulence-specific proteins B and C described originally by Hansen et al. (6) correspond to the P90 and P40 protein products of MPN142, but a definitive relationship has not been previously established. Thus, the possibility remained that the cytadherence defect in mutant III-4 for example was not the direct result of the absence of proteins B and C. Furthermore, the inability to identify the gene for protein A has contributed some measure of uncertainty regarding cause and effect. Here we demonstrate conclusively that the genetic defect responsible for the loss of cytadherence in mutant III-4 is a frameshift in the gene MPN142. This defect can be rescued by a second-site mutation that restores the MPN142 reading frame, or by complementation with the wild-type MPN142 allele.

Our attempts to determine the identity of the protein A spot, which is seen in wild-type *M. pneumoniae* only by 2-D PAGE using isoelectric focusing in the first dimension (6) and is absent in mutant III-4 (11), were unsuccessful. While the identity of protein A remains unclear, we speculate that its loss may be a secondary consequence of the mutation in MPN142, perhaps in the same manner that loss of cytadherence-associated protein HMW2 is accompanied by reduced steady-state levels of HMW1, HMW3 (17), and P65 (8).

Finally, the construction of pKV258 and pKV264 and the ability to express their recombinant proteins in and complement a mutant background provide opportunities for more detailed studies into the nature of proteins B, C, and P1. For example, recombinant derivatives of each can be examined in wild-type and mutant *M.*

pneumoniae for structure-function analyses of these proteins, their inter-relationships, and the mechanisms of their processing and maturation.

Acknowledgements

We thank Richard Herrmann for the kind gift of P90- and P40-specific antisera in addition to pCOS_E07. The plasmid containing MPN141 was a gift from Joel Baseman. Kyungok Lee provided invaluable technical assistance in the construction of pKV258. This work was supported by the Public Health Service research grant AI23362 from the National Institute of Allergy and Infectious Diseases (D.C.K.) and a National Science Foundation Research Training Grant in Prokaryotic Diversity (NSF BIR9413235) (R.H.W.).

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FIG. 3.1. Sequence comparisons of MPN142 of wild-type and *M. pneumoniae*, cytadherence mutant III-4, and revertant III-4-R1.

(A) Nucleotide sequence. Nucleotide one is the annotated start of the gene. The TAAA acquired in mutant III-4 and the T lost in the reversion of III-4 to III-4-R1 are shown in bold. **(B)** Deduced amino acid sequence. Dashes show an alignment gap. Ellipsis mark denotes a break in the representation. Asterisk denotes a translational stop. Note the order of the strains differs between A and B for convenience in alignment.

A

```
                    .         .         .         .         .
WT          1 ATGAAATCGAAGC----TAAAGTTAAAACGTTATTTACTGTTTTTACCAC 46
              ||||||||||||    ||||||||||||||||||||||||||||||||
III-4       1 ATGAAATCGAAGCTAAATAAAGTTAAAACGTTATTTACTGTTTTTACCAC 50
              |||||||||||||||||||||||||||||||||||| |||||||||
III-4-R1    1 ATGAAATCGAAGCTAAATAAAGTTAAAACGTTATTTACTG-TTTTACCAC 49
```

B

```
                  .         .         .         .
WT          1 MKSKL-KLKRYLLFLPLLPLGTLSLANTYLLQDHNTLTPY           39
              ||||| | |     |||||||||||||||||||||||||
III-4-R1    1 MKSKLNKVKTLFTVLPLLPLGTLSLANTYLLQDHNTLTPY           40
              ||||||||||||||
III-4       1 MKSKLNKVKTLFTVFTTFTARDVVTSQHLPPPRPQHPHPLH...EFGSK* 99
```

FIG. 3.2. Qualitative hemadsorption screening of *M. pneumoniae* colonies.

(A) Wild-type (WT), mutant III-4, and revertant (III-4-R1). **(B)** Transformants (III-4 + pKV258 or pKV264).

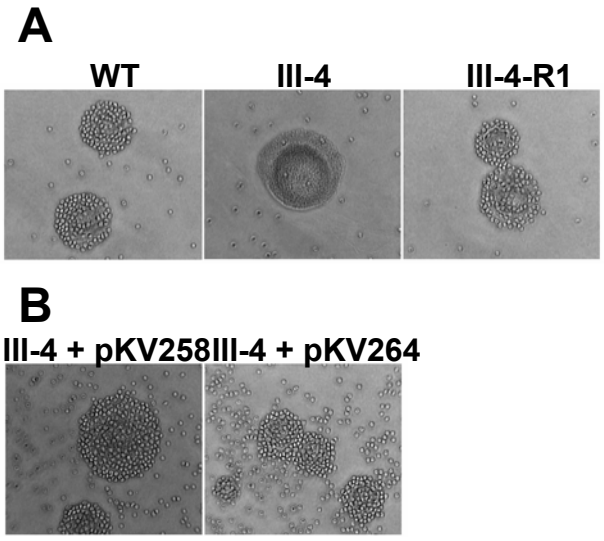

A
WT
III-4
III-4-R1
B
III-4 + pKV258
III-4 + pKV264

FIG. 3.3. Creation of pKV258 and pKV264.

1 The 7.5-kb *Sph*I-*Bam*HI fragment from pKV208 was subcloned into the corresponding sites of pALTER-1 (Promega). **2** Into this was cloned the 1.5-kb *Bam*HI fragment spanning the 5' end of MPN141. **3** A 7.7-kb *Bsi*WI fragment was removed to reduce the size of the plasmid temporarily for efficient silent mutagenesis. **4** The 2nd and 3rd *Eco*RI sites in MPN142 were silently changed to GGATTC using the AlteredSites® II mutagenesis kit (Promega), and the *Sph*I site was replaced with an *Mfe*I site using complementary synthetic linkers (5'-CAATTGTCATG and 5'-ACAATTGCATG). **5** The resulting 1.4-kb *Bam*HI-*Mfe*I fragment was cloned into the *Bam*HI and *Eco*RI sites of the Tn*4001*mod-bearing plasmid pKV74 (5). **6** The 7.7-kb *Bsi*WI fragment was then restored to yield pKV258. To create pKV264, the 4.6-kb *Bsa*BI-*Stu*I fragment in MPN141 was removed to create a massive internal in-frame deletion, as described in the text. B, *Bam*HI; Bi, *Bsi*WI; Bs, *Bsa*BI; E, *Eco*RI; e, destroyed *Eco*RI site; S, *Stu*I; Sm/P, *Sma*I/*Pme*I junction; M/E, *Mfe*I/*Eco*RI junction at the former *Sph*I site. Open arrow from Tn*4001*mod in pKV74 shows the location and direction of the P_{OUT} promoter. Note that the inserts are inverted when cloned into Tn*4001*mod. Figure is not created to scale.

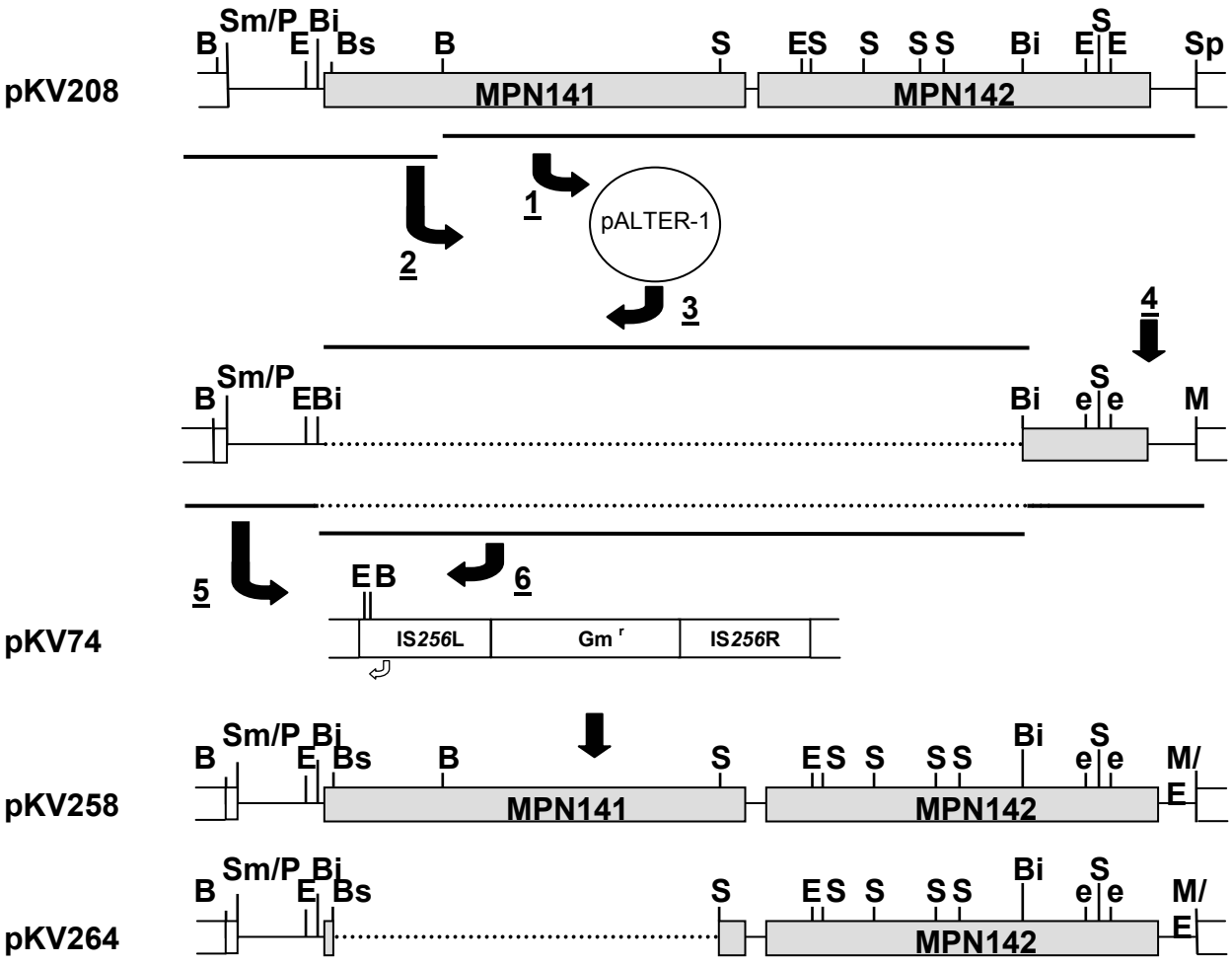

pKV208
B
Sm/P
Bi
E
Bs
B
S
ES
S
S
S
Bi
S
E
E
Sp
MPN141
MPN142
1
2
pALTER-1
3
4
B
Sm/P
EBi
Bi
S
e
e
M
5
E
B
6
pKV74
IS256L
Gm r
IS256R
pKV258
B
Sm/P
Bi
E
Bs
B
S
ES
S
S
S
Bi
S
e
e
M/
E
MPN141
MPN142
pKV264
B
Sm/P
Bi
E
Bs
S
ES
S
S
S
Bi
S
e
e
M/
E
MPN142

FIG. 3.4. Western immunoblot analysis of wild-type (WT), mutant (III-4), revertant (III-4-R1) and transformant *M. pneumoniae* lysates.

Two independent transformants are shown for each transposon/background combination. 40 μg protein used per lane. Anti-P90 (upper) and anti-P40 (lower) sera used at a dilution of 1:1000.

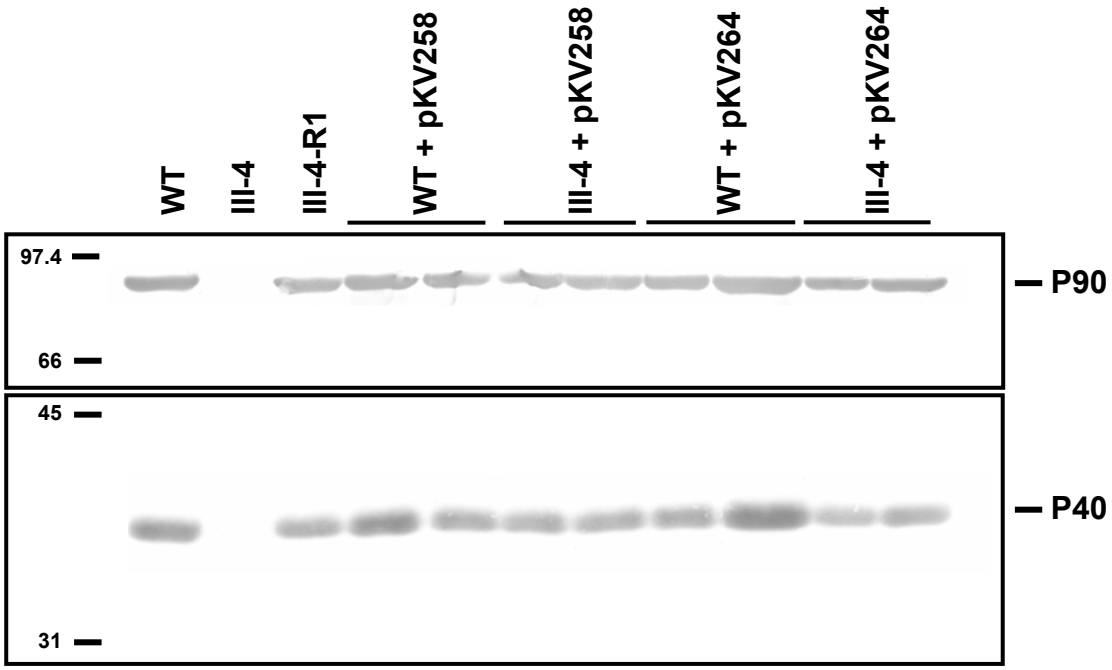
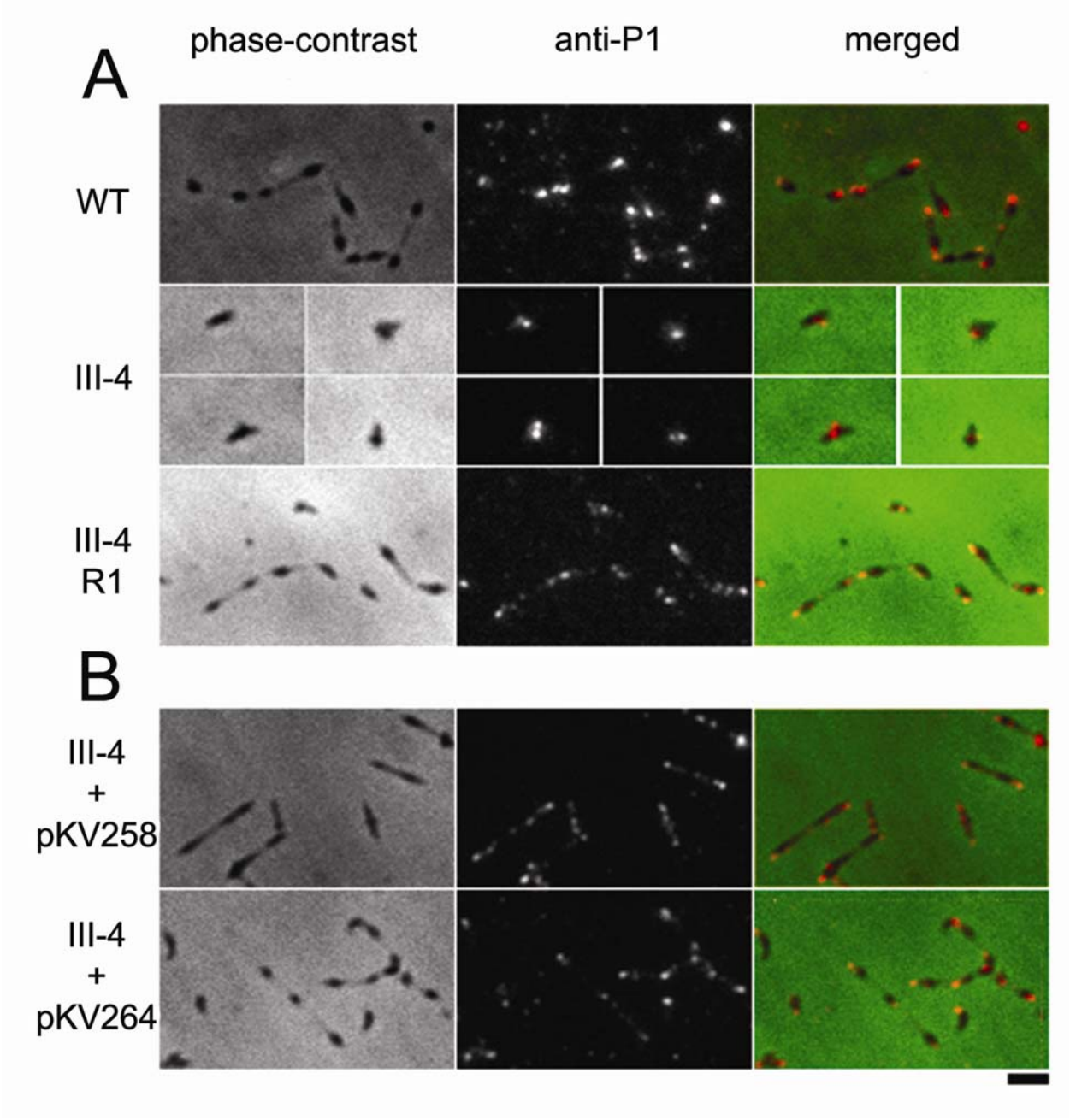

WT
III-4
III-4-R1
WT + pKV258
III-4 + pKV258
WT + pKV264
III-4 + pKV264
97.4
66
P90
45
31
P40

FIG. 3.5. Phase-contrast, anti-P1 immuno-fluorescence, and merged micrographs of *M. pneumoniae*.

(A) Wild-type, mutant III-4, and revertant III-4-R1. Due to the poor growth of mutant III-4 and its sparse distribution on the slide, individual cells are shown in separate panels.

(B) Mutant III-4 transformed with pKV258 or pKV264. Bar is 2 μm.


A
phase-contrast
anti-P1
merged
WT
III-4
III-4
R1
B
III-4
+
pKV258
III-4
+
pKV264

CHAPTER 4

SYNTHESIS, STABILITY, AND FUNCTION OF CYTADHESIN P1 AND ACCESSORY PROTEINS B/C OF *MYCOPLASMA PNEUMONIAE*[3]

[3] Waldo RH 3rd and Krause DC. 2005. To be submitted. J Bacteriol.

Abstract

In *Mycoplasma pneumoniae* the genes MPN141 (*p1*) and MPN142 (ORF6), encode the major adhesin P1 and the cytadherence-related B/C proteins (P90/P40), respectively. Using reverse-transcriptase PCR we found that ORFs MPN140-142 constitute a polycistronic transcriptional unit. Cytadherence mutant IV-22 has a frameshift mutation in MPN141 and does not possess the P1/B/C proteins. Recombinant MPN141 and/or MPN142 were introduced into mutant IV-22 by transposon delivery in several configurations, and levels of the P1/B/C proteins were assessed by immunoblot. MPN142 in mutant IV-22 has a wild-type nucleotide sequence, but MPN141 alone added to mutant IV-22, while capable of resoring P1, was not sufficient to restore protein B/C levels. However, recombinant MPN141 and MPN142 delivered by transposon *in cis* or *in trans* to each other were sufficient to restore all three proteins. These data indicate that some but not all synthesis of B/C was dependent on coupling to the translation of P1 immediately upstream of MPN142. Finally, proteins B and C were not stable in the absence of P1. The linkage of MPN141 and MPN142 at the levels of transcription, translation, and protein stability, in addition to their previously demonstrated co-localization and the requirement of B/C for P1 function, reinforces the conclusion that these proteins constitute a multi-protein complex that functions in receptor binding.

Introduction

The cell wall-less bacterium *Mycoplasma pneumoniae* is a common agent of respiratory infections, including tracheobronchitis, pharyngitis, and atypical or ‘walking’

pneumonia in humans (27). A crucial event in the initiation of infection is the intimate attachment of the bacteria to the respiratory epithelium (cytadherence). This process is essential to disease, and cytadherence mutants are avirulent (16). *M. pneumoniae* possesses a differentiated polar extension of the cell-body called the attachment organelle, which also plays a role in cell division and gliding motility (23). Many of the proteins that are important for cytadherence are located at the attachment organelle, and their proper localization is important for attachment organelle function (13).

P1 is a large integral membrane protein found clustered primarily, but not exclusively, at the attachment organelle of *M. pneumoniae* and is a major cytadhesin (3, 4, 10). Localization of P1 to the attachment organelle is necessary but not sufficient for function, and the generation of properly localized and functional P1 appears to be a complex process requiring many accessory proteins (13). The gene encoding P1 (MPN141) is the second of three genes thought to be expressed from a single promoter found upstream of the first gene and having a predicted stem-loop terminator following the third (Fig. 4.1a) (11). MPN140, the first gene of the three and also known as ORF4, encodes a predicted phosphoesterase (1). The presence but not the predicted biochemical activity of the MPN140 gene product has been demonstrated (12). Immediately downstream of MPN141 is MPN142, also known as ORF6. The MPN142 gene product has a predicted size of 130-kDa, but is cleaved to yield two polypeptides of sizes 90- and 40-kDa (17, 24), also known as P90 and P40 or B and C, respectively. Interestingly, the gene products of MPN142 homologs in the closely related *Mycoplasma genitalium* and *Mycoplasma gallisepticum* are present as single polypeptides (19, 21), indicating that the roles of B and C are highly interrelated.

Proteins B and C are integral membrane proteins located at the attachment organelle in proximity to P1 (5, 18). These are required for P1 function, and with P1 likely constitute a major adhesin complex.

Ribosome-binding sites (RBS) in *Mycoplasma* are poorly defined. Only five nucleotides separate the stop codon of MPN141 and the start of MPN142 (11), raising the possibility that the two are translationally linked. This scenario is supported by both the linked nature of the P1/B/C complex, which implies a role for stoichiometric protein levels, and the observation that a non-cytadherent *M. pneumoniae* mutant with a frameshift in MPN141 (mutant IV-22) which does not make P1, also lacks B or C. Non-cytadherent mutant III-4 has a mutation in MPN142 and lacks B and C but produces wild-type levels of P1 (Table 4.1). Mutants III-4 and IV-22 also each lack the 70-kDa protein A (8), for which the gene is unknown. However, the non-cytadherent phenotype of mutant III-4 is not due to the loss of A (28), and the same is probably true for mutant IV-22.

In this study we examined in greater detail the relationship of MPN141 and MPN142. Analysis by reverse-transcriptase polymerase chain reaction (RT-PCR) demonstrated that MPN140-142 are transcribed as a unit. Furthermore, using a series of genetic studies with recombinant MPN141 and MPN142 in mutants IV-22 and III-4 we examined the factors involved in their translation initiation and the interrelated fates of the P1/B/C proteins. Results strongly suggested that some but not all translation of MPN142 is coupled to translation of MPN141, and proteins B and C are unstable and quickly degraded in the absence of P1.

Materials and Methods

Bacterial strains and culture conditions.

The *M. pneumoniae* strains used in this study included wild-type M129 (broth passage 18) and non-cytadherent mutants III-4 and IV-22 derived from M129 (16). Mutant IV-22 was initially found to produce trace levels of the B and C proteins (data not shown). Upon filter cloning (26), this phenotype disappeared and was likely due to a sub-population of spontaneous revertants within the population. The filter-cloned strain was used for all experiments. Cultures were grown and harvested in Hayflick broth and PPLO agar, as described previously (6). Transformations with Tn*4001*mod-bearing suicide vectors were performed as previously described (9). Gentamicin (Gm) was included at 18 μg/ml for selection and maintenance of Tn*4001*mod (9), while chloramphenicol (Cm) was included at 12 or 24 μg/ml for selection and maintenance, respectively, of Tn*4001*cat (7). To generate strains carrying both transposons, *M. pneumoniae* cells were electroporated and transformants isolated sequentially, first with Tn*4001*mod and then Tn*4001*cat constructs. The *Escherchia coli* strains used to construct plasmids were Sure (Stratagene, La Jolla, Calif.) and JM109 (Promega, Madison Wisc.), and ampicillin resistance was selected on Luria media with 50 μg/ml of antibiotic.

RNA preparation and RT-PCR.

All RNA experiments used RNase-free reagents, and equipment was treated to eliminate RNase. RNA was purified from 10 ml mid-logarithmic-phase cultures using the RNAqueous®-4PCR kit (Ambion, Texas) according to the instructions, save the DNase treatment required 10% vol/vol enzyme incubated for 2 h. The boundaries of the *p1* transcriptional unit and linkage of its genes were analyzed by RT-PCR using the Access RT-PCR kit (Promega) and amplicons visualized using agarose gel electrophoresis and ethidium bromide staining. PCR primers were designed to amplify regions sized 407-812 nt internal to each gene of the predicted transcriptional unit, spanning their junctions, or outside its predicted limits (Table 4.2 and Fig. 4.1a). Controls without reverse transcriptase were used to detect residual DNA contamination. RNA yields were below the limit of detection of UV spectroscopy, and the amounts necessary for RT-PCR were determined empirically with equal volumes used in each set of reactions for a given preparation.

Sequencing of MPN142.

The MPN142 gene of mutant IV-22 was sequenced following PCR amplification using genomic DNA from the mutant as template. PCR primer pairs were designed to amplify the gene and flanking sequence in five overlapping segments. These PCR products were then either sequenced directly by the same primers or cloned into pCR®II using the TA Cloning® Kit (Invitrogen, Carlsbad, Cal.) and sequenced using the T7 or SP6 primers of pCR®II (sequencing by IBL, University of Georgia).

Plasmid construction.

Recombinant genes can be introduced into *M. pneumoniae* using a modified Tn*4001* (6). The plasmids used in this study are shown in Fig. 4.2. Plasmids pKV258 and pKV264, whose construction is described elsewhere (28), were used here extensively. In pKV258, MPN141 and MPN142 are expressed from the P_{OUT} promoter of Tn*4001*. In pKV264, MPN142 is likewise expressed from P_{OUT}. The capability to couple the translation of MPN142 to MPN141 is preserved, but 95% of the MPN141 gene is missing due to an internal in-frame deletion. For pKV265, the 1.5-kb *Bam*HI fragment of pKV258 was removed and the remainder of the plasmid was religated, leaving MPN142 and a large region of the upstream DNA intact, but disrupting the 5' end of MPN141 along with any of its transcription initiation signals. Plasmid pKV104 carries a modified version of Tn*4001* (Tn*4001*cat) for which the original gentamicin resistance gene was replaced with the chloramphenicol resistance gene (7). A 5.7-kb *Eco*RI fragment bearing MPN141 in pUC19 (a gift from J. Baseman) was released and subjected to a Klenow fill-in reaction, then ligated into the *Sma*I site of pKV104 oriented such that MPN141 is under the control of the P_{OUT} promoter of Tn*4001* to generate pKV299.

Analysis of cytadherence proteins and cytadherence-related phenotypes.

Wild-type, mutant, and transformant cells were analyzed for the restoration of several phenotypes related to cytadherence which were lacking in the mutants. Western blotting was performed as described previously (6) with polyclonal rabbit anti-P1 (1:1000), anti-protein B (1:2000), and anti-protein C (1:1000). Anti-P1 serum was

raised as described previously (14). Qualitative hemadsorption assays were performed as previously described (6), except that sheep blood was used.

Results

Transcriptional analysis of the *p1* locus:

RT-PCR analysis of wild-type *M. pneumoniae* RNA using primer pairs internal to or spanning the junctions between genes, or extending past the boundaries of the predicted *p1* transcriptional unit (Fig. 4.1a, Table 4.2) demonstrated that regions of the message transcribed from MPN140-142 are linked and likely present as a single unit. No product was detected with primer pair A, which flanks the promoter, and a weakly positive PCR reaction was seen using primer pair G, which flanks the predicted end of the unit. Comparably abundant products were seen with all other primer pairs (Fig. 4.1b). Similar results were seen using RNA from mutant IV-22 (data not shown), however quantitative comparisons were not pursued.

Restoration of proteins P1/B/C in mutant IV-22:

When mutant IV-22, which has a frameshift mutation in MPN141, was transformed with pKV258 (wild-type MPN141 & MPN142, Fig. 4.2), recombinant proteins P1, B, and C were oberved at approximately wild-type levels (Fig. 4.3). However, when mutant IV-22 was transformed with pKV264 (near total in-frame deletion in MPN141 and wild-type MPN142, Fig. 4.2), proteins B and C were present at greatly reduced levels while P1, as expected, remained absent (Fig. 4.3). In contrast, mutant III-4 transformed with pKV264 produced wild-type levels of B and C (Fig. 4.3) (28).

Thus, the recombinant MPN142 in pKV264 is functional and expressed at approximately wild-type levels, but the fate of B and C differed between the III-4 and IV-22 mutant backgrounds.

Restoration of MPN141 alone in mutant IV-22:

Because mutant IV-22 lacks proteins B and C while the gene for them, MPN142, is apparently wild-type (data not shown), we explored whether production of recombinant P1 alone *in trans* was sufficient to restore B and C to wild-type levels. When mutant IV-22 was transformed with pKV299 (wild-type MPN141, Fig. 4.2), production of P1 was restored, and trace amount of proteins B and C could be detected (Fig. 4.4). When mutant IV-22 was transformed with both pKV264 and pKV299 (recombinant MPN142 and MPN141, respectively), proteins P1, B, and C were fully restored (Fig. 4.4).

Expression of MPN142 without translation of MPN141:

The observation that low levels of B and C were seen in mutant IV-22 transformed with pKV299 alone suggested that some translation of B/C was possible independent of translational coupling. This possibility was examined further using pKV265 (wild-type MPN142 but untranslatable MPN141, Fig. 4.2). Low levels of B/C were detected in mutant III-4 transformed with pKV265, while no B/C was detected in mutant IV-22 transformants (Fig. 4.5).

Discussion

Cytadherence in *M. pneumoniae* is a complex process requiring many proteins that function in roles beyond simple receptor binding. The major adhesin P1 is crucial but not sufficient for attachment. A series of events requiring cytadherence-accessory proteins is required for P1 function (13). These include, not necessarily in order: insertion into the cytoplasmic membrane, trafficking to the attachment organelle, folding into the correct conformation, and interacting (stably or transiently) with partner proteins. The interrelationships between cytadherence-accessory proteins in processes leading to the formation of a functional attachment organelle and establishment of proper cellular morphology and functional P1 have been examined in detail (13). However, little is known about the relationship between P1 and the proteins B and C, except that the spatial arrangement of the genes (11) and gene products (18) suggest that these proteins are linked functionally. Here we show that proteins P1, B, and C are related at the levels of transcription, translation, and protein stability.

Analysis of MPN140-142 in *M. pneumoniae* by RT-PCR showed that they are a transcriptionally linked operon as suggested by others previously (11). However, the non-quantitative nature of simple RT-PCR limits the conclusions that might be drawn about transcription and prompted our genetic approach. Proteins P1, B, and C were restored to wild-type levels in mutant IV-22 by the introduction of recombinant MPN141 and MPN142 by transposon delivery *in cis* (pKV258, Fig. 4.3) or *in trans* (pKV264 & pKV299, Fig. 4.4). When wild-type MPN142 was present downstream of translatable but non-functional MPN141 having a major in-frame deletion (pKV264), recombinant proteins B and C were observed at wild-type levels in the mutant III-4 background (Fig.

4.3) (28). However, when introduced into P1-less background (mutant IV-22), the same construct yielded only low levels of B and C. We conclude that recombinant MPN142 was expressed at normal levels in both backgrounds, but in the absence of P1, proteins B and C were unstable. The converse was not seen, however, as P1 is stable in the absence of B and C, as with mutant III-4 (16). We were unable to identify B or C using pulse-chase radio-immunoprecipitation analysis to track the fate of newly synthesized B and C (data not shown). However, transposon delivery of wild-type MPN141 *in trans* (pKV299) into mutant IV-22 transformed with pKV264 yielded wild-type levels of B and C (Fig. 4.4), supporting the interpretation that recombinant MPN142 is indeed expressed normally from pKV264 in mutant IV-22, and that the stability of B and C requires the presence of P1. This non-reciprocal requirement for stability is similar to that seen with HMW3 and the two proteins HMW1 and HMW2. In that case, HMW3 is unstable if HMW1 or HMW2 levels are reduced (22, 29), but HMW1 and HMW2 are present at wild-type levels in the absence of HMW3 (30).

The MPN142 gene in mutant IV-22 has a wild-type nucleotide sequence (data not shown), but the restoration of just recombinant MPN141 *in trans* using pKV299 was not sufficient to restore levels of B and C fully (Fig. 4.4), with only trace amounts of B and little or no C detected. This is similar to what is seen in the closely related *M. gallisepticum*, where co-expression of the MPN141 and MPN142 homologs is necessary (20). The MPN141 gene in mutant IV-22 encodes a drastically truncated protein (46 vs. 1627 residues) due to a frameshift (25). Once the translation machinery has encountered the premature stop codon in the resulting MPN141 message, the ribosome likely disassociates from the mRNA. In contrast, in wild-type *M. pneumoniae*,

the translation of MPN142 probably begins before the ribosome can disassociate. This coupling of translation between MPN141 and MPN142 would likely ensure near-equimolar amounts of the two gene products. However, when wild-type MPN141 is delivered to mutant IV-22 *in trans*, small amounts of B and C were detected and could only come from the native allele, suggesting one or more of the following: (i) translation of MPN142 can proceed at low levels despite premature termination of MPN141 translation, (ii) limited translation re-initiation occurs independent of coupling, or (iii) the untranslated message is less stable resulting in less protein than would otherwise be produced. Regardless, the read-through products could only be seen in the presence of P1, indicating that the stability of B and C is dependent on the presence of the adhesin. In order to distinguish the factors driving the limited translation of MPN142 seen in mutant IV-22, we produced a construct possessing wild-type MPN142 and a large portion of upstream sequence but lacking the 5' end of MPN141, including any likely translation initiation signals (pKV265). When pKV265 was introduced into mutant IV-22, no MPN142 products were detected (Fig. 4.5), as expected in the absence of P1. Mutant III-4 transformed with pKV265 produced less than wild-type amounts of B and C, probably the result of translation initiation independent of coupling to MPN141, with the balance of B/C synthesis due to coupling.

When P1, B, and C were restored in mutant IV-22 by transformation with pKV258 or both pKV264 and pKV299, the transformants remained hemadsorption-negative (data not shown). The phenotype of these transformants is clearly distinct from both wild-type and mutant IV-22 *M. pneumoniae*, with an intermediate ability to adhere to plastic and a morphologically distinct appearance by microscopic observation (data not

shown). The critical role of proteins B and C in hemadsorption has recently been confirmed (28). Cytadherence and virulence in an avirulent high-passage *M. gallisepticum* strain can be restored by the introduction of recombinant versions of the MPN141 and MPN142 homologs (20), suggesting that restoration of protein levels should be sufficient to confer hemadsorption in an otherwise wild-type strain. There are several possible explanations for the failure to restore hemadsorption in mutant IV-22. First, transposon insertion could have affected a gene essential for cytadherence. This seems unlikely given that multiple transformants with pKV258 were examined. Alternatively, and more likely, a secondary mutation may exist in the gene for a possibly undescribed cytadherence-related protein in the filter-cloned mutant IV-22 used in these experiments, as this strain has wild-type levels of all other known cytadherence-accessory proteins for which antibody is available (2). The lack of P1/B/C in mutant IV-22 would obscure such a secondary mutation, but its effects would be observable in the pKV258 or pKV264/299 transformants. This conclusion is supported by the observation that spontaneous hemadsorption positive revertants of mutant IV-22 were obtained (15) from the non-filter cloned population. Lower passage non-filter cloned stocks of mutant IV-22 must be examined to isolate what we believe to be the original mutant IV-22. Regardless, the current study significantly expands our understanding of the relationship between MPN141 and MPN142 and their products.

A model of the protein interactions necessary of the formation of the *M. pneumoniae* attachment organelle and cytadherence competence has been proposed (13). In that model, several protein interactions are shown to be important for the proper localization of functional P1, B, and C, but these three are treated as a single

unit and little about possible interactions involving P1/B/C is known. Here evidence is presented supporting the hypothesis that P1/B/C are closely tied in function and possibly act in concert to confer cytadherence, because MPN141 and MPN142 are connected at the levels of transcription and translation. Furthermore, it is shown that there are distinct, measurable, and asymmetric characteristics of these proteins. Namely that B and C require the presence of P1, but the reciprocal does not hold, although B/C are required for P1 function. It is possible that interaction with P1 promotes the proper folding of B/C. This finding and the genetic tools used here now allow an exploration of the regions of P1 necessary for the stabilization and thus likely interaction and binding with B/C.

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FIG. 4.1. RT-PCR analysis of the *p1* operon of *M. pneumoniae*.

(A) Map of the *p1* operon of *M. pneumoniae*. Arrow indicates transcriptional promoter upstream of MPN140. Twelve nt separate MPN140 and MPN141, and five separate MPN141 and MPN142. A predicted stem-loop terminator begins 17 nt downstream of MPN142. Locations corresponding to annealing sites of oligonucleotide primer sets A-G (Table 4.2) used for RT-PCR are shown below the operon map. Minus primer (-) used for cDNA synthesis and negative strand PCR amplification of the cDNA and plus primer (+) used for positive strand amplification. Restriction enzyme sites for *Pme*I (P) and *Sph*I (Sp) referenced in Figure 2 are shown. Scale bar is 1 kb. **(B)** Agarose gel electrophoresis of RT-PCR products. RT-PCR reactions for primer pairs A-G with wild-type *M. pneumoniae* RNA template are shown. Reverse transcriptase was included (+) or omitted (-). Locations of DNA markers and their sizes in basepairs are shown.

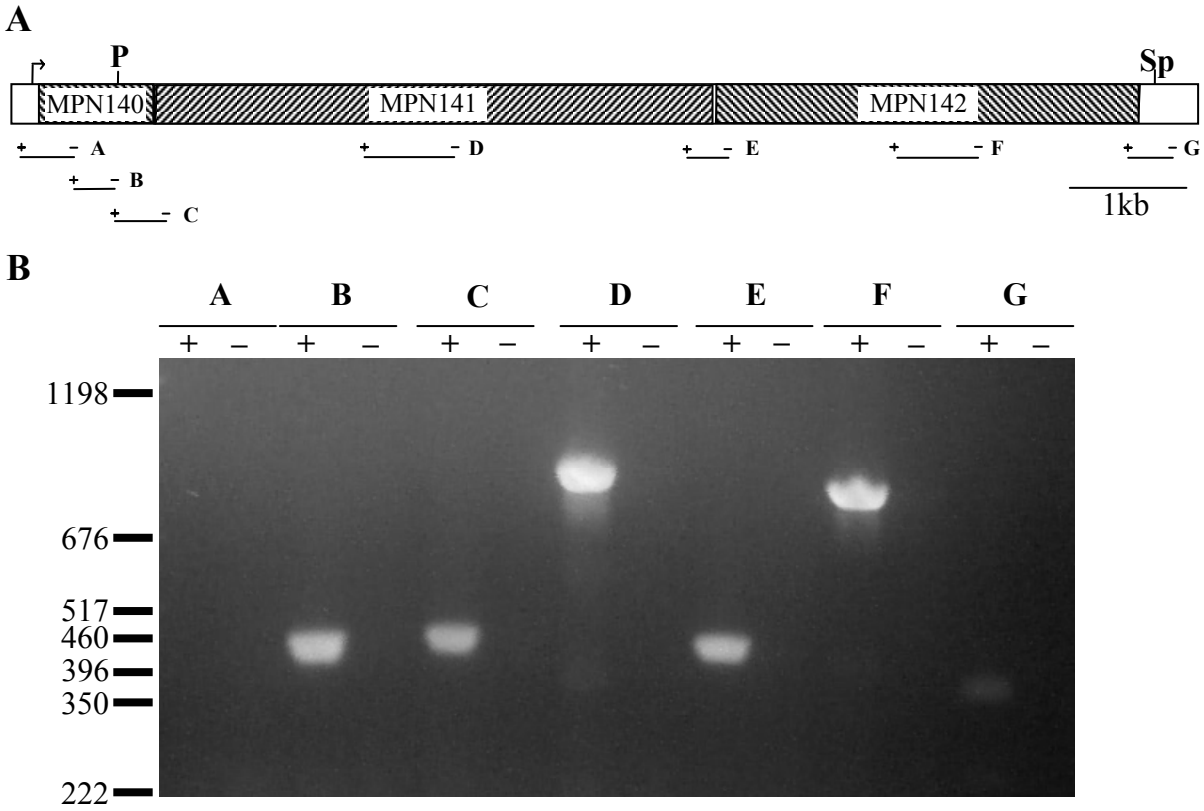
A
P
Sp
MPN140
MPN141
MPN142
A
B
C
D
E
F
G
1kb
B
A
B
C
D
E
F
G
+ – + – + – + – + – + – + –
1198
676
517
460
396
350
222

FIG. 4.2. Map of the DNA fragments cloned into the unique cloning sites in IS256L of Tn*4001*mod of pKV74 or Tn*4001*cat of pKV104 to create pKV258, pKV264, pKV265, and pKV299.

Save for the IS elements, all open reading frames are shown with the 5' end on the left. The region encoding the 3' end of MPN140 is not indicated. B, *Bam*HI; Bs, *Bsa*BI; E, *Eco*RI; S, *Stu*I; Sm/P, *Sma*I/*Pme*I junction; M/E/Sp, former *Sph*I site of the *p1* operon replaced by *Mfe*I linker and present as an *Mfe*I/*Eco*RI junction; E or Sm, *Eco*RI in pKV74, *Sma*I in pKV104; barred symbol indicates a silent change to destroy the site. Gentamicin resistance (Gm^r) is found in pKV74 and chloramphenicol resistance (Cm^r) in pKV104. Arrow from Tn*4001*mod/cat shows the location and direction of the P_{OUT} promoter. Note that the orientation of the inserts is inverted when cloned into Tn*4001*mod/cat.

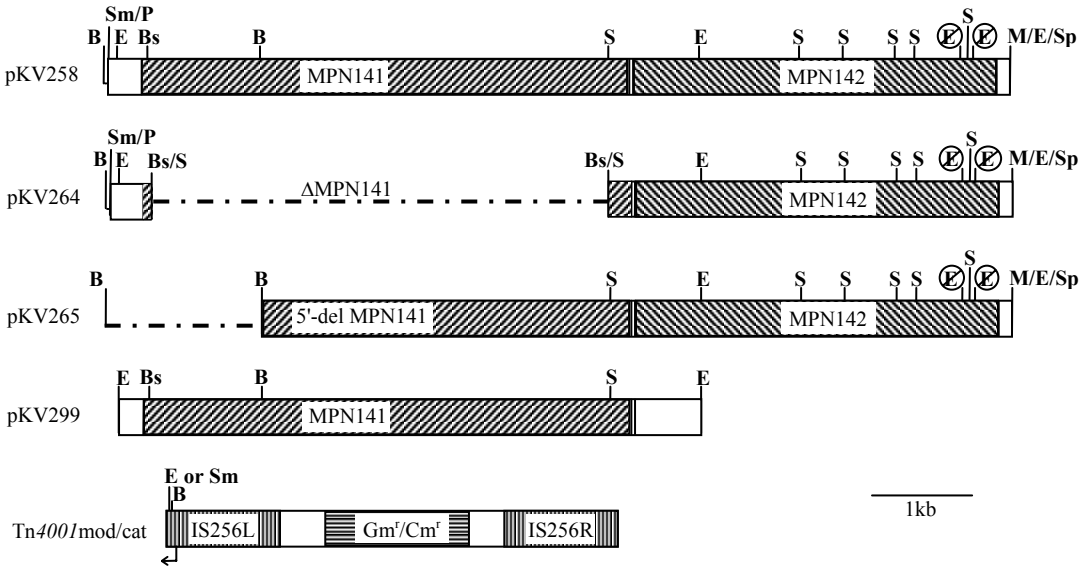
Sm/P
B
E
Bs
B
S
E
S
S
S
S
E
E
S
M/E/Sp
pKV258
MPN141
MPN142
Sm/P
B
E
Bs/S
Bs/S
E
S
S
S
S
E
E
S
M/E/Sp
pKV264
ΔMPN141
MPN142
B
B
S
E
S
S
S
S
E
E
S
M/E/Sp
pKV265
5'-del MPN141
MPN142
E
Bs
B
S
E
pKV299
MPN141
E or Sm
B
Tn4001mod/cat
IS256L
Gmr/Cmr
IS256R
1kb

FIG. 4.3. Western blot analysis of lysates of wild-type (WT) or mutant (IV-22 or III-4) *M. pneumoniae* untransformed or transformed with pKV258 or pKV264.

Two independent transformants are shown for each plasmid/background combination. 60 μg protein used per lane. Blots probed with antiserum against P1, B, or C as indicated.

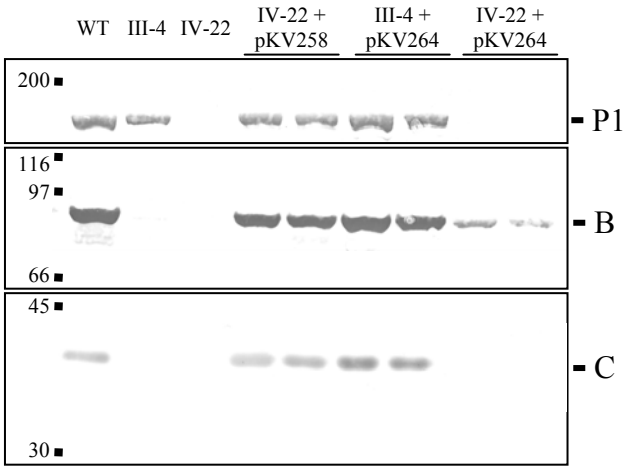
WT
III-4
IV-22
IV-22 + pKV258
III-4 + pKV264
IV-22 + pKV264
200
116
97
66
45
30
P1
B
C

FIG. 4.4. Western blot analysis of lysates of wild-type (WT) or mutant (IV-22) *M. pnumoniae* untransformed or transformed with pKV264, pKV299, or pKV264 and pKV299.

Three independent transformants are shown for each plasmid in the mutant IV-22 background save IV-22 + pKV264 (see Fig. 4.3). 60 μg protein used per lane. Blots probed with antiserum against P1, B, or C as indicated.

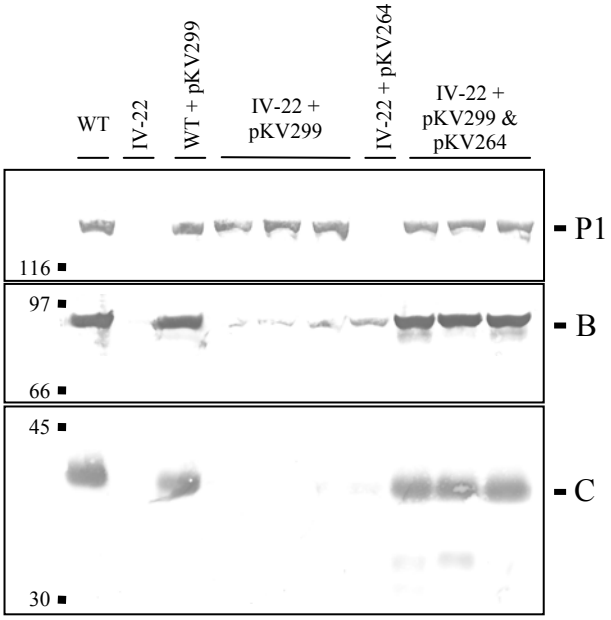
WT
IV-22
WT + pKV299
IV-22 + pKV299
IV-22 + pKV264
IV-22 + pKV299 & pKV264
116
97
66
45
30
P1
B
C

FIG. 4.5. Western blot analysis of lysates of wild-type (WT) or mutant (IV-22 or III-4) *M. pnumoniae* untransformed or transformed with pKV265.

Two independent transformants are shown for each plasmid/background combination. 60 μg protein used per lane. Blots probed with antiserum against P1, B, or C as indicated.

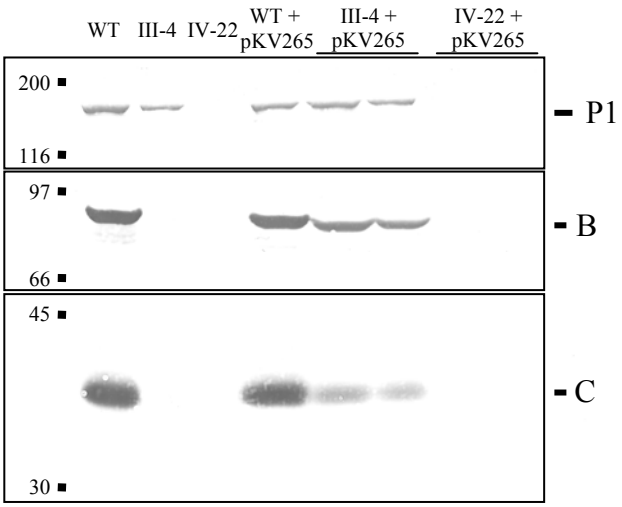
WT
III-4
IV-22
WT + pKV265
III-4 + pKV265
IV-22 + pKV265
200
116
97
66
45
30
P1
B
C

Table 4.1. Strains examined in this study

Strain	*p1* operon defect	Relevant phenotype		References
		P1 (MPN141)	B/C (MPN142)	
Wild-type	None	++	++	
III-4	frameshift in MPN142	++	–	16, 28
IV-22	frameshift in MPN141	–	–	16, 25
III-4 + pKV258	None	++	++	28
III-4 + pKV264	None	++	++	28
III-4 + pKV265	Reduced translation of MPN142	++	+	This study
IV-22 + pKV258	None	++	++	This study
IV-22 + pKV264	MPN141^{-}	–	+/-	This study
IV-22 + pKV265	MPN141^{-}	–	–	This study
IV-22 + pKV299	Reduced translation of MPN142	++	+/-	This study
IV-22 + pKV264 & pKV299	None	++	++	This study

++ protein present at or near wild-type levels
+ protein present at reduced levels
+/- protein present at trace levels
- protein undetected
Transposon from plasmid pKV258 has recombinant MPN141 & MPN142
Transposon from plasmid pKV264 has recombinant MPN142
Transposon from plasmid pKV265 has recombinant MPN142
Transposon from plasmid pKV299 has recombinant MPN1412

Table 4.2. Summary of RT-PCR analysis of the *p1* operon

	Location	nt[a]	Primer sequence	Results			
				Size (bp)	RT-PCR +RT	RT-PCR -RT	PCR
A	MPN140-upstream	(+) -73 (–) 395	5'-TTTTTGCACCAAAATGGCG 5'-CATCAACGAAGTTATGGGTCG	468	+	–	+
B	MPN140-internal	(+) 332 (–) 739	5'-ACCAAAACCAGTTTAAGGCAG 5'-TGCACATTCTAAGGGTGTTAC	407	+	–	+
C	MPN140-141-junction	(+) 708 (–) 1128	5'-GGCCGATAAGAAGTTCCAAAAG 5'-GGGTGTAGCTAAATTGCTGG	420	+	–	+
D	MPN141-internal	(+) 2876 (–) 3688	5'-TGCAACTCTTCACACCCTAC 5'-GCCTTATCATTCCTTCACCC	812	+	–	+
E	MPN141-142-junction	(+) 5642 (–) 6055	5'-CAATGCACAAGAACAAACAGG 5'-CGTATGAccccTGTAAATGGG[b]	414	+	–	+
F	MPN142-internal	(+) 7424 (–) 8218	5'-GACCTGATGAGCGAAAACC 5'-GCTAATCTTCACCCCATTCC	794	+	–	+
G	MPN142-downstream	(+) 9478 (–) 9838	5'-AAGCACCAGTTAAACCAGC 5'-ACCTTCTTTTTCCAGTAAGACC	360	+/-	–	+

[a] nucleotide position of 5' end of primer in relation to the first +1 transcript position

[b] Due to an oversight, the minus-strand primer was synthesized with the incorrect sequence. Each lower case nucleotide (c) should be G. However, the amplification worked and the results are reported.

DISSERTATION SUMMARY

Cytadherence in *M. pneumoniae* is a complex process requiring at least eight proteins found in three operons or gene clusters distributed throughout the chromosome. Gene regulation in *Mycoplasma* is generally poorly understood due to technical difficulties inherent in the system and the apparent divergence in genetic mechanisms from better understood organisms such as *Escherichia coli* and *Bacillus subtilis*. In spite of the availability of genome sequences from several closely and distantly related *Mycoplasma* species, basic genetic elements such as ribosome-binding sites, promoter sequences, and regulatory regions are at best poorly defined. This work presents the different mechanisms by which the so-called *hmw* gene cluster and *p1* operon are transcribed and translated. The overlapping transcripts of the *hmw* gene cluster and the translational coupling of MPN141 and MPN142 of the *p1* operon provide insight into how the levels of protein encoded by these genes may be regulated. This information about relative protein amounts gives clues as to the function of these proteins and how they might interact. Furthermore, these studies greatly increase the available information about general genetic mechanisms in *M. pneumoniae*, and the molecular tools developed and described here provide a basis for future work.

The *hmw* gene cluster contains four promoter elements that each drive transcription within the cluster. The mRNA synthesized downstream overlaps transcript from upstream promoters within the cluster, as determined by nuclease protection and

reverse-transcriptase PCR (RT-PCR), but the extent of this overlap is uncertain. I have shown that the expression of a given gene is not necessarily the additive result of transcription from all the promoters found upstream, and in the case of *p30* and *hmw3*, expression depends on PromP21 much more heavily than PromP32 found farther upstream. If multiple promoters can drive transcription of certain genes within the gene cluster, this indicates a possible mechanism of regulating the relative amount or temporal expression of proteins involved in the formation of the attachment organelle, a structure requiring the interaction of several proteins and the synthesis of which is linked to cell-division. Each of the four transcripts in the gene cluster has an upstream region corresponding to the -10 Pribnow Box of a promoter element. This eight-nucleotide sequence (TTAAAATT) is similar to the consensus *E. coli* σ^{70} sequence (TATAAT). By examination the genome sequence of *M. pneumoniae* it was found that the eight-nucleotide sequence was found upstream of ORFs at a much higher frequency than would be expected by chance, and this sequence may be indicative of an important type of promoter in *M. pneumoniae*. Comparison of the promoters found in the *hmw* gene cluster and other reported transcriptional promoters showed that no similarity in the -35 region could be found. This was the first report that the DNA-dependent RNA polymerase of *M. pneumoniae* might not use a -35 recognition sequence, and this result has been supported by the subsequent findings. Finally, this study shows the first use of chloramphenicol acetyl-transferase (*cat*) as a reporter gene in *M. pneumoniae*.

While the genetic defect in mutant IV-22 has long been known, the factor(s) responsible for the phenotype of mutant III-4 was unreported. In order to study the interactions of the genes and proteins of the *p1* operon, a better characterization of

mutant III-4, which lacks proteins B and C, thought to be encoded by a gene in that operon, was necessary. In addition, protein A is missing in mutants IV-22 and III-4, but any possible gene encoding protein A is unknown. In order to study cytadherence proteins in these mutants, the loss of protein A must be defined or (as demonstrated here) shown to have no direct role in cytadherence. I have shown that mutant III-4 has a frameshift mutation in MPN142. Restoration of MPN142 either by spontaneous reversion or delivery of recombinant wild-type MPN142 restored cytadherence and a wild-type morphology. Furthermore, the 90-kDa protein B and the 40-kDa protein C were restored and shown to correspond to the previously described P90 and P40 products of the MPN142 gene.

RT-PCR showed the three genes of the *p1* operon are co-transcribed and the transcript does not extend past the predicted stem-loop terminator. The constructs studied in mutant III-4 in addition to newly created ones were used in mutant IV-22 to examine the nature of the relationship between P1 (encoded by MPN141) and B/C. Mutant IV-22 has a defective MPN141 but a wild-type MPN142; nonetheless, proteins B and C are missing in this mutant. Delivery of recombinant MPN141 restored P1, but not B/C in a mutant IV-22 background. MPN141 and MPN142 *in cis* or *in trans* were sufficient to restore P1/B/C. However, these transformants were not cytadherent, indicating a secondary mutation affecting cytadherence is probably present in this mutant. The absence of B/C in mutant IV-22 is partially the result of the disruption of translational coupling of MPN142 to MPN141 in the mutant. However, limited translation of MPN142 occurs without coupling. I found that in the absence of P1, B/C is unstable and degraded by *M. pneumoniae*. This accounts for the absence of B/C in

mutant IV-22. The linkage of P1/B/C at the levels of DNA, transcription, and translation in addition to the previously described co-localization of the proteins and the requirement for B/C for P1 function supports the view of these proteins as components of a larger major adhesin complex. Furthermore, the requirement of P1 for B/C stability suggests interaction of the proteins in this putative complex and allows for some differentiation between them and structure-function analysis of their sites of binding and/or interaction.

Information about the genetics of *M. pneumoniae* is scarce. The results presented here about the expression of two of the three known clusters of cytadherence-related proteins increase our understanding of the expression of their genes and possibly shed light onto the nature of the interactions between the encoded proteins. Furthermore, critical facts about two important cytadherence mutants are described. This knowledge along with the genetic tools created for these studies enable the future elucidation of the nature of the interaction of P1/B/C in the major adhesin complex and the attachment organelle and insight into the function of these proteins and their role in cytadherence.